BIOSYNTHESIS OF PHENAZINES BY
PSEUDOMONAS AERUGINOSA

by
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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

BIOSYNTHESIS OF PHENAZINES BY PSEUDOMONAS AERUGINOSA

Ph.D. Pin-Chuan Chang Microbiology

Pseudomonas aeruginosa Mac 436 was found to produce simultaneously pyocyanine, phenazine-κ-carboxylic acid and oxychlororaphine in several defined media.

Glycerol-1,3-C\textsuperscript{14}, glycerol-2-C\textsuperscript{14}, urea-C\textsuperscript{14} and acetate-1-C\textsuperscript{14} were used as substrates for the study of pyocyanine biogenesis, and acetate-1-C\textsuperscript{14}, acetate-2-C\textsuperscript{14}, glycerol-1,3-C\textsuperscript{14}, glycerol-2-C\textsuperscript{14}, succinate-1,4-C\textsuperscript{14}, succinate-2,3-C\textsuperscript{14}, glucose-1-C\textsuperscript{14}, glucose-2-C\textsuperscript{14}, glucose-6-C\textsuperscript{14} and shikimate-1,6-C\textsuperscript{14} were used to study the simultaneous formation of pyocyanine, phenazine-κ-carboxylic acid and oxychlororaphine.

The pigments were isolated and degraded to the extent that 7 of the 13 carbons were assessed. Incorporation data and the results of degradation suggest that the aromatic rings of phenazine pigments did not solely originate through the shikimate pathway nor the acetate pathway. The carboxyl carbons of phenazine-κ-carboxylic acid and oxychlororaphine did not originate from the carboxylic carbon of shikimic acid.

Using radioactive phenazine-κ-carboxylic acid, P. chlororaphis and P. aeruginosa were found to synthesize oxychlororaphine from phenazine-κ-carboxylic acid.
ACKNOWLEDGMENTS

The author would like to extend his sincere gratitude to Dr. A. C. Blackwood, Chairman of the Department of Microbiology, for his continued inspiration and guidance throughout the course of this investigation and in the preparation of the manuscript.

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CLAIMS OF CONTRIBUTION TO KNOWLEDGE

The simultaneous formation of pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine was followed in a defined medium with *P. aeruginosa* Mac 436 using radioactive substrates.

The following observations related to these studies constitute a contribution to knowledge:

1. *P. aeruginosa* Mac 436 was found to produce pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine in the defined media of Frank and DaMoss (1959) and of Ingram and Blackwood (1962).

2. A basal salts solution containing 0.41% MgCl₂, 6H₂O, 0.71% Na₂SO₄, 0.0005% FeSO₄·7H₂O, 0.2% urea and 0.010% K₂HPO₄ was devised which, upon the addition of 2% acetate, glycerol or glucose as a sole source of carbon, supported the formation of pyocyanine, phenazine-α-carboxylic acid, and oxychlororaphine by *P. aeruginosa* Mac 436.

3. Degradation schemes for phenazine-α-carboxylic acid and oxychlororaphine were established whereby 7 carbons out of 13 carbons can be assessed in one single carbon and three pairs of carbons.

4. Incorporation data and the results of degradations suggest that:

   (a) The aromatic rings of the phenazine nuclei of pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine did not originate solely through the conventional...
shikimate pathway nor through the acetate pathway.

(b) The carboxyl carbons of phenazine-κ-carboxylic acid and oxychlororaphine did not originate from the carboxyl carbon of shikimic acid.

5. Oxychlororaphine is biosynthesized from phenazine-κ-carboxylic acid by both *P. chlororaphis* and *P. aeruginosa*. 
INTRODUCTION

Various substituted phenazines are produced by bacteria in the genera *Pseudomonas* and *Brevibacterium*, by myxobacteria of genus *Sorangium*, and by actinomycetes of the genus *Streptomyces*, *Waksmania* and *Microbispora*.

The production of various of these pigments by the genus *Pseudomonas*, especially *P. aeruginosa* which produces the phenazine derivatives; pyocyanine, phenazine-1-carboxylic acid, chlororaphine, oxychlororaphine and aeruginosin A and B, make this species an ideal tool for elucidation of the biosynthetic pathway.

In this project, trials were made to find appropriate conditions for pigment production by *P. aeruginosa* Mac 436. Then, radioactive pyocyanine, phenazine-1-carboxylic acid and oxychlororaphine were biosynthesized by feeding specifically labeled radioactive acetate, glycerol, succinate, glucose and shikimic acid. The pigments were isolated and degraded to establish the mechanisms of biogenesis of these pigments.
LITERATURE REVIEW

GENERAL

Among the naturally occurring phenazines, pyocyanine was the first to be discovered. A strain of Pseudomonas aeruginosa producing the pigment was isolated by Gessard (1890). The chemical structure of the pigment was studied by Wrede and Strack (1929), Michaelis (1931), and Hillemann (1938):

Later, the green pigment, chlororaphine and a yellow pigment, oxychlororaphine, produced by P. chlororaphis were discovered by Lasseur (1911). The structures of these pigments were reported by Kögl and Postowsky (1930).

A violet, copper-glittering pigment, iodinin, was isolated by Clemo and MacIlwain (1938) from Chromobacterium iodinum (latter named as Pseudomonas iodina, Sneath, 1956), and was found to be produced also by Brevibacterium crystalloidinum (Irie et al., 1960).

Phenazine-α-carboxylic acid, a yellow pigment, was isolated from P. aureofaciens, as early as 1936, in Prof. A. J. Kluiver's laboratory in Delft. Publication was made simultaneously in 1956 by Kluiver, and also by Haynes et al. who isolated the same phenazine independently.

Isono et al. (1958) isolated phenazine-α-carboxylic acid from Streptomyces misikiensis. Following this finding, other phenazine producing actinomycetes were found. S.
griseoluteus (Yagishita, 1960) produces griseoluteic acid, griseolutein A and B, 1-hydroxymethyl-6-carboxy-phenazine, and 1-methoxy-4-methyl-9-carboxy-phenazine. Iodinin and 1,6-phenazinediol are produced by S. thioluteus (Akabori et al., 1959), by Waksmania aerata (Gerber et al., 1964), and also by Microbispora aerata (Lechavalier, 1965). 1,6-Phenazinesdiol-5-oxide was isolated from S. thioluteus, M. aerata and P. iodinum (Lechevalier, 1965; Gerber et al., 1965).

Aeruginosin A and B are water soluble red pigments produced by P. aeruginosa and reported to be phenazine derivatives by Holliman (1957), and Bentley and Holliman (1966).

A second phenazine derivative produced by P. aureofaciens was identified as $\beta$-hydroxyphenazine-$\delta$-carboxylic acid by Toohey et al. (1965), and as $\beta$-hydroxyphenazine by Levitch et al. (1966).

A red phenazine derivative, 1-hydroxy-6-methoxy-4,9-dioxide, was isolated from a Sorangium spp. by Edward and Gillespie (1966).

The list of naturally occurring phenazines are summarized in Table 1.

PIGMENTS PRODUCED BY PSEUDOMONAS AERUGINOSA

P. aeruginosa produces, aside from pyocyanine, various pigments of indicator and redox types which lead to the famous "chameleon phenomenon," a multiple color change along a scratch created by drawing a needle through a heavily grown agar culture. The pigments produced are listed in Table II and these pigments, pyocyanine, phenazine-1-
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<th>Organism</th>
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<td><img src="image1" alt="Chemical structure" /></td>
<td><em>Pseudomonas</em></td>
<td>Wells et al. (1955) *Hilleman (1956)</td>
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<td>Phenamine-6-carboxylic acid</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td><em>P. aeruginosa</em></td>
<td>Kluyver (1956) *P. aeruginosa *Enterobacter cloacae</td>
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<td><img src="image3" alt="Chemical structure" /></td>
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<td>Kepi et al. (1951) *Kepi et al. (1950)</td>
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<td>Oxychlorophenamine (phenamine-1-carboxamide)</td>
<td><img src="image4" alt="Chemical structure" /></td>
<td><em>P. aeruginosa</em></td>
<td>Kepi et al. (1950) *Kepi et al. (1947)</td>
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<td>Iodin (1,6-dihydroxy-phenazine-3-carboxamide)</td>
<td><img src="image5" alt="Chemical structure" /></td>
<td><em>P. aeruginosa</em></td>
<td>Tsurum et al. (2021) *Abbert et al. (1964) *Giese et al. (1963) *Lefevre (1969)</td>
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<td>1,6-dihydroxy-phenazine-3-carboxamide</td>
<td><img src="image6" alt="Chemical structure" /></td>
<td><em>P. aeruginosa</em></td>
<td>Giese et al. (1963) *Lefevre (1969)</td>
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<td>1,6-dihydroxyphenazine</td>
<td><img src="image7" alt="Chemical structure" /></td>
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<td>Giese et al. (1963) *Lefevre (1969)</td>
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<td><img src="image8" alt="Chemical structure" /></td>
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<td>Tsuchiya et al. (1966)</td>
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<td><img src="image10" alt="Chemical structure" /></td>
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<td>Williams (1961) *Tsuchiya et al. (1966)</td>
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<td>Greigiolidendrin A (1-hydroxy-2-hydroxyethyl-4-hydroxyphenol)</td>
<td><img src="image12" alt="Chemical structure" /></td>
<td><em>S. griseolus</em></td>
<td>Yagihiita (1960)</td>
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<td>Greigiolidendrin B (1-hydroxy-4-(1,2-dihydroxyethyl-4-hydroxyphenol)</td>
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<td><img src="image16" alt="Chemical structure" /></td>
<td><em>S. griseolus</em></td>
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<td>1-acetyl-4-hydroxyphenol-3-carboxyphenol</td>
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<td><em>S. griseolus</em></td>
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<td>Spalin (1-acetyl-4-methoxyphenol-3-carboxyphenol)</td>
<td><img src="image18" alt="Chemical structure" /></td>
<td>Normania spp.</td>
<td>Edwards et al. (1964)</td>
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carboxylic acid, chlororaphine and oxychlororaphine will be covered in the later section of this review.

Korth (1962) reported that _P. aeruginosa_ produces golden-yellow colored pigments, dihydrophenazine-1-carboxylic acid and phenazine-1-carboxylic acid, in a glucose and (NH₄)₂ SO₄ medium. Whereas, in an alkaline peptone medium, the same strain produces pyocyanine.

Fluorescein* or bacteriofluorescein is a green fluorescent pigment, yellow in acid, red in alkali. It emits a blue fluorescence at neutral pH. Chemical formulae assigned are C₃₂H₄₁O₈N₇ by Turfeijer (1941) and C₄H₇ON by Turfitt (1937). Recently, Japanese workers found that _P. aeruginosa_ produces two fluorescent pigments, fluoresceine I and II (Osawa et al., 1963). Fluoresceine II was crystallized later and assigned the formula C₁₇H₂₃₋₂₅O₂N by Osawa et al. (1965).

Pyorubin, a red pigment of non-indicator type, was studied by Leonard (1924) and Meader et al. (1925). It was precipitable by alkaline CS₂ as orange mass. Chernomordik (1956) reported a red pigment which was insoluble in all organic solvents. Post (1959) isolated a red pigment which contained 47% ash and the organic portion consisted of C₆H₁₂NO₄. He believed that pyorubin was a Mg salt of a phenazine derivative.

Aeruginosin A and B, water soluble red pigments, were isolated from a red pigmented strain of _P. aeruginosa_ by Holliman (1961). Aeruginosin A was characterized first as

*Fluorescein is 9-(6-carboxyphenyl)-6-hydroxy-3-isoxanthenone
TABLE II
Pigments produced by *P. aeruginosa*.

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<td>1-Hydroxy-5-methylphenazine</td>
<td>Wrede <em>et al.</em> (1929)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hilleman (1938)</td>
</tr>
<tr>
<td>Dihydro-1-phenazine-carboxylic acid</td>
<td></td>
<td>Korth (1962)</td>
</tr>
<tr>
<td>Phenazine-1-carboxylic acid</td>
<td></td>
<td>Takeda (1958a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Korth (1962)</td>
</tr>
<tr>
<td>Chlororaphine</td>
<td>molecular comp'd of phenazine-1-carboxamide and its dihydro-derivative</td>
<td>Birkofer (1947)</td>
</tr>
<tr>
<td>Oxychlororaphine</td>
<td>phenazine-1-carboxamide</td>
<td>Birkofer <em>et al.</em> (1948)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Takeda (1958a)</td>
</tr>
<tr>
<td>Aeruginosin A</td>
<td>2-Amino-6-carboxyphenazinium zwitterion</td>
<td>Bentley <em>et al.</em> (1966)</td>
</tr>
<tr>
<td>Aeruginosin B</td>
<td>2-Amino-6-carboxy-10-methylphenazinium betaine</td>
<td>Herbert <em>et al.</em> (1964)</td>
</tr>
<tr>
<td>Fluorescénin</td>
<td></td>
<td>Young (1947)</td>
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<tr>
<td>Pyorubin</td>
<td></td>
<td>Post (1959)</td>
</tr>
<tr>
<td>Melanine-like</td>
<td></td>
<td>Idu (1962)</td>
</tr>
<tr>
<td>Pyo</td>
<td>C₁₄O₁₀N₂O₂</td>
<td>Hay <em>et al.</em> (1945)</td>
</tr>
<tr>
<td></td>
<td>C₁₀H₈NSO₃</td>
<td></td>
</tr>
<tr>
<td>Pyoluteorine</td>
<td>C₁₄H₇Cl₂O₃</td>
<td>Takeda (1958a)</td>
</tr>
</tbody>
</table>
2-amino-6-carboxy-10-methylphenazinium zwitterion (Herbert and Holliman, 1964), but later, corrected to 2-amino-6-carboxy-phenazine (Bentley and Holliman, 1966). Aeruginosin B is 2-amino-6-carboxy-8-sulfo-10-methylphenazinium betaine (Herbert and Holliman, 1964).

A black melanine-like pigment was reported to be produced by non-motile varieties of P. aeruginosa transduced by phage (Liu, 1962).

In extensive fractionations of cell extracts, Hay et al. (1945) isolated yellow crystals of composition C14H10N2O2 and C10H8NSO3, the so-called pyo compounds of interest as antibiotics.

Takeda (1958a, 1959a) reported that P. aeruginosa strain T359 produces pyoluteorine, C11H7Cl2O3, or 2-(2,6-dihydroxybenzol)-x-x-dichloropyrrol, a chlorine containing yellow pigment.

PYOCYANINE

Fordos (1860) reported the isolation of a blue pigment by chloroform extraction from a blue tinted bandage covering a pus filled wound, and the causal agent, P. aeruginosa (Bacillus pyocyaneus), was isolated (Gessard, 1882), and investigated. The method of extraction was improved by shaking the chloroform solution with dilute mineral acid and obtaining a red aqueous solution of the salt from which the blue base could be extracted again with chloroform after adding alkali. Little was known about the chemical
nature of the pigment until 1923, when Mccombie and Scarborough (1923) failing to obtain the base in a pure state, prepared and analyzed a series of different salts, from which they deduced that pyocyanine had the empirical formula, $C_{26}H_{28}O_3N_4$. In the following year, Wrede and Strack (1929) reported the formula to be $C_{26}H_{20}O_2N_4$, which is double the now-accepted molecular formula. The bimolecular structure was corrected to the single molecular formula by Michaelis (1931), and the relative position of the $N$-methyl group and the oxygen atom in the pyocyanine was established by Hillemann (1938).

**Media for Pyocyanine Production**

To obtain high yields of pyocyanine various media have been recommended. With complex media, ranging from bouillon horse flesh and human placenta (Wrede and Strack, 1924) to peptone (Gessard, 1891; Klinge, 1959), supplemented with salts, the following substrates were shown to increase the yield of pyocyanine; glycerol (Gessard, 1891; Jelinek and Hof, 1938; Sellers et al., 1962), sodium citrate, mannitol, fructose and propylene glycol (Sellers et al., 1962).

A defined medium was proposed first by Goris and Liot (1922), and Liot (1923). On mineral agar many amino acids could induce pigmentation, but ammonium salts were the best and their anions were unimportant. Glycine did not promote any pyocyanine production, but glycerol, glucose, fructose and mannitol gave good pigmentation. Jordan
(1899) reported that the ammonium salts of succinic, acetic, or citric acids could serve as adequate carbon and nitrogen sources for pyocyanine production. However, the yields from synthetic media had never been found to equal the yields from the complex glycerol-peptone agar of Gessard (Robinson, 1932; Seleen and Stark, 1943).

Burton et al. (1947) found that hydrolyzed casein with added glycerol and salts gave a yield of pyocyanine equal to that obtained on the Gessard's peptone-glycerol agar. By fractionation of casein-hydrolysate, glycine, DL-alanine, DL-valine or L-tyrosine were shown to produce pyocyanine, when employed as a sole source of nitrogen with glycerol as carbon source. Based on this finding, synthetic media to give maximum yields of pyocyanine by P. aeruginosa, ATCC 9027, were proposed; one contained glycerol, L-leucine and glycine, the other substituted DL-alanine for glycine.

Following Burton et al.'s synthetic medium, various synthetic media were used; Hellinger's (1951), Ingram and Blackwood's (1962), MacDonald's (1963), Sheikh and MacDonald's (1964), Frank and DeMoss' (1959), Kurachi's (1959), Valette et al. (1964), and Takeda et al.'s (1959b).

Hellinger's, Blackwood and Neish's, Ingram and Blackwood's were essentially the modification of one of the Burton et al.'s media, to which CaCO₃ was incorporated, and changes were made in the K₂HPO₄ and amino acid concentrations. MacDonald and Frank and DeMoss dropped
L-leucine from the medium. In Sheikh and MacDonald's medium, quinic acid was added as an extra carbon source. Kurachi's (1959c) and Takeda et al.'s (1959b) media employed glycerol as carbon source and urea as nitrogen source. Valette et al. (1964) used the media using ammonium salt as the sole source of nitrogen and succinic acid as the sole source of carbon. Succinic acid could be replaced by methylsuccinate, malate, and fumarate.

Mineral Requirements for Pyocyanine Production

The mineral requirement for pyocyanine production was studied by several authors. The following were found essential: \( \text{Mg}^{++} \) (Robinson, 1932; Burton et al., 1948; Frank and DeMoss, 1959; Robinson, 1932), \( \text{SO}_4^{=} \) (Burton et al., 1948; Frank and DeMoss, 1959), \( \text{K}^{+} \) (Burton et al., 1948; Takeda et al., 1959), \( \text{Fe}^{++} \) (Burton et al., 1948; Kurachi, 1958), \( \text{HCO}_3^{-} \) was found to enhance the yield (Heßlinger, 1951). According to the graph presented by Frank and DeMoss (1959), \( \text{Mg}^{++}, \text{PO}_4^{=} \) and \( \text{SO}_4^{=} \) were not essential, but enhance pigment formation. The requirement of phosphate for maximum pyocyanine production was found to be much lower for shaken cultures than stationary cultures (MacDonald, 1966).

However, there are reports that pyocyanine is produced on agar (Goris and Liot, 1921; Liu, 1952).

The pH necessary for pyocyanine production lies between 7.4–8.4 (Kurachi, 1958a). Young (1947) reported that sufficient glucose to establish and maintain an acid
reaction will prevent pigmentation. Higher concentrations of carbon sources delayed or decreased pyocyanine formation (Kurachi, 1958a).

The optimum temperature for pyocyanine production was reported as 37°C. (Seelen et al., 1943; Kurachi, 1958a).

**Inhibitors of Pyocyanine Production**

Some enzyme inhibitors, antibiotics and chemicals were tested for their effects on pyocyanine production by *P. aeruginosa*. The following were reported to be inhibitory: KCN (Grosswicz et al., 1957; Frank and DeMoss, 1959; Park et al., 1963), NaN₃ (Grosswicz et al., 1957; Frank and DeMoss, 1959), Na₂HAsO₄ (Frank and DeMoss, 1959), chloramphenicol (Fugita, 1954; Scheneierson et al., 1960; Grosswicz et al., 1957; Frank and DeMoss, 1959), streptomycin (Kurachi, 1959), erythromycin (Schneierson et al., 1960; Park et al., 1963), oleandomycin (Schneierson et al., 1960), neomycin (Park et al., 1963), and polyphenols (Kurachi, 1959c). Some compounds were reported to stimulate pyocyanine production: streptomycin and kanamycin (Park et al., 1963), bacitracin, neomycin, nitrofurantoin, and sulfadiazine (Schneierson et al., 1960).

**Isolation of Pyocyanine**

Pyocyanine is easily extractable into chloroform from aqueous alkaline solution. Gessard (1890) improved the method of isolation by an aqueous acid extraction of the chloroform solution, thus obtaining a red aqueous
solution of the salt. Wrede and Strack (1928) purified pyocyanine as picrate. Blackwood and Neish (1957) purified pyocyanine by adsorption chromatography in an alumina column. MacDonald (1963) removed a green pigment associated with pyocyanine by ion-exchange resin. Pyocyanine was recently purified as a platinum salt (Vining, 1965).

Biosynthetic Studies

Although, nutritional studies have not succeeded in finding "an obligate intermediate" (Davis, 1955), various nutritional studies and radioactive tracer experiments, either by direct incorporation or by isotopic competition, revealed that the following substances are precursors (as defined by Davis, 1955), of pyocyanine: DL-alanine (Frank and DeMoss, 1958; MacDonald, 1963), DL-valine (Burton et al., 1948), glutamic acid (Grosswicz et al., 1957; Frank and DeMoss, 1958), γ-aminobutyric acid (Grosswicz et al., 1957; Frank and DeMoss, 1958), pyrrolidine carboxylic acid (Grosswicz et al., 1957), glutamine (Grosswicz et al., 1957; Frank and DeMoss, 1958), aspartic acid (Grosswicz et al., 1957), proline (Grosswicz et al., 1957; Frank and DeMoss, 1958), hydroxyproline (Grosswicz et al., 1957), histidine (Grosswicz et al., 1957), leucine (Blackwood and Neish, 1957; Arora, 1964), isoleucine (Blackwood and Neish, 1957; Frank and DeMoss, 1958), anthranilic acid (Kurachi, 1959c, 1959d; Millican, 1962; Arora, 1964; Hollstein et al., 1966), glycerol (Gessard, 1890; Jelinsk
and Hof, 1938; Blackwood and Neish, 1957; Frank and De-Moss, 1957; Macdonald, 1963; Kurachi, 1958a), dihydroxy-acetone (Blackwood and Neish, 1957), glyceric acid (Macdonald, 1963), succinic acid (Grosswicz et al., 1957; Valette et al., 1964), citric acid (Seller et al., 1962), fumaric acid (Grosswicz et al., 1957; Valette et al., 1964), pyruvate (Grosswicz et al., 1957), glucose (Goris and Liot, 1922; Young, 1947; Macdonald, 1963; Kurachi, 1958a), fructose (Goris and Liot, 1922; Macdonald, 1963; Kurachi, 1958a), mannitol (Goris and Liot, 1922; Macdonald, 1963), shikimic acid (Millican, 1962; Macdonald, 1963), ribose (Macdonald, 1963), quinic acid (Macdonald, 1963), 3-ketogluconate (Macdonald, 1963), N-acetylglucosamine (Macdonald, 1963), kynurenine (Hollstein et al., 1966), 3-hydroxy-kynurenine (Hollstein et al., 1966).

A possible synthetic pathway for pyocyanine production was proposed first by Blackwood and Neish (1957), and advanced further by Ingram and Blackwood (1962). The proposal is based on the facts that glycerol is incorporated more readily compared to other radioactive substrates, including amino-acids with preformed rings, into pyocyanine, and from the results of degradation of pyocyanine where 7 out of 13 carbons were assayed. The theory is that pyocyanine is formed from glycerol or from a product closely related to it by the condensation of either two carbon units or by the condensation of four and two carbon units. The shikimic acid pathway was
suggested by Millican (1962) from the finding that during the period of pyocyanine biosynthesis, the ratio of specific activity of pyocyanine to shikimic acid remained constant. Degradation of pyocyanine, biosynthesized by feeding shikimic acid-U-C\textsuperscript{14}, showed that the N-methyl group was not labeled and was further evidence. From the ratio of carbon atoms entering 1-hydroxy-phenazine from carbon 1 plus 3 of glycerol as compared to carbon 2 of glycerol, Macdonald (1963) suggested that shikimic acid or a metabolite similar to shikimic acid is an intermediate. Anthranilic acid or its methylated compound was suggested by Kurachi (1959c, 1959d) as an intermediate in biogenesis of pyocyanine. Anthranilic is a normal metabolite of \textit{P. aeruginosa} and overcame the inhibition of pigment production by the addition of polyphenol. Takeda et al. (1959c) showed that anthranilic acid accumulated in the earlier stages of fermentation and decreased with formation of phenazines. Radioactive experiments of Millican (1962), and Hollstein \textit{et al.} (1966), showed that ring-labeled anthranilic acid was incorporated into the pyocyanine, but the per cent incorporation was low.

Kurachi (1959a) showed that methionine, choline and betaine could act as methyl donors for pyocyanine formation. Sheikh and MacDonald (1964) demonstrated the incorporation of methyl-C\textsuperscript{14}-methionine into N-methyl group of pyocyanine.
Kurachi (1959a) reported that a degradation product from pyocyanine by Aspergillus oryzae, A. niger and Penicillium spp. made an apyocyanogenic mutant of P. aeruginosa pyocyanogenic. The intermediate of pyocyanine was also claimed to accumulate in the presence of streptomycin, Ag⁺, Hg⁺, H₂O₂, and HCHO, however, the nature of the compound was not determined (Kurachi, 1959b). Carbobenzyloxyamino acids were shown to make apyocyanogenic mutants to produce pyocyanine (Azuma et al., 1964).

**PHENAZINE-α-Carboxylic Acid**

*Pseudomonas aureofaciens* produces a yellow pigment, phenazine-α-carboxylic acid. The organism was isolated from a sample of clay treated with kerosene and the pigment isolated and identified by Kluyver (1956). The organism was isolated also from kerosene treated soil by Haynes et al. (1956), and from a "barren ring" by Toohey et al. (1965).

*P. aeruginosa* was reported to produce phenazine-α-carboxylic acid by Takeda (1958b), and by Korth (1962). Isono et al. (1958) reported that *Streptomyces misakiensis* produces this pigment.

**Media for Phenazine-α-carboxylic acid Production**

Complex media containing peptone or yeast extract, plus glycerol and salts for production of phenazine-α-carboxylic acid by *P. aureofaciens* were reported (Kluyver, 1956; Haynes et al., 1956; Toohey et al., 1965). *P. aeruginosa* produces phenazine-α-carboxylic acid in a bouillon
medium (Takeda, 1958b), in synthetic media consisting of glycerol and urea (Takeda et al., 1959c) or glucose and ammonium sulfate (Korth, 1962). Isono et al. (1958) used a complex medium containing starch and dried yeast.

**Inhibition of Phenazine-β-carboxylic Acid Production**

Biogenesis of phenazine-β-carboxylic acid by _P. aureofaciens_ was shown to be inhibited by o-anisidine, aniline, N-formylaniline, acetaldehyde, 2-phenylenediamine, 2,3-dihydroxy-quinoline, pyrogallol, o-amino-phenol, anthranilic acid and phosphate ion by Levitch et al. (1964).

Production of phenazine-β-carboxylic acid by _P. aeruginosa_ was inhibited by DL-alanine, β-alanine, L-glutamic acid, L-aspartic acid, anthranilic acid and Ca++ (Takeda et al., 1959c).

**Isolation of Phenazine-β-carboxylic Acid**

Phenazine-β-carboxylic acid is extractable into many organic solvents from acidified broth. Crude acid was further purified by column-chromatography on _Al_2_O_3_ (Kluyver, 1956; Isono et al., 1958), by paper-chromatography (Toohey et al., 1965), by bubbling through CO_2_ (Haynes et al., 1958), by repeated recrystallization (Levitch et al., 1964), or by vacuum sublimation (Arora, 1965).

**Biosynthetic Studies**

Takeda et al. (1959c) reported that L-cysteine, DL-tryptophan and DL-tyrosine stimulated phenazine-β-carboxylic acid production. Levitch et al. (1964) reported that
glycerol was the preferred carbon source and a large number of amino acids could serve equally well as the nitrogen source. Levitch et al. also showed that glycerol-1,3-\(^{14}\)C and glycerol-2-\(^{14}\)C produced the highest specific radioactive pigment with shikimic acid-\(^{14}\)C giving lower specific activities. Anthranilic-(ring)-\(^{14}\)C was shown to be incorporated into the pigment (Arora, 1965; Hollstein et al., 1966). Ring-labeled kynurenine sulfate and 3-hydroxy-kynurenine were also incorporated into phenazine-1-carboxylic acid (Hollstein et al., 1966). By competition experiments, unlabeled shikimic acid was shown to dilute the incorporation of glycerol-1,3-\(^{14}\)C (Levitch et al., 1964; Arora, 1965).

Of the possible synthetic pathways of phenazine-\(^{14}\)C-carboxylic acid, the shikimic acid pathway was suggested for biogenesis of one or both carboxylic rings (Levitch et al., 1964; Arora, 1965). Hollstein et al. (1966) concluded that oxidative coupling of anilines and phenolic anilines was the mechanism of the formation of all pigments.

**OXYCHLORORAPHINE**

*Pseudomonas chlororaphis* was isolated from worms by Guignard et al. (1894), from spring and well-water by Lasseur (1911). The organism produces a green or yellow crystals in broth. The yellowish pigment, oxychlororaphine was characterized by Kögl et al. (1930) to be phenazine-1-carboxamide. The greenish crystals were shown to be a molecular compound of phenazine-1-carboxamide and its
dihydroderivative in the ratio of 3 to 1 (Dufraisse et al., 1952).

*P. aeruginosa* was reported to produce chlororaphine and oxychlororaphine (Birkofer, 1947; Birkofer et al., 1948; Sierra et al., 1958; Takeda, 1958b).

**Media for Oxychlororaphine Production**

Both synthetic medium and complex medium were used to produce oxychlororaphine with *P. chlororaphis*. The synthetic medium (Lasseur et al., 1911) contained glycerol and asparagin. Haynes et al.'s medium (1956) containing peptone and glycerol was used by Carter and Richards (1961) and Arora (1965).

*P. aeruginosa* can produce oxychlororaphine in Lasseur et al.'s medium (Birkofer, 1947), in a medium containing glycerol and urea (Takeda et al., 1959c), in a complex medium containing bouillon (Takeda et al., 1958). Sierra et al. (1958) grew *P. aeruginosa* in a medium containing glycerol, but the composition was not given.

**Isolation of the Pigment**

Chlororaphine is separable by filtration (Kögl et al., 1930; Arora, 1965) by centrifugation (Sierra et al., 1958), or by ether extraction (Takeda, 1958b; Carter and Richards, 1961). Chlororaphine is separable from oxychlororaphine by crystallization from acetone in an oxygen-free atmosphere, but in the presence of air, chlororaphine is entirely oxidized to phenazine-1-carboxamide (Kögl et al.,
1930). Oxychlororaphine was purified by column-chromatography on alumina (Carter and Richards, 1961; Arora, 1965), by sublimation (KögI et al., 1930; Arora, 1965), or by repeated recrystallization (Sierra et al., 1958).

**Biosynthetic Studies**

Phenazine-α-carboxylic acid, picolinic acid, pyöcatechol, and pyocathecol-3-carboxylic acid was shown to stimulate oxychlororaphine production by *P. chlororaphis* (KögI et al., 1932).

DL-γ-aminobutyric acid and L-aspartic acid stimulated the oxychlororaphine production by *P. aeruginosa* (Takeda et al., 1959c).

In radioactive incorporation experiments, Carter and Richards (1961) showed that anthranilic acid-carboxyl-C\(^{14}\), sodium carbonate-C\(^{14}\), alanine-1-C\(^{14}\), alanine-2-C\(^{14}\) was incorporated. Arora (1965) showed that the incorporation of radioactivity from labeled substrates into the pigment in the order of decreasing efficiency was as follows: glycerol-2-C\(^{14}\), glycerol-1,3-C\(^{14}\), C\(^{14}\)-ring-labeled anthranilic acid, glycerol-2-\(\text{H}^{3}\). A competition experiment showed that unlabeled shikimic acid did dilute the incorporation of glycerol-1,3-C\(^{14}\) into the pigment, but the extent of dilution was very small (Arora, 1965).

A possible pathway of biosynthesis of oxychlororaphine was suggested by Carter and Richards (1961), involving shikimic acid and anthranilic acid. The suggestion was based on the fact that anthranilic acid-carboxyl-C\(^{14}\)
labeled mainly the carboxyl group of the pigment, and that sodium carbonate-$^{14}$C labeled mainly the ring of the pigment. However, Arora (1965) showed that the ratio of incorporation of glycerol-1,3-$^{14}$C to that of glycerol-2-$^{14}$C differed from the ratio expected, if the shikimic acid pathway was involved. Arora (1965) also found the radioactivity of oxychlororaphine labeled from $^{14}$C-ring-labeled anthranilic acid was found mainly in carboxamide group, and concluded that anthranilic acid was not incorporated as an intact molecule.
MATERIALS AND METHODS

ORGANISMS

Pseudomonas aeruginosa Mac 436 was obtained from Dr. N. Grosswicz, Department of Bacteriology, The Hebrew University, Jerusalem, Israel. P. aureofaciens, Mac 371 was received from Dr. W. C. Haynes, Northern Utilization Research Branch, U.S. Department of Agriculture, Peoria, Illinois, U.S.A., numbered NRRL-1543-C. Also used was P. chlororaphis Mac 370, ATCC 9447.

MEDIA

P. aeruginosa was maintained on nutrient agar (Difco), and P. chlororaphis and P. aureofaciens on trypti-case-soy-agar (BBL).

Organisms were grown on nutrient broth (Difco), trypticaso-soy-broth (BBL), or on a medium containing 5% DL-alanine, 0.5% glycerol and Vogel et al.'s salts (1955) which consisted of 0.02% MgSO4·7H2O, 0.2% citric acid monohydrate, 1% K2HPO4 and 0.35% NaNH4HPO4·4H2O.

Three inorganic salts combinations, Ingram and Blackwood's (1962), Kurachi's (1959d), and Frank and DeMoss' (1959) were compared in studies of the requirements for inorganic salts. The composition of salts is as follows: Ingram and Blackwood's (1962) - 0.01% K2HPO4, 0.2% MgSO4, 0.001% FeSO4·7H2O, 0.1% CaCO3, pH7.4; Kurachi's (1959) -
0.05% MgSO₄·7H₂O, 0.05% K₂HPO₄, 0.0005% Fe₂(SO₄)₃, PH7.4; Frank and DeMoss' (1959) - 0.0139% K₂HPO₄, 0.406% MgCl₂, 6H₂O, 1.42% Na₂SO₄, 0.01% ferric citrate, PH7.0-7.2. 

For radioactive experiments with a single substrate, the medium contained 0.41% MgCl₂·6H₂O, 0.71% Na₂SO₄, 0.01% K₂HPO₄, 0.0005% FeSO₄·7H₂O, 0.2% urea plus 0.2% substrate. The pH was adjusted to 7.0-7.2.

In studies on phenazine-α-carboxylic acid, oxychlororaphine and pyocyanine production by *P. aeruginosa* with labeled substrates, Frank and DeMoss' medium (1959) was used.

**GROWTH CONDITIONS**

For pyocyanine production, incubation on rotary shaker at 30° or 37°C. was employed. When phenazine-1-carboxylic acid, oxychlororaphine and pyocyanine were produced together by *P. aeruginosa*, 30°C. incubation on the rotary shaker was necessary.

Inoculum was prepared from cells harvested from a 24-hour culture in nutrient broth or other media incubated at 30°C. on rotary shaker, and used directly or used after preparing a cell suspension. Washed cell suspensions were prepared by washing cells three times with 0.9% saline and resuspending in 10 ml. of saline per litre of medium. Direct inoculation from the 24-hour culture on nutrient agar incubated at 30°C. was also employed where specified.

For preparation of cell-free extracts washed cells were suspended in cold 0.1 M tris-buffer at pH 7.4, and
were sonicated for intervals of five minutes using a MSE ultrasonic disintegrator. The sonicate was centrifuged at 9,000 g for 15 minutes to remove cells and cell debris. For studies where all living cells had to be removed, the supernatant from the centrifugation was filtered through a Millipore filter (GSWP 02500).

When radioactive experiments were conducted, air was passed first through a scrubber to remove CO₂ before passing the air through the fermentation flask containing the incubation medium. The CO₂ produced in the fermentation was scrubbed from the effluent air using the train described by Neish (1952).

RADIOISOTOPES AND CHEMICALS

Glycerol-1,3-C¹⁴, glycerol-2-C¹⁴, acetate-1-C¹⁴, acetate-2-C¹⁴, urea-C¹⁴, D-glucose-1-C¹⁴, D-glucose-2-C¹⁴ and D-glucose-6-C¹⁴ were obtained from the Radiochemical Center. D-glucose-U-C¹⁴, succinic acid-1,4-C¹⁴, and succinic acid-2,3-C¹⁴ were the product of the Merck Radiochemicals. Shikimic acid-1,6-C¹⁴ was supplied by the Schwarz Bio-research Inc.

Carrier pyocyanine was prepared according to McIlwain (1937). Radioactive phenazine-β-carboxylic acid was biosynthesized by growing P. aeruginosa in a defined medium, containing DL-alanine and glycerol, to which radioactive glucose-U-C¹⁴ was added. Phenazine-β-amine was synthesized according to Albert et al. (1947) or by a modified Hofmann's reaction (Wallis and Lane, 1946). The
additional carrier phenazine-α-amine was a generous gift from Dr. A. Taurins, Department of Chemistry, McGill University. Carrier phenazine-α-carboxylic acid was biosynthesized according to Haynes et al. (1956), but the accompanying pigment was hard to remove (see Appendix I), and carrier was obtained also through the acid hydrolysis of oxychlororaphine biosynthesized by *P. chlororaphis* in medium containing trypticase-soy-broth (BBL) plus 2% glycerol incubated at 25°C on the shaker. Carrier quinoxaline dicarboxylic acid was prepared according to Chattaway and Humphrey (1929). Pyrazine-tetra-carboxylic acid dipotassium salt was prepared by Dr. J. Ingram (Ingram and Blackwood, 1962). Other chemicals were obtained from commercial sources.

**ISOLATION OF PRODUCTS**

Pyocyanine, phenazine-1-carboxylic acid, and oxychlororaphine were separated by the method of Takeda et al. (1958b). The portion containing pyocyanine was extracted and column chromatographed according to Blackwood and Neish (1957), and purified further by the ion-exchange chromatography as described by Macdonald (1963).

Phenazine-1-carboxylic acid was purified by thin-layer chromatography on silica-gel G twice, developing first with CHCl₃:MeOH (9:1) followed by acetone. The crude acid was recrystallized from methanol.
Oxychlororaphine was sublimed under vacuum twice before thin-layer chromatographic separation. Crude oxychlororaphine was recrystallized from an acetone:H₂O mixture.

When the quantities of phenazine-1-carboxylic acid and oxychlororaphine were large, they were separated on Al₂O₃ column. Oxychlororaphine was eluted first with CHCl₃:MeOH (9:1), and the phenazine-1-carboxylic acid remaining was collected by elution with ammoniated methanol (MeOH:NH₄OH, 100:1). The separation scheme is summarized in Figure 1.

QUANTITATIVE ASSAYS

Pyocyanine

An aliquot of fermentation liquor was acidified and centrifuged. The supernatant was made alkaline by adding appropriate amounts of 5 M borate buffer (pH 10), and extracted twice with 10 ml. of chloroform. The chloroform extract was back-extracted with 10 ml. of 0.2N HCl twice and made to 25 ml. by the addition of 0.2N HCl. Pyocyanine was determined by reading %T at 520 μM using a Coleman spectrophotometer, model 6A, with 0.2N HCl as blank. A standard curve is given in Figure 2.

Phenazine-α-carboxylic Acid

Twenty ml. of fermentation liquor was withdrawn, acidified to pH 2 with 4N HCl and ether extracted in a continuous extractor overnight. The ether fraction was
Figure 1. Scheme for separation and purification of three phenazine pigments.

1. Broth
   - Partition Chromatography (Al₂O₃, CHCl₃:MeOH (9:1))
   - 4N HCl to pH 2
   - CHCl₃ or R₂O ext'n
   - H₂O layer
   - NaHCO₃
   - CHCl₃ or R₂O layer
   - 5% NaHCO₃
   - CHCl₃ or R₂O
   - 4N HCl to pH
   - CHCl₃ or R₂O
   - H₂O layer
   - NaHCO₃
   - CHCl₃ or R₂O
   - CHCl₃ ext'n
   - CHCl₃ layer
   - 0.2N HCl
   - 0.2N HCl layer
   - CHCl₃ layer
   - H₂O layer
   - NaHCO₃
   - CHCl₃ or R₂O
   - CHCl₃ ext'n
   - CHCl₃ layer
   - 0.2N HCl
   - CHCl₃ layer
   - 0.2N HCl
   - CHCl₃ layer
   - Ion exchange chromatography (Dowex 50, 4N HCl)
   - T.L.C. (silica gel G, CHCl₃:MeOH (9:1))
   - Cut & elute corresponding band
   - T.L.C. (silica gel G, acetone)
   - *Cut & elute corresponding band
   - Phenazine-α-carboxylic acid
   - Oxychlororaphine
   - Pyocyanine

2. Amide
   - MeOH:NH₄OH (100:1)
   - Acid
   - evaporate to dryness
   - 1N HCl
   - CHCl₃ ext'n
   - CHCl₃*
   - evaporate to dryness
   - sublimation (170-180°C. vac.)
   - T.L.C. (silica gel G, CHCl₃:MeOH (9:1))
   - Cut & elute corresponding band
   - Cut & elute corresponding band

Pyocyanine

Figure 1. Scheme for separation and purification of three phenazine pigments.
Figure 2  Standard curve for colorimetric determination of pyocyanine, 520 µg, in 0.2 N HCl.
extracted with 1N NaOH. Oxychlororaphine remained in the ether fraction. The alkaline fraction containing phenazine-1-carboxylic acid was acidified to pH2 with 4N HCl and further treated by extraction with chloroform. The chloroform extract was dried over Na₂SO₄; after removal of the Na₂SO₄, the filtrate was evaporated to dryness. The residue was thin-layer chromatographed on silica-gel G developed with CHCl₃:MeOH (9:1). The band corresponding to phenazine-1-carboxylic acid was eluted with methanol containing 1% conc. NH₄OH. The methanol extract was used directly for an estimation of phenazine-1-carboxylic acid by measuring O.D. at 252 mμ with a Zeiss PMQ II spectrophotometer. The crystalline phenazine-1-carboxylic acid was assayed in chloroform at 252 mμ. Standard curves were recorded in Figure 3 and Figure 4.

Oxychlororaphine

The fraction containing the amide described above in the separation of phenazine-1-carboxylic acid was dried over Na₂SO₄. After drying, the Na₂SO₄ was removed by filtration, and the filtrate evaporated to dryness on a steam bath. The residue was thin-layer chromatographed on silica-gel G, using CHCl₃:MeOH (9:1) as the solvent. The band corresponding to oxychlororaphine was removed and eluted with CHCl₃. The amount of oxychlororaphine was determined by measuring O.D. at 250 mμ with a Zeiss PMQ II spectrophotometer. A standard curve is given in Figure 5.
Figure 3 Standard curve for colorimetric determination of phenazine-1-carboxylic acid, 252 μg, in methanol containing 1% conc. NH₄OH.
Figure 4 Standard curve for determination of phenazine-1-carboxylic acid, 252 μg, in chloroform.
Figure 55 Standard curve for determination of oxychlororaphine, 250 µg, in chloroform.
**α-Amino-phenazine**

α-Amino-phenazine prepared as a sublimate was dissolved in CHCl₃, and assayed by measuring O.D. at 285 μm with a Zeiss PMQ II spectrophotometer. A standard curve is given in Figure 6.

**Phenazine-1-ol**

Phenazinol prepared as the sublimate was dissolved in CHCl₃, and assayed by measuring O.D. at 265 μm with a Zeiss PMQ II spectrophotometer. A standard curve is given in Figure 7.

**Quinoxaline dicarboxylic acid**

Quinoxaline dicarboxylic acid was dissolved in 0.2N Na₂CO₃, and assayed by measuring O.D. at 327 μm, using a Zeiss PMQ II spectrophotometer. A standard curve is given in Figure 8.

**Pyrazine-tetra-carboxylic acid**

Pyrazine-tetra-carboxylic acid was dissolved in 1N HCl, and the quantity of the acid was determined by measuring O.D. at 282 μm with a Zeiss PMQ II spectrophotometer. A standard curve is given in Figure 9.

**Quinoxaline**

Quinoxaline was converted to the mercuric chloride salt, and purified by sublimation under vacuum. The sublimate was dissolved in 1N HCl, and the amount of the acid was determined by measuring O.D. at 325 μm with a Zeiss
Figure 6 Standard curve for colorimetric determination of 1-aminophenazine, 285 μμ, in chloroform.
Figure 7 Standard curve for colorimetric determination of hemipyocyanine, 265 mp, in chloroform.
Figure 8 Standard curve for determination of quinoxalinedicarboxylic acid, 327 µg, in 0.2N Na₂CO₃.
Figure 9 Standard curve for colorimetric determination of pyrazine tetracarboxylic acid dipotassium salt, 282 μA, in 1N HCl.
Figure 10 Standard curve for determination of quinoxaline, 325 μg, in 1N HCl.
PMQ II spectrophotometer. A standard curve is given in Figure 10.

DEGRADATION

Pyocyanine was degraded to hemipyocyanine by the method of Macdonald (1963). 1-Hydroxyphenazine was oxidized to quinoxaaline-dicarboxylic acid and pyrazine-tetra-carboxylic acid according to the method of Ingram and Blackwood (1962). The separation of quinoxaaline-dicarboxylic acid and pyrazine-tetra-carboxylic acid was carried out on Dowex-50Wx4 (H⁺) as described by Vining (1965). Quinoxaaline-dicarboxylic acid was decarboxylated to quinoxaaline according to the method of Ingram and Blackwood (1962).

Oxychlororaphine was degraded to 1-amino-phenazine by a modified Hofmann's reaction described by Wallis and Lane (1946). α-Amino-phenazine was oxidized directly with KMnO₄ to quinoxaaline dicarboxylic acid and pyrazine-tetra-carboxylic acid according to the method of Ingram and Blackwood (1962).

Phenazine-α-carboxylic acid was converted first to the acid chloride and then to the amide according to the procedure of Kögl. and Postowsky (1930). The resulting product, oxychlororaphine, was treated as described above.

DETERMINATION OF RADIOACTIVITY

All samples were dissolved in appropriate solvents,
and aliquots were removed and dried on a small square of absorbent tissue (Kleenex) with the aid of an infrared lamp and hair dryer. The dried samples were combusted and the resulting CO₂ absorbed by 5 ml. each of 1N Hyamine hydroxide (Packard) and of ethanol according to Arora's modification (1965) of Kelly et al.'s procedure (1961). An aliquot of the Hyamine hydroxide was added to 15 ml. of phosphor containing 4.0 gm. 2,5-diphenyloxazole (PPO) and 300 mgm. 1,4-bis-2-(5-phenyloxazolyl) 2-benzene (POPOP) per liter of scintillation grade toluene (Matheson, Coleman and Bell).

Counts were made in a Packard Tricarb liquid scintillator model 314EX with a preset time of 10 minutes and preset count of 10,000. The instrument was at the predetermined balancing point. Counting was repeated ten times, and the data were treated statistically as outlined in Nuclear Chicago Bulletin No. 14, using a program prepared for the IBM 1620 (see Appendix II).
Figure 11. Pyocyanine production by *P. aeruginosa* Mac 436

Fermentation conditions. 200 ml. of medium (Frank and DeMoss, 1959) in a 500 ml. Erlenmeyer flask was inoculated with 1.0 ml. of culture grown on nutrient broth for 24 hrs. Incubation was at 37°C. on a rotary shaker. 10 ml. samples were withdrawn at the times specified and centrifuged and washed three times with 0.9% saline and resuspended in 10 ml. saline and the turbidity recorded at 470 mμ. The amount of pyocyanine was estimated on 1 ml. samples.
It

pH

7.6

0.0

Z-O

M'lm 1.

0.0

1.0

400

PYOCYANINE

200

100

7 day
Figure 12. Effect of inoculum on pyocyanine production

Fermentation conditions. 200 ml. of medium (Frank and DeMoss, 1959) in a 500 ml. Erlenmeyer flask was inoculated directly from 24 hr. culture on nutrient agar at 30°C. then incubated at 37°C. on a rotary shaker. Samples were withdrawn as indicated, and O.D. and pyocyanine concentration estimated.

x _______ x indicates inoculation directly from a nutrient agar slant incubated at 30°C. for 24 hrs.

x .......... x indicates inoculation with 10 ml. of a 24 hr. culture in Frank and DeMoss' medium incubated at 37°C. on a rotary shaker.
Figure 13. Effect of amount of inoculum on pyocyanine production

Fermentation conditions. 500 ml. of medium (Frank and DeMoss, 1959) in a 2L Erlenmeyer flask was inoculated with a washed cell suspension harvested from 24 hr. culture grown in nutrient broth incubated at 30°C, on a rotary shaker.

\[
\begin{align*}
\times \quad x \quad & \text{10 ml. of washed cell suspension added as inoculum.} \\
\times \quad \ldots \ldots \quad x \quad & \text{5 ml. of washed cell suspension added as inoculum.}
\end{align*}
\]
Pyocyanine
Fermentation conditions. 500 ml. Erlenmeyer flask containing 100 ml. of modified Kurachi's medium (1959) with the phosphate level reduced to 0.01%, was inoculated and incubated at 30°C. on a rotary shaker. Inoculum was prepared by harvesting cells grown in nutrient broth at 30°C. on a rotary shaker at the times specified and 1 ml. of a washed cell suspension was added per flask.

- x ______ x 24 hr. inoculum.
- . ____ . 48 hr. inoculum.
- o ____ o 3-day inoculum.
- △ ______ △ 4-day inoculum.
Figure 15. Effect of phosphate level on pyocyanine production

Fermentation conditions. 500 ml. Erlenmeyer flask containing 100 ml. of Kurachi's medium (1959a) with the concentration of phosphate varied were inoculated as follows:

A. One ml. of a washed cell suspension harvested from a 24 hr. culture grown in nutrient broth at 30°C. on a rotary shaker was added per flask.

B. Flasks were inoculated directly with 1 ml. of a 24 hr. culture grown at 30°C. on a rotary shaker in nutrient broth.

x -------- x  Concentration of K_2HPO_4  0.005%
.-.-.-.-.-.  Concentration of K_2HPO_4  0.010%
@ -------- @  Concentration of K_2HPO_4  0.015%
△ -------- △  Concentration of K_2HPO_4  0.050%
TABLE III

Effect of inorganic ions on pyocyanine production

<table>
<thead>
<tr>
<th>D-quinic acid</th>
<th>Na₂SO₄</th>
<th>MgCl₂</th>
<th>Na₂HPO₄</th>
<th>KCl</th>
<th>Yield of pyocyanine µg/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M 0.5 ml.</td>
<td>0.5 ml.</td>
<td>0.1 ml.</td>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0 µg/ml.</td>
</tr>
<tr>
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<td>0 ml.</td>
<td>0 ml.</td>
<td>0 ml.</td>
<td>0 ml.</td>
<td>10 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0 ml.</td>
<td>0 ml.</td>
<td>0 ml.</td>
<td>8 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0.1 ml.</td>
<td>0 ml.</td>
<td>0 ml.</td>
<td>7.5 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0.1 ml.</td>
<td>0.2 ml.</td>
<td>0 ml.</td>
<td>8.5 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0.1 ml.</td>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>6 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0 ml.</td>
<td>0.1 ml.</td>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>5 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0 ml.</td>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>6 µg/ml.</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>0.5 ml.</td>
<td>0.1 ml.</td>
<td>0 ml.</td>
<td>0.5 ml.</td>
<td>7.5 µg/ml.</td>
</tr>
</tbody>
</table>

Fermentation conditions. The medium contained 1.0 ml. of 1M NH₄Cl and other constituents as specified in the protocol with a final volume of 5 ml. after inoculating with 1 ml. of washed cell suspension. Incubation was at 37°C. on a rotary shaker for 24 hrs., a washed cell suspension was used as inoculum and was prepared from cells grown in a medium containing 0.5% Dl-alanine, 0.5% glycerol plus Vogel et al.'s salts (1956) for 24 hrs. at 30°C. on a rotary shaker.
TABLE IV

Effect of concentration of inorganic ions on pyocyanine production

<table>
<thead>
<tr>
<th>D-quinic acid</th>
<th>Na₂SO₄</th>
<th>MgCl₂</th>
<th>Na₂HPO₄</th>
<th>Yield mg/ml.</th>
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Fermentation conditions. As specified in Table III.
### TABLE V

Pyocyanine production on various media

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<tr>
<th>Substrate</th>
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<th>Cells grown in nutrient broth</th>
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<td>*</td>
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<td>*</td>
<td>*</td>
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<td>*</td>
<td>*</td>
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<td></td>
<td>4</td>
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TABLE V (continued)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time</th>
<th>CaCO3</th>
<th>Cells grown in nutrient broth</th>
<th>Cells grown in trypticase-soy-broth</th>
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</thead>
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<tr>
<td></td>
<td>days</td>
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<td>Composition of salts</td>
<td>Composition of salts</td>
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<td></td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>212.5</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>200</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Final pH</td>
<td>+</td>
<td>8.8</td>
<td>8.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>8.3</td>
<td>8.4</td>
<td>8.0</td>
</tr>
</tbody>
</table>
TABLE V (continued)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (days)</th>
<th>CaCO₃</th>
<th>Composition of salts</th>
<th>Composition of salts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>+ 1</td>
<td></td>
<td>I 2 3.8 3.8 5</td>
<td>I 3.8 5 5</td>
</tr>
<tr>
<td></td>
<td>- 1</td>
<td></td>
<td>II 53.8 7.5 2.5 *</td>
<td>II 5 5 * 5</td>
</tr>
<tr>
<td></td>
<td>+ 2</td>
<td></td>
<td>III 86.3 7.5 5</td>
<td>III 8.8 * 8.8</td>
</tr>
<tr>
<td></td>
<td>- 2</td>
<td></td>
<td>* 53 12.5 3.8 * 10 *</td>
<td>* 10</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>- 3</td>
<td></td>
<td>* 78.8 5 2.5 * 6.3</td>
<td>* 6.3</td>
</tr>
<tr>
<td></td>
<td>* 3</td>
<td></td>
<td>* 52.5 10 2.5 * 8.8</td>
<td>* 8.8</td>
</tr>
<tr>
<td></td>
<td>+ 4</td>
<td></td>
<td>* 77.5 5 3.8 * 6.3</td>
<td>* 6.3</td>
</tr>
<tr>
<td></td>
<td>- 4</td>
<td></td>
<td>* 50 8.8 2.5 * 5</td>
<td>* 5</td>
</tr>
<tr>
<td></td>
<td>* 5</td>
<td></td>
<td>* 81.3 7.5 3.8 1.25</td>
<td>* 1.25 6.3</td>
</tr>
<tr>
<td></td>
<td>* 5</td>
<td></td>
<td>* 48.8 8.8 1.3 1.25</td>
<td>* 2.5</td>
</tr>
<tr>
<td>Final pH</td>
<td>+</td>
<td>8.3</td>
<td>8.6 8.3 8.4 8.5</td>
<td>8.5 8.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>8.2</td>
<td>8.7 8.2 8.4 8.6</td>
<td>8.5 8.5</td>
</tr>
</tbody>
</table>

#Yield of pyocyanine was given in μg/ml.

Composition of salts

| I Ingram & Blackwood (1962) minus 0.1% CaCO₃ |
| II Kurachi (1959)                             |
| III Frank & DeMoss (1959)                     |

*No visible pyocyanine in supernatant of acidified broth

Fermentation conditions. 50 ml. Erlenmeyer flask containing 20 ml. of medium was inoculated with 1 ml. of washed cell suspension and incubated at 30°C. on rotary shaker. At time intervals, a 1 ml. sample was removed for pyocyanine assay.

The substrate concentration was 1%. For non-nitrogenous substrates (NH₄)₂SO₄ was added to final concentration of 0.2%. The level of CaCO₃ added was 0.1%.
### TABLE VI

**Effect of carbon source on pyocyanine production***

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation Time</th>
<th>Composition of salts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td><strong>I</strong></td>
</tr>
<tr>
<td>Glycerol</td>
<td>1</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>4.2</td>
</tr>
<tr>
<td>γ-NH₂-butyric acid</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.2</td>
</tr>
<tr>
<td>K-gluconate</td>
<td>1</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>3.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.0</td>
</tr>
<tr>
<td>ω-OH-ω-methyl-glutaric acid</td>
<td>1</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.2</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>1</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.0</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.3</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td>8.6</td>
</tr>
<tr>
<td>L-tryptophane</td>
<td>1</td>
<td>31.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37.5</td>
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<tr>
<td></td>
<td>Final pH</td>
<td>8.4</td>
</tr>
<tr>
<td>L-leucine</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
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</tr>
<tr>
<td>DL-alanine</td>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td></td>
<td>Final pH</td>
<td></td>
</tr>
<tr>
<td>ω-Keto-glutaric acid</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Final pH</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>Incubation Time</td>
<td>Composition of salts **I</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 22.5</td>
</tr>
<tr>
<td>Indole</td>
<td>1</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 6.9</td>
</tr>
<tr>
<td>Phenyl-alanine</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 52.5</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>1</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 6.0</td>
</tr>
<tr>
<td>Catechol</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 1.3</td>
</tr>
<tr>
<td>Na-acetate</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 18.8</td>
</tr>
<tr>
<td>O-amino-phenol</td>
<td>1</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Final pH 7.7</td>
</tr>
</tbody>
</table>

*Concentration of pyocyanine is recorded as μg/ml.

**Compositions of salts

I Ingram & Blackwood (1962) minus 0.1% CaCO₃
II Frank and DeMoss (1959)

***No visible pyocyanine was observed in supernatant of acidified broth, therefore no quantitative assay was made.

Fermentation conditions. 50 ml. Erlenmeyer flask containing 20 ml. of medium was inoculated with 1 ml. of washed cell suspension prepared from the cells grown in the medium containing 0.5% DL-alanine, 0.5% glycerol plus Vogel et al.'s salts (1956), and incubated at 30°C. on rotary shaker. At time intervals, 1 ml. of sample was withdrawn for pyocyanine assay. The substrate concentration for the following was 1%; glycerol, γ-amino-butyric acid, K-gluconate, glucose, β-hydroxy-β-methyl-glutaric acid. For other substrates, 0.5% was employed. For non-nitrogenous substrates, 0.2% (NH₄)₂SO₄ was added.
TABLE VII

Yields of pyocyanine from acetate, glycerol and glucose#

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>NaAC·3H₂O</th>
<th>Glycerol</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>42.5</td>
<td>158.8</td>
<td>83.8</td>
</tr>
<tr>
<td>3</td>
<td>37.5</td>
<td>156.3</td>
<td>76.3</td>
</tr>
<tr>
<td>4</td>
<td>36.5</td>
<td>150</td>
<td>76.3</td>
</tr>
<tr>
<td>5</td>
<td>33.8</td>
<td>140</td>
<td>75</td>
</tr>
</tbody>
</table>

#Yield in µg/ml.

*No visible pyocyanine

Fermentation conditions. 20 ml. medium in 50 ml. Erlenmeyer flask was inoculated with a drop of culture grown in nutrient broth at 30°C. on rotary shaker for 24 hrs., and incubated at 37°C. on shaker. 1 ml. of sample was withdrawn at the time specified and amount of pyocyanine assayed. The medium contained 0.41% MgCl₂·6H₂O; 0.71% Na₂SO₄; 0.0005% FeSO₄·7H₂O; 0.2% urea; 0.010% K₂HPO₄; 1% substrate. The pH was adjusted to 7.0-7.2.
TABLE VIII

Optimum substrate concentration of acetate and glucose for pyocyanine production*

<table>
<thead>
<tr>
<th>Substrate Conc'n.</th>
<th>NaAC.3H₂O</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5</td>
<td>72.5</td>
</tr>
<tr>
<td>2</td>
<td>160.0</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>147.5</td>
<td>132.5</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>62.5</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>67.5</td>
</tr>
</tbody>
</table>

*Yield in μg/ml.

Fermentation conditions. 20 ml. medium in 50 ml. Erlenmeyer flask was inoculated with a drop of culture grown in nutrient broth at 30°C. on rotary shaker for 24 hrs., and incubated at 37°C. on shaker. Fermentations were terminated after 48 hrs. and amount of pyocyanine assayed. The medium contained 0.41% MgCl₂.6H₂O; 0.71% Na₂SO₄; 0.0005% FeSO₄.7H₂O; 0.2% urea; 0.010% K₂HPO₄ and substrate in varying concentration. The pH of medium was adjusted to 7.0-7.2.
TABLE IX

Production of pyocyanine, phenazine-1-carboxylic acid and oxychlororaphine by *P. aeruginosa* Mac 436*

<table>
<thead>
<tr>
<th>Media</th>
<th>Phenazine -(\text{N})-carboxylic acid</th>
<th>Oxychlororaphine</th>
<th>Pyocyanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frank &amp; DeMoss (1959)</td>
<td>26.1</td>
<td>76.1</td>
<td>195</td>
</tr>
<tr>
<td>Ingram &amp; Blackwood (1962)</td>
<td>0.3</td>
<td>2.2</td>
<td>210</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>32.1</td>
<td>22.4</td>
<td>35</td>
</tr>
<tr>
<td><strong>Na acetate</strong></td>
<td>15.6</td>
<td>4.6</td>
<td>84</td>
</tr>
<tr>
<td><strong>Glycerol</strong></td>
<td>30.3</td>
<td>Trace</td>
<td>188</td>
</tr>
<tr>
<td>Sellens et al. (1962)</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Haynes et al. (1956)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas agar <em>p</em>**</td>
<td>0</td>
<td>0</td>
<td>74</td>
</tr>
</tbody>
</table>

*Yield in mgm/L; all phenazines were recorded as crystalline preparations.

**The medium contained 0.41% MgCl\(_2\).6H\(_2\)O; 0.71% Na\(_2\)SO\(_4\); 0.01% KH\(_2\)PO\(_4\); 0.2% urea plus 0.2% substrate mentioned. The pH was adjusted to 7.0-7.2.

***Pseudomonas agar *P* (Difco, no agar added).

Fermentation conditions. 500 ml. medium in a 2L Erlenmeyer flask was inoculated with 1 ml. of washed cell suspension prepared from cells grown in nutrient broth, and incubated at 30°C. on rotary shaker for 6 days. Volume of each test was 1 L.
Figure 16. A time study of the production of pyocyanine, phenazine-1-carboxylic acid and oxychlororaphine.

Fermentation conditions. 500 ml. of medium (Frank and De-Moss, 1959) in a 2L Erlenmeyer flask was inoculated with 5 ml. of washed cell suspension and incubated at 30°C. on a rotary shaker. The cells were harvested from 24 hr. culture of P. aeruginosa Mac 436 grown in nutrient broth at 30°C. on a rotary shaker. Samples were removed at the times specified and assayed.

-  
  -  
  x  

Pyocyanine
Phenazine-1-carboxylic acid
Oxychlororaphine
TABLE X

Percentage of radioactivity among fermentation products from a single carbon source

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Pyocyanine</th>
<th>CO₂</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol-1,3-C¹⁴</td>
<td>0.32</td>
<td>33.26</td>
<td>1.64</td>
</tr>
<tr>
<td>Glycerol-2-C¹⁴</td>
<td>-</td>
<td>16.35</td>
<td>1.64</td>
</tr>
<tr>
<td>Urea-C¹⁴</td>
<td>0.01</td>
<td>61.58</td>
<td>0.26</td>
</tr>
<tr>
<td>NaAC-1-C¹⁴</td>
<td>0.01</td>
<td>22.97</td>
<td>3.06</td>
</tr>
</tbody>
</table>

Fermentation conditions. To 500 ml. Erlenmeyer flask containing 50 ml. of two-fold strength modified Kurachi's medium was added a millipore-filtrate of 0.05 mc. of radioactive substrate in 50 ml. of water. The flask was inoculated with 1 ml. of 24 hr. culture of P. aeruginosa Mac 436 grown in nutrient broth at 30°C. on rotary shaker, and incubated at 37°C. on rotary shaker for 5 days.
### TABLE XI

**Pyocyanine formation from a single carbon source**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity, $\mu$Ci/mM</th>
<th>Pyocyanine Incorporation, %</th>
<th>Phenazine Incorporation, %</th>
<th>$x$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrates</td>
<td>Radio activity</td>
<td>Substrate</td>
<td>Radio-active carbon</td>
</tr>
<tr>
<td>Glycerol-1,3-C$^{14}$</td>
<td>1.825</td>
<td>7.388</td>
<td>4.114</td>
<td>0.32</td>
</tr>
<tr>
<td>Glycerol-2-C$^{14}$</td>
<td>1.825</td>
<td>* -</td>
<td>4.397</td>
<td>-</td>
</tr>
<tr>
<td><strong>Urea-C$^{14}$</strong></td>
<td>3.030</td>
<td>0.346</td>
<td>0.264</td>
<td>0.01</td>
</tr>
<tr>
<td>NaAC-1-C$^{14}$</td>
<td>4.545</td>
<td>2.419</td>
<td>0.206</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Sample for combustion was too small.*

$x$-Ratio = \( \frac{\text{Incorporation of carbon 1+3 of glycerol into phenazine}}{\text{Incorporation of carbon 2 of glycerol into phenazine}} \)

**Glycerol was used as carbon source.**

Sample calculation:

\[
\text{% Incorporation of glycerol-1,3-C}^{14}\text{ into pyocyanine} = \left( \frac{7.388}{1.825} \right) \times 100 = 405
\]

\[
\text{% Incorporation of carbon 1+3 of glycerol into pyocyanine} = \left( \frac{7.388}{1.825} \right) \times \frac{3}{2} \times 100 = 270
\]
TABLE XII

Incorporation of glycerol into phenazine ring via various metabolic pathways

![Diagram of metabolic pathways]

<table>
<thead>
<tr>
<th>Pathway</th>
<th>No. of carbon derived from*</th>
<th>Specific activities** from labelled glycerol</th>
<th>Ratio***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1+3</td>
<td>Gly-1,3-C(^{14})</td>
<td></td>
</tr>
<tr>
<td>A Shikimic acid</td>
<td>22/3 14/3</td>
<td>lla 14a</td>
<td>Incorporation</td>
</tr>
<tr>
<td>B Acetate</td>
<td>6 6</td>
<td>9a 18a</td>
<td>6/6 = 1</td>
</tr>
<tr>
<td>C Glycerol</td>
<td>8 4</td>
<td>12a 12a</td>
<td>8/4 = 2</td>
</tr>
</tbody>
</table>

* Number of carbon derived from carbon 1+3 of glycerol is equivalent to incorporation of carbon 1+3 of glycerol

** Specific activities of glycerol-1,3-C\(^{14}\) and glycerol-2-C\(^{14}\) are a \(\mu\)c/\(\mu\)M of glycerol

*** Ratio of incorporation =

\[
\frac{\text{Incorporation of carbon 1+3 of glycerol into phenazine}}{\text{Incorporation of carbon 2 of glycerol into phenazine}}
\]

Ratio of specific activity =

\[
\frac{\text{Specific activity of phenazine labelled by glycerol-1,3-C}^{14}}{\text{Specific activity of phenazine labelled by glycerol-2-C}^{14}}
\]

Assumption made on the pathways
A shikimic acid pathway: (According to MacDonald, 1963)
9 glycerol → phenazine nucleus + shikimic acid + fructose-6-phosphate
B acetate pathway: 6 glycerol → phenazine nucleus + 6CO\(_2\)
C glycerol pathway: 4 glycerol → phenazine nucleus
**TABLE XIII**

Percentage distribution of radioactivity from labeled substrates among fermentation products

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyocyanine</th>
<th>Oxycholoro-raphine</th>
<th>Phenazine-α-carboxylic acid</th>
<th>CO₂</th>
<th>Cells</th>
<th>Total activity added mc</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaAC-1-C</td>
<td>2.09x10⁻³</td>
<td>5.18x10⁻⁴</td>
<td>1.44x10⁻³</td>
<td>71.26</td>
<td>4.30</td>
<td>0.2</td>
</tr>
<tr>
<td>NaAC-2-C</td>
<td>1.19</td>
<td>0.02</td>
<td>0.01</td>
<td>61.71</td>
<td>9.86</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycerol-1,3-C₁⁴</td>
<td>1.59</td>
<td>0.08</td>
<td>0.21</td>
<td>35.60</td>
<td>4.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycerol-2-C₁⁴</td>
<td>1.30</td>
<td>0.11</td>
<td>0.37</td>
<td>23.34</td>
<td>4.02</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycerol-1,2-C₁⁴</td>
<td>1.68</td>
<td>0.18</td>
<td>0.20</td>
<td>16.24</td>
<td>9.84</td>
<td>0.022</td>
</tr>
<tr>
<td>Succinate-1,4-C₁⁴</td>
<td>0.01</td>
<td>3.09x10⁻³</td>
<td>1.42x10⁻³</td>
<td>27.93</td>
<td>3.22</td>
<td>0.1</td>
</tr>
<tr>
<td>Succinate-2,3-C₁⁴</td>
<td>0.11</td>
<td>0.01</td>
<td>0.01</td>
<td>34.60</td>
<td>9.77</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose-1-C₁⁴</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>52.36</td>
<td>3.56</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose-2-C₁⁴</td>
<td>0.94</td>
<td>0.02</td>
<td>0.01</td>
<td>13.03</td>
<td>2.38</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose-6-C₁⁴</td>
<td>0.11</td>
<td>0.02</td>
<td>0.03</td>
<td>36.31</td>
<td>5.51</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose-6-C₁⁴</td>
<td>1.68</td>
<td>0.18</td>
<td>0.20</td>
<td>16.24</td>
<td>9.84</td>
<td>0.1</td>
</tr>
<tr>
<td>Shikimic acid</td>
<td>2.22</td>
<td>0.06</td>
<td>0.11</td>
<td>7.39</td>
<td>8.96</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Fermentation conditions. 2L Erlenmeyer flask containing 500 ml. of Frank and DeMoss' medium (1959) was inoculated with 5 ml. of washed cell suspension prepared from cells grown in nutrient broth, and incubated at 30°C. on rotary shaker. Radioactive substrate was prepared in the concentration of 0.1 mc. per 100 ml. and sterilized by millipore-filtration. Half of total radioactive substrate was added to a single flask after one day's incubation and fermentation was stopped after 5 days. Each experiment involved two fermentations which were pooled for analyses.
### TABLE XIV

Pyocyanine formation from various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity</th>
<th>Pyocyanine</th>
<th>Phena-</th>
<th>Incorporation, %</th>
<th>Phenazine</th>
<th>Radioactive</th>
<th>Radioactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate mc/mM</td>
<td>Pyocyanine mc/M</td>
<td>Phena-</td>
<td>Pyocyanine Substrate mc/mM</td>
<td>Radioactive carbon</td>
<td>Phenazine Substrate mc/mM</td>
<td>Radioactive carbon</td>
</tr>
<tr>
<td>NaAC-1-Cl\textsubscript{14}</td>
<td>29.0</td>
<td>0.005</td>
<td>0.014</td>
<td>17x10^{-6}</td>
<td>9x10^{-6}</td>
<td>48x10^{-6}</td>
<td>24x10^{-6}</td>
</tr>
<tr>
<td>NaAC-2-Cl\textsubscript{14}</td>
<td>33</td>
<td>0.339</td>
<td>0.369</td>
<td>1.03x10^{-3}</td>
<td>514x10^{-6}</td>
<td>1.12x10^{-3}</td>
<td>559x10^{-6}</td>
</tr>
<tr>
<td>Glycerol -1,3-Cl\textsubscript{14}</td>
<td>0.365x10^{-3}</td>
<td>1.329</td>
<td>0.606</td>
<td>364</td>
<td>243</td>
<td>166</td>
<td>111</td>
</tr>
<tr>
<td>Glycerol -2-Cl\textsubscript{14}</td>
<td>0.365x10^{-3}</td>
<td>0.884</td>
<td>1.262</td>
<td>242</td>
<td>81</td>
<td>346</td>
<td>115</td>
</tr>
<tr>
<td>Succinate -1,4-Cl\textsubscript{14}</td>
<td>10.7</td>
<td>0.052</td>
<td>0.005</td>
<td>486x10^{-6}</td>
<td>243x10^{-6}</td>
<td>47x10^{-6}</td>
<td>24x10^{-6}</td>
</tr>
<tr>
<td>Succinate -2,3-Cl\textsubscript{14}</td>
<td>13.9</td>
<td>0.074</td>
<td>0.029</td>
<td>532x10^{-6}</td>
<td>266x10^{-6}</td>
<td>209x10^{-6}</td>
<td>105x10^{-6}</td>
</tr>
<tr>
<td>Glucose -1-Cl\textsubscript{14}</td>
<td>35.9</td>
<td>0.034</td>
<td>0.031</td>
<td>95x10^{-6}</td>
<td>16x10^{-6}</td>
<td>86x10^{-6}</td>
<td>14x10^{-6}</td>
</tr>
<tr>
<td>Glucose -2-Cl\textsubscript{14}</td>
<td>33.4</td>
<td>0.114</td>
<td>0.068</td>
<td>341x10^{-6}</td>
<td>57x10^{-6}</td>
<td>204x10^{-6}</td>
<td>34x10^{-6}</td>
</tr>
<tr>
<td>Glucose -6-Cl\textsubscript{14}</td>
<td>27.5</td>
<td>0.114</td>
<td>0.083</td>
<td>415x10^{-6}</td>
<td>69x10^{-6}</td>
<td>302x10^{-6}</td>
<td>50x10^{-6}</td>
</tr>
<tr>
<td>Shikimic acid -1,6-Cl\textsubscript{14}</td>
<td>10.15</td>
<td>1.930</td>
<td>1.553</td>
<td>19.01x10^{-3}</td>
<td>5.43x10^{-3}</td>
<td>15.22x10^{-3}</td>
<td>4.35x10^{-3}</td>
</tr>
</tbody>
</table>
Table XIV (continued)

Ratios:

\[
\begin{align*}
\text{Incorporation of carboxyl carbon of acetate into phenazine} & = \frac{24}{559} = 0.043 \\
\text{Incorporation of methyl carbon of acetate into phenazine} & = \frac{40}{559} = 0.072 \\
\text{Incorporation of carbon 1+3 of glycerol into phenazine} & = \frac{111}{115} = 0.965 \\
\text{Incorporation of carbon 2 of glycerol into phenazine} & = \frac{113}{115} = 0.992 \\
\end{align*}
\]
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity</th>
<th>Incorporation, %</th>
<th>RADIOACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mc/mM</td>
<td>mc/M</td>
<td>mc/M</td>
</tr>
<tr>
<td>NaAC-1-C¹⁴</td>
<td>29.0</td>
<td>0.015</td>
<td>0.008</td>
</tr>
<tr>
<td>NaAC-2-C¹⁴</td>
<td>33</td>
<td>0.401</td>
<td>0.132</td>
</tr>
<tr>
<td>Glycerol -1,3-C¹⁴</td>
<td>0.365x10⁻³</td>
<td>1.205</td>
<td>0.500</td>
</tr>
<tr>
<td>Glycerol -2-C¹⁴</td>
<td>0.365x10⁻³</td>
<td>1.183</td>
<td>1.094</td>
</tr>
<tr>
<td>Succinate -1,4-C¹⁴</td>
<td>10.7</td>
<td>0.008</td>
<td>0.015</td>
</tr>
<tr>
<td>Succinate -2,3-C¹⁴</td>
<td>13.9</td>
<td>0.065</td>
<td>0.042</td>
</tr>
<tr>
<td>Glucose -1-C¹⁴</td>
<td>35.9</td>
<td>0.075</td>
<td>0.029</td>
</tr>
<tr>
<td>Glucose -2-C¹⁴</td>
<td>33.4</td>
<td>0.136</td>
<td>0.095</td>
</tr>
<tr>
<td>Glucose -6-C¹⁴</td>
<td>27.5</td>
<td>0.084</td>
<td>0.052</td>
</tr>
<tr>
<td>Shikimic acid -1,6-C¹⁴</td>
<td>10.15</td>
<td>1.885</td>
<td>0.858</td>
</tr>
</tbody>
</table>
### TABLE XV (continued)

Ratios:

<table>
<thead>
<tr>
<th>Description</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of carboxyl carbon of acetate into phenazine</td>
<td>14/200 = 0.07</td>
</tr>
<tr>
<td>Incorporation of methyl carbon of acetate into phenazine</td>
<td></td>
</tr>
<tr>
<td>Incorporation of carbon 1+3 of glycerol into phenazine</td>
<td>91/100 = 0.91</td>
</tr>
<tr>
<td>Incorporation of carbon 2 of glycerol into phenazine</td>
<td></td>
</tr>
<tr>
<td>Incorporation of carbon 1+3 of glycerol into phenazine-(\alpha)-carboxylic acid</td>
<td>2.04</td>
</tr>
<tr>
<td>Incorporation of carbon 2 of glycerol into phenazine-(\alpha)-carboxylic acid</td>
<td></td>
</tr>
</tbody>
</table>
TABLE XVI
Incorporation of glycerol into phenazine-α-carboxylic acid and oxychlororaphine via shilmanic acid pathway

![Chemical structures and reactions]

<table>
<thead>
<tr>
<th>Compounds</th>
<th>No. of carbon* derived from</th>
<th>Specific activities** from labelled glycerol</th>
<th>Ratios***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1+3</td>
<td>2</td>
<td>glycerol-1,3-C^{14}</td>
</tr>
<tr>
<td>Phenazine-α-carboxylic acid</td>
<td>25/3</td>
<td>14/3</td>
<td></td>
</tr>
<tr>
<td>Oxychlororaphine</td>
<td>25/3</td>
<td>14/3</td>
<td>25/2a</td>
</tr>
</tbody>
</table>

* Number of carbon derived from carbon 1+3 of glycerol is equivalent to incorporation of carbon 1+3 of glycerol.

** Specific activity of glycerol-1,3-C^{14} and glycerol-2-C^{14} are a μc/μM of glycerol.

*** Ratio of incorporation into phenazine-α-carboxylic acid

\[ \text{Ratio of specific activity of phenazine-α-carboxylic acid} \]

= \( \frac{\text{Incorporation of carbon 1+3 of glycerol into phenazine-α-carboxylic acid}}{\text{Incorporation of carbon 2 of glycerol into phenazine-α-carboxylic acid}} \)

Specific activity of phenazine-α-carboxylic acid formed from glycerol-1,3-C^{14}

= Specific activity of phenazine-α-carboxylic acid formed from glycerol-2-C^{14}
Figure 17. Major contributions of glucose carbon atoms to shikimate biosynthesis (from Srinivasan et al., 1956)

Beside each carbon atom of shikimate each number in parentheses represents the fraction of that atom derived from a given carbon atom of glucose, whose number is denoted beside the parentheses.
TABLE XVII

Oxychlororaphine formation from various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity</th>
<th>Oxychlororaphine</th>
<th>Incorporation, %</th>
<th>Phenazine-α-amine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate</td>
<td>Oxy-</td>
<td>Phena-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mc/mM</td>
<td>chloro-</td>
<td>zine-α-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Substrate</td>
<td>raphine</td>
<td>amine mc/M</td>
<td>Radioactive</td>
</tr>
<tr>
<td></td>
<td>mc/M</td>
<td>mc/M</td>
<td></td>
<td>carbon Substrate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Radioactive</td>
</tr>
<tr>
<td></td>
<td>Substrate</td>
<td></td>
<td></td>
<td>carbon</td>
</tr>
<tr>
<td>NaAC-1-C\textsuperscript{14}</td>
<td>29.0</td>
<td>0.020</td>
<td>0.008</td>
<td>69x10^{-6}</td>
</tr>
<tr>
<td>NaAC-2-C\textsuperscript{14}</td>
<td>33</td>
<td>0.453</td>
<td>0.285</td>
<td>1.373x10^{-3}</td>
</tr>
<tr>
<td>Glycerol-1,3-C\textsuperscript{14}</td>
<td>0.365x10^{-3}</td>
<td>1.102</td>
<td>0.673</td>
<td>302</td>
</tr>
<tr>
<td>Glycerol-2-C\textsuperscript{14}</td>
<td>0.365x10^{-3}</td>
<td>1.326</td>
<td>1.112</td>
<td>363</td>
</tr>
<tr>
<td>Succinate-1,4-C\textsuperscript{14}</td>
<td>10.7</td>
<td>0.012</td>
<td>0.004</td>
<td>112x10^{-6}</td>
</tr>
<tr>
<td>Succinate-2,3-C\textsuperscript{14}</td>
<td>13.9</td>
<td>0.044</td>
<td>0.033</td>
<td>317x10^{-6}</td>
</tr>
<tr>
<td>Glucose-1-C\textsuperscript{14}</td>
<td>35.9</td>
<td>0.093</td>
<td>0.038</td>
<td>259x10^{-6}</td>
</tr>
<tr>
<td>Glucose-2-C\textsuperscript{14}</td>
<td>33.4</td>
<td>0.208</td>
<td>0.243</td>
<td>623x10^{-6}</td>
</tr>
<tr>
<td>Glucose-6-C\textsuperscript{14}</td>
<td>27.5</td>
<td>0.072</td>
<td>0.070</td>
<td>262x10^{-6}</td>
</tr>
<tr>
<td>Shikimic acid-1,6-C\textsuperscript{14}</td>
<td>10.15</td>
<td>1.108</td>
<td>0.986</td>
<td>10.916x10^{-3}</td>
</tr>
</tbody>
</table>

9.714x10^{-3} 2.775x10^{-3}
### TABLE XVII (continued)

**Ratios:**

<table>
<thead>
<tr>
<th>Incorporation of carboxyl carbon of acetate into phenazine nucleus</th>
<th>Incorporation of methyl carbon of acetate into phenazine nucleus</th>
<th>= 14/432 = 0.03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation of carbon 1+3 of glycerol into phenazine nucleus</td>
<td>Incorporation of carbon 2 of glycerol into phenazine nucleus</td>
<td>= 123/102 = 1.21</td>
</tr>
<tr>
<td>Incorporation of carbon 1+3 of glycerol into oxychlororaphine</td>
<td>Incorporation of carbon 2 of glycerol into oxychlororaphine</td>
<td>= 1.66</td>
</tr>
</tbody>
</table>
### TABLE XVIII
Specific activities of degradation products of phenazine ring from pyocyanine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activities</th>
<th>dpm/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenazine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formula Carbon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quinoxaline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formula Carbon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quinoxaline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formula Carbon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyrazine-tetra-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formula Carbon</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phenazine</th>
<th>Quinoxaline</th>
<th>Pyrazine-tetra-carboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formula Carbon</td>
<td>Formula Carbon</td>
<td>Formula Carbon</td>
</tr>
<tr>
<td></td>
<td>Carbon</td>
<td>Carbon</td>
<td>Carbon</td>
</tr>
<tr>
<td>Acetate-1-C(^{14})</td>
<td>30</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>Acetate-2-C(^{14})</td>
<td>816</td>
<td>68</td>
<td>865</td>
</tr>
<tr>
<td>Glycerol-1,3-C(^{14})</td>
<td>1,344</td>
<td>112</td>
<td>1,135</td>
</tr>
<tr>
<td>Glycerol-2-C(^{14})</td>
<td>2,220</td>
<td>185</td>
<td>2,951</td>
</tr>
<tr>
<td>Glucose-1-C(^{14})</td>
<td>69</td>
<td>5.8</td>
<td>-</td>
</tr>
<tr>
<td>Glucose-2-C(^{14})</td>
<td>150</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>Glucose-6-C(^{14})</td>
<td>184</td>
<td>15.3</td>
<td>125</td>
</tr>
<tr>
<td>Shikimate-1,6-C</td>
<td>3,450</td>
<td>288</td>
<td>4,055</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE XIX

Specific activities of degradation product of oxychlororaphine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxychlororaphine Molecule Carbon</th>
<th>Phenazine-(\alpha)-amine Molecule Carbon</th>
<th>Quinoxaline di-carboxylic acid Molecule Carbon</th>
<th>Quinoxaline Molecule Carbon</th>
<th>Pyrazine-tetra-carboxylic acid Molecule Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol-1, 3-(^{14})C</td>
<td>2,445 188</td>
<td>1,493 124</td>
<td>1,525 153</td>
<td>339* 42</td>
<td>1,675 209</td>
</tr>
<tr>
<td>Glycerol-2-(^{14})C</td>
<td>2,945 227</td>
<td>2,470 206</td>
<td>2,926 293</td>
<td>791 99</td>
<td>1,734 217</td>
</tr>
<tr>
<td>Shikimic acid-1,6-(^{14})C</td>
<td>2,459 189</td>
<td>2,190 183</td>
<td>1,702 170</td>
<td>801** 100</td>
<td>2,343 293</td>
</tr>
</tbody>
</table>

*Exploded during ignition

**Combustion not complete

Specific activity in dpm/\(\mu\)M.
TABLE XX

Ratios of specific activities of the degradation products from pyocyanine and oxychloro-raphine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phenazine nucleus Quinoxaline-dicarboxylic acid</th>
<th>Phenazine nucleus Pyrazine-tetra-carboxylic acid</th>
<th>Quinoxaline dicarboxylic acid Pyrazine-tetra-carboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol -1,3-C\textsubscript{14}</td>
<td>1.18</td>
<td>0.98</td>
<td>1.51</td>
</tr>
<tr>
<td>Glycerol -2-C\textsubscript{14}</td>
<td>0.75</td>
<td>0.84</td>
<td>2.73</td>
</tr>
<tr>
<td>Shikimate -1,6-C\textsubscript{14}</td>
<td>0.85</td>
<td>1.29</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Sample calculation:

The ratio of the specific activity of phenazinol to that of quinoxaline dicarboxylic acid from the degradation of radioactive pyocyanine labeled with glycerol-1,3-C\textsubscript{14} was:

\[
\frac{1.344}{1.135} = 1.18
\]
**TABLE XXI**  
Biogenesis of oxychlororaphine from labeled phenazine-\(\alpha\)-carboxylic acid by *P. chlororaphis* Mac 370

<table>
<thead>
<tr>
<th>Oxychlororaphine</th>
<th>Cell suspension</th>
<th>Sonicated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (\mu g/100 \text{ ml.})</td>
<td>460</td>
<td>330</td>
</tr>
<tr>
<td>Sp. activity mc/M</td>
<td>0.53</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fermentation conditions. L Erlenmeyer flasks containing 100 ml. Frank and DeMoss' medium (1959) plus 14.5 mgm of neutralized phenazine-\(\alpha\)-carboxylic acid of specific activity 0.58 mc/M in 5 ml. solution, were inoculated with either 5 ml. of washed cell suspension in 0.1 Tris-buffer at pH 7.6, or with equal amounts of sonicated cells, and incubated at 28°C on rotary shaker for two days.
## TABLE XXII

Biogenesis of oxychlororaphine from labeled phenazine-κ-carboxylic acid by *P. aeruginosa* Mac 436

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Yield (mgm)</th>
<th>Specific activity (mc/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxychlororaphine</td>
<td>2.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Phenazine-κ-carboxylic acid</td>
<td>9.9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Fermentation conditions. 1 L Erlenmeyer flask containing 250 ml. Frank and DeMoss’ medium (1959) plus 7.9 mgm of phenazine-κ-carboxylic acid of specific activity 0.87 mc/M in 10 ml. solution was inoculated with 5 ml. of washed suspension, and incubated at 30°C. on rotary shaker for 3 days.
RESULTS

FACTORS AFFECTING PYOCYANINE PRODUCTION

Effects of Inoculum on Pyocyanine Production

Pseudomonas aeruginosa (Mac 436) grew very rapidly in the medium described by Frank and DeMoss (1959) and at about twelve hours, after direct inoculation from a 24-hour culture grown on nutrient agar, logarithmic growth ended, pyocyanine production started, and the pH increased (Figure 12). Inoculation from a nutrient slant gave better yields than inoculum produced by growing cells in the same medium (Figure 12). A larger inoculum (Figure 13) gave earlier production of pyocyanine but the maximum yield was lower (Figure 13). The heavier inoculum also affected the pH and there was no decrease in early stages of growth as was found when smaller amounts of inoculum were used (Figures 11, 13 and 16). The pH decreased initially then increased about 1 pH unit and remained constant. The age of the inoculum also had an effect and as is shown in Figure 14, inoculum grown for more than two days gave lower yields on modified Kurachi's medium. The type of inoculum affected pyocyanine production in Kurachi's medium, also. No pyocyanine was produced when a washed cell suspension was used as inoculum (Figure 15A, Table V), but when inoculum grown on nutrient broth was used, pyocyanine was formed (Figure 15B).
In all trials, production of pyocyanine reached a maximum and then the concentration gradually decreased (Figures 11, 13 and 16).

**Effects of Salts on Pyocyanine Production**

The optimum concentration of \( K_2HPO_4 \) in Kurachi's medium was found to vary between 0.010% using a washed cell suspension as an inoculum and 0.015% using nutrient broth as an inoculum (Figures 15A and 15B). In trials where various ions were omitted (Table III), no essential requirements of ions were found for pyocyanine production. The results in Table IV indicate that optimum concentration for pyocyanine production was 0.0002M \( HPO_4^2^- \) and 0.08M \( SO_4^{2-} \).

**Effects of Carbon Source on Pyocyanine Production**

The yield of pyocyanine depended not only on the carbon source but also on the inoculum and on the composition of the salts in the medium (Table V). The addition of calcium carbonate appeared to increase the yield of pyocyanine. This was especially evident where potassium gluconate was incubated with cells recovered from nutrient broth into a medium containing Ingram and Blackwood's salts (Table V). Media which supported pyocyanine production were all alkaline after incubation, except those with catechol and glycerol (Table VI), but the reverse was not true. Acetate supported both trypticase-soy-broth-grown cells and cells grown in the medium containing 0.5% DL-alanine, 0.5% glycerol plus Vogel et al.'s salts (1956), in Ingram
and Blackwood's salts (1962) and Frank and DeMoss' salts (1959) to produce small amounts of pyocyanine (Tables V and VI). With glycerol and Ingram and Blackwood's salts as a medium, a washed cell suspension grown on 0.5% alanine, 0.5% glycerol plus Vogel et al.'s salts produced pyocyanine (Table VI). Also with glycerol as substrate, inoculum grown in trypticase-soy-broth produced pyocyanine when Ingram and Blackwood's and Frank and DeMoss' salts were added, but not when Kurachi's salts were included (Table V). Kurachi's medium (glycerol plus Kurachi's salts) did not support pyocyanine production when a washed cell suspension prepared from nutrient broth grown cells was inoculated. The fact that acetate, glycerol and glucose could serve as substrates for pyocyanine production was further investigated and the results are given in Table VII. The optimum substrate concentrations of acetate and glucose for pyocyanine production were found to be 2-3% (Table VIII). Some substrates with a preformed benzene ring, e.g., tyrosine (Table VI), tryptophane, phenylalanine and catechol (Table VI) did contribute to pyocyanine production, whereas o-amino-phenol, indol and anthranilic acid did not support pyocyanine formation (Table VI). Glutamine, glutaric acid, 2-keto-glutaric acid supported the pigmentation, but the yield of pigment from 2-keto-glutaric acid was lower. Though L-leucine supported pyocyanine production, 2-hydroxy-
2-methyl-glutaric acid was much weaker in its capability to enhance pigment formation (Table VI).
This strain of *P. aeruginosa* produced three phenazines in Frank and DeMoss' medium (1959), in Ingram and Blackwood's medium (1962), as well as in the media containing glucose, acetate or glycerol as a single carbon source (Table IX), but Haynes et al.'s medium (1956), Sellers et al.'s medium (1962), and *Pseudomonas* agar P (Difco with no agar added) did not support production of phenazine-1-carboxylic acid and oxychlororaphine.

A time-study of biogenesis of phenazines in Frank and DeMoss' medium (1959) showed that yields of pyocyanine reached a maximum at 3-4 days, then decreased gradually. Phenazine-1-carboxylic acid and oxychlororaphine were produced after a lag of about 24 hours and reached maximum concentrations after 5 days incubation (Figure 16). Based on these results, radioactive substrates were added after a 24-hour incubation period in the radioactive experiments.

**BIOSYNTHESIS OF PHENAZINES FROM LABELED SUBSTRATES**

**Pyocyanine Formation From a Single Carbon Source**

Incorporation of the radioactivity from all substrates tested was quite low (Table X). Labeled glycerols were better incorporated into pyocyanine than other substrates tried (Table XI). Urea-C\textsuperscript{14} appeared to be incorporated into both the methyl carbon and the phenazine nucleus, whereas the carboxyl carbon of acetate-1-C\textsuperscript{14} was found
mainly in the methyl carbon of the pyocyanine. The ratio for the carbon atoms entering phenazinol from carbons 1 plus 3 of glycerol as compared to carbon 2 of glycerol was 1.88. This value is higher than 1.57, the theoretical value expected, assuming that glycerol was used to synthesize shikimic acid and that two molecules of shikimic acid condensed to form the aromatic rings of pyocyanine. The ratio is also higher than the theoretical value of 1, assuming that glycerol was transformed into acetate, and three moles of acetate condensed head-to-tail to form the aromatic ring, and that two aromatic rings were converted into the aromatic rings of pyocyanine. But the ratio is lower than the theoretical value of 2, assuming that six moles of glycerol form the carbon skeleton of the phenazine ring of pyocyanine (Table XII).

The ratio of specific activity found in the phenazine nucleus formed from glycerol-1,3-C\textsuperscript{14} to that formed from glycerol-2-C\textsuperscript{14} was 0.94. The value is not in agreement to any one of the theoretical values listed in Table XII. Calculating on a molar basis, the per cent incorporation of glycerol-1,3-C\textsuperscript{14} into pyocyanine was 405, that is approximately 4 moles of glycerol were used to synthesized one mole of pyocyanine. The methyl group of pyocyanine was highly labeled when glycerol-1,3-C\textsuperscript{14} was used as the substrate.

Formation of Pyocyanine, Phenazine-\(\text{N}\)-carboxylic Acid and Oxychlororaphine from labeled substrates

While the incorporation of radioactivity into the
pigments was quite low, the percentage incorporation into pyocyanine was generally higher than into oxychlororaphine or into phenazine-α-carboxylic acid. Shikimic acid-1,6-\(^{14}\)C, glucose-U-\(^{14}\)C, glycerol-U-\(^{14}\)C and glycerol-2-\(^{14}\)C were incorporated into pyocyanine more efficiently than labeled acetate, succinate or glucose. The radioactive substrates were metabolized by the organism and high levels of radioactivity were found in respiratory CO\(_2\) and in the cells (Table XIII).

The percentage incorporation of substrate into phenazine nucleus of pyocyanine decreased in efficiency as follows: glycerol-2-\(^{14}\)C > glycerol-1,3-\(^{14}\)C > shikimic acid-1,6-\(^{14}\)C > acetate-2-\(^{14}\)C > glucose-6-\(^{14}\)C > succinate-2,3-\(^{14}\)C > glucose-2-\(^{14}\)C > glucose-1-\(^{14}\)C > acetate-1-\(^{14}\)C and succinate-1,4-\(^{14}\)C. However, the percentage incorporation of radioactive carbon into phenazine nucleus in the decreasing order was as follows: glycerol-2-\(^{14}\)C > glycerol-1,3-\(^{14}\)C > shikimic acid-1,6-\(^{14}\)C > acetate-2-\(^{14}\)C > succinate-2,3-\(^{14}\)C > glucose-6-\(^{14}\)C > glucose-2-\(^{14}\)C > acetate-1-\(^{14}\)C and succinate-1,4-\(^{14}\)C > glucose-1-\(^{14}\)C (Table XIV).

The methyl carbon of pyocyanine was labeled when glycerol-1,3-\(^{14}\)C, succinate-1,4-\(^{14}\)C, succinate-2,3-\(^{14}\)C, glucose-2-\(^{14}\)C, glucose-6-\(^{14}\)C and shikimic acid-1,6-\(^{14}\)C were supplied as substrates. Acetate-2-\(^{14}\)C and glucose-1-\(^{14}\)C, gave slightly radioactive N-methyl carbon, whereas Acetate-1-\(^{14}\)C and glycerol-2-\(^{14}\)C did not give radioactivity to this particular carbon (Table XIV).
The ratio of incorporation of the carboxyl carbon of acetate into the phenazine nucleus of pyocyanine to that incorporated from the methyl carbon of acetate should be one but the data in Table XIV show that the methyl carbon of acetate is incorporated about 25 times more than the carboxyl carbon of acetate. The conclusion is that the acetate pathway is not involved in the synthesis of phenazine nucleus of pyocyanine. Similar calculations given in Table XIV for the ratio of incorporation of glycerol carbon indicate that the shikimic acid pathway is not involved in the synthesis of phenazine nucleus either.

The percentage incorporation of substrate into phenazine nucleus of phenazine-1-carboxylic acid decreased as follows: glycerol-2-C\textsuperscript{14} > glycerol-1,3-C\textsuperscript{14} > shikimic acid-1,6-C\textsuperscript{14} > acetate-2-C\textsuperscript{14} > succinate-2,3-C\textsuperscript{14} > glucose-2-C\textsuperscript{14} glucose-6-C\textsuperscript{14} > succinate-1,4-C\textsuperscript{14} > glucose-1-C\textsuperscript{14} and acetate-1-C\textsuperscript{14}. However, percentage incorporation of radioactive carbon into the phenazine nucleus in the decreasing order was as follows: glycerol-2-C\textsuperscript{14} > glycerol-1,3-C\textsuperscript{14} > shikimic acid-1,6-C\textsuperscript{14} > acetate-2-C\textsuperscript{14} > succinate-2,3-C\textsuperscript{14} > succinate-1,4-C\textsuperscript{14} > glucose-2-C\textsuperscript{14} > glucose-6-C\textsuperscript{14} > glucose-1-C\textsuperscript{14} and acetate-1-C\textsuperscript{14} (Table XV).

The carboxyl group of phenazine-1-carboxylic acid was labeled by acetate-1-C\textsuperscript{14}, glycerol-1,3-C\textsuperscript{14}, succinate-2,3-C\textsuperscript{14}, glucose-1-C\textsuperscript{14}, glucose-2-C\textsuperscript{14}, glucose-6-C\textsuperscript{14} and shikimic acid-1,6-C\textsuperscript{14} (Table XV).

Various values given in Table XV for the ratio of
incorporation of acetate carbon and glycerol carbon into the phenazine nucleus of phenazine-α-carboxylic acid indicate that neither the acetate pathway nor the shikimic acid pathway is involved in the synthesis of phenazine nucleus of phenazine-α-carboxylic acid.

If the assumption is made that two moles of shikimic acid are condensed to form a mole of phenazine-1,6-dicarboxylic acid, which then is decarboxylated to form phenazine-α-carboxylic acid, the ratio for the carbon atoms entering phenazine-α-carboxylic acid from carbons 1 plus 3 of glycerol as compared to carbon 2 of glycerol is theoretically 1.79 (Table XVI). The value from the Table XV is 2.04. This means that the carboxyl group of phenazine-α-carboxylic acid does not originate from the carboxyl carbon of shikimic acid. The fact that glucose-1-C\textsubscript{14}, glucose-2-C\textsubscript{14}, glucose-6-C\textsubscript{14} and shikimic acid-1,6-C\textsubscript{14} gave a radioactive carboxyl group also support this conclusion (Figure 17).

The percentage incorporation of substrate into the phenazine nucleus of oxychlororaphine in the decreasing order was as follows: glycerol-2-C\textsubscript{14} > glycerol-1,3-C\textsubscript{14} > shikimic acid-1,6-C\textsubscript{14} > acetate-2-C\textsubscript{14} > glucose-2-C\textsubscript{14} > glucose-6-C\textsubscript{14} > succinate-2,3-C\textsubscript{14} > glucose-1-C\textsubscript{14} > succinate-1-C\textsubscript{14} > acetate-1-C\textsubscript{14}. However, the percentage incorporation of radioactive carbon into the phenazine nucleus decreased as follows: glycerol-1,3-C\textsubscript{14} > glycerol-2-C\textsubscript{14} > shikimic acid-1,6-C\textsubscript{14} > acetate-2-C\textsubscript{14} > succinate-2,3-C\textsubscript{14} > glucose-2-C\textsubscript{14} > glucose-6-C\textsubscript{14} > succinate-1,4-C\textsubscript{14} > glucose-1-C\textsubscript{14} > acetate-1-C\textsubscript{14} (Table XVII).
The carboxyl carbon of oxychlororaphine was labeled by acetate-1-C\textsubscript{14}, acetate-2-C\textsubscript{14}, glycerol-1,3-C\textsubscript{14}, glycerol-2-C\textsubscript{14}, succinate-1,4-C\textsubscript{14}, succinate-2,3-C\textsubscript{14}, glucose-1-C\textsubscript{14}, and shikimic acid-1,6-C\textsubscript{14} (Table XVII).

Various values given in Table XVII for the ratio of incorporation of acetate carbon, glycerol carbon into the phenazine nucleus of phenazine-\(\alpha\)-carboxamide indicate that neither the acetate pathway nor the shikimic acid pathway is involved in the biosynthesis of phenazine nucleus of oxychlororaphine.

The ratio for the carbon atoms entering phenazine-\(\alpha\)-carboxamide from carbons 1 plus 3 of glycerol as compared to carbon 2 of glycerol was 1.66. This value is lower than the theoretical 1.79 (Table XVI). The conclusion is that the carboxyl group of phenazine-\(\alpha\)-carboxamide does not originate from the carboxyl group of shikimic acid. The fact that the carboxyl group of oxychlororaphine biosynthesized from shikimic acid-1,6-C\textsubscript{14} and glucose-1-C\textsubscript{14} was radioactive gives further evidence.

Judging from the specific activities of the degradation product of pyocyanine, carbons 2 and 3 of phenazine ring were labeled from glycerol-1,3-C\textsubscript{14} and glucose-6-C\textsubscript{14}; carbons 1 and 4 of phenazine ring were labeled from acetate-2-C\textsubscript{14}, glycerol-2-C\textsubscript{14}, and shikimate-1,6-C\textsubscript{14}; carbons 7 and 8 were labeled from glycerol-1,3-C\textsubscript{14}, glycerol-2-C\textsubscript{14} and glucose-6-C\textsubscript{14} (Table XVIII).

Comparison of the radioactivities of the degradation
products of oxychlororaphine revealed that the carboxyl groups was labeled from glycerol-1,3-C\textsuperscript{14}, glycerol-2-C\textsuperscript{14} and shikimic acid-1,6-C\textsuperscript{14}. Carbons 2 and 3 of phenazine ring were labeled from shikimate-1,6-C\textsuperscript{14}. Carbons 1 and 4 were labeled from glycerol-2-C\textsuperscript{14}. Carbons 7 and 8 were labeled from glycerol-2-C\textsuperscript{14} (Table XIX).

Ratios of specific activities of the degradation products of the phenazine nucleus of pyocyanine formed from various labeled substrates differed from ratios of specific activities of the degradation products of the phenazine nucleus of oxychlororaphine (Table XX).

Biogenesis of Oxychlororaphine from Labeled Phenazine-\textalpha-carboxylic Acid

Incubation of radioactive phenazine-\textalpha-carboxylic acid with \textit{P. chlororaphis} and with \textit{P. aeruginosa} in Frank and DeMoss' medium resulted in formation of radioactive oxychlororaphine (Tables XXI and XXII). The fact that the specific activity of phenazine-\textalpha-carboxylic acid and that of oxychlororaphine was identical (Table XXII) suggests that phenazine-\textalpha-carboxylic acid was incorporated as an intact unit and is the precursor of oxychlororaphine.
DISCUSSION

All attempts to synthesize pyocyanine with acetone-dried cells, and cell-free extracts of Pseudomonas aeruginosa (Mac 436) were negative. Likewise, attempts to shorten the lag period before pigment formation with resting and sonicated cells preparations were negative although NADP, NADPH, ATP, yeast extracts, and fractions of an apyocyanogenic strain of P. aeruginosa (Azuma and Witter, 1964) prepared according to Gordon (1962) were tested. During these experiments, the criterion for production was the detection of pyocyanine by paper chromatography as described by Frank and DeMoss (1959). Experiments with the medium of Ingram and Blackwood (1962) revealed that DL-alanine disappeared after 45-hour incubation, L-leucine after 116-hour, and glycerol was detectable even after 10 days of incubation. This suggested that L-leucine was not necessary and omission of L-leucine from the Ingram and Blackwood's medium supported pyocyanine production.

A defined medium was used previously by Frank and DeMoss (1959) which contained DL-alanine and glycerol. Production of pigment in this medium was studied. Sodium citrate was reported by Sellers et al. (1962) to enhance pyocyanine production, therefore, 1% sodium citrate was incorporated in Frank and DeMoss' medium (1959), but results indicated that pyocyanine production was delayed.
When a 24-hour culture grown in the medium of Frank and DeMoss was used as inoculum, pyocyanine was delayed considerably which might be explained by pyocyanine carried-over in the inoculum.

Various substrates were incubated with cells recovered from different media in the different salts solutions. The yield of pyocyanine depends not only on the carbon source, but also on the inoculum and the composition of the salts in the medium. Acetate, glycerol or glucose did support the pigmentation in certain combinations of factors. Substrates with preformed benzene ring, such as tryptophane, phenylalanine, or catecol supported pyocyanine formation whereas o-aminophenol, indol, anthranilic acid did not act as precursors under the conditions tested. Since catechol, succinate, acetate, and DL-alanine supported pigmentation, it is difficult to decide whether those substrates with preformed benzene rings were incorporated into pyocyanine in toto, or after degradation.

Kurachi's medium (1959) contains glycerol as the carbon source, and urea as the nitrogen source, and has a much simpler composition compared to other defined media. The fact that Kurachi's medium did not support pigmentation when a washed cell suspension was used as an inoculum, was a surprise. However, when a washed cell suspension was used as an inoculum, the phosphate level in the medium should be reduced to 0.010% to produce pyocyanine. Based on this finding, and on the results of studies on the
inorganic ion requirements, a basal salts solution containing 0.41% MgCl₂·6H₂O, 0.71% Na₂SO₄, 0.0005% FeSO₄·7H₂O, 0.2% urea, and 0.010% K₂HPO₄ was devised, and designated as 'modified' Kurachi's salts. The medium was adjusted to pH 7.0-7.2 after the addition of substrate. With this salts solution pyocyanine is produced from acetate and glucose at optimum substrate concentrations of 2-3% NaAC·3H₂O or 2-3% of glucose. As will be pointed out later, this salts solution supports not only the formation of pyocyanine, but also the formation of phenazine-α-carboxylic acid, and oxychlororaphine from glycerol, acetate or glucose. Kurachi's salts thus modified were used for radioactive experiments using glycerol-1,3-C¹⁴, glycerol-2-C¹⁴, and acetate-1-C¹⁴.

No essential inorganic ions were required for the washed cell suspension to produce pyocyanine from D-quinic acid, however, the optimum concentration of HPO₄⁻ was found to be 0.0002 M, and that of SO₄²⁻ 0.08M. This finding seems to be contrary to Burton et al.'s (1948). However, according to Kurachi (1958a), K⁺, HPO₄⁻, SO₄²⁻, Mg⁺⁺ and Fe⁺⁺⁺ were required for growth, but not for pyocyanine production. Frank and DeMoss (1959) also reported that while Mg⁺⁺⁺, HPO₄⁻ and SO₄²⁻ were not essential, pigmentation was enhanced.

Certain strains of P. aeruginosa were reported to produce oxychlororaphine (Birkofer, 1947; Sierra et al., 1958; Takeda, 1958b) and phenazine-α-carboxylic acid (Korth, 1962; Takeda, 1958b). Takeda (1958b) reported
that the *P. aeruginosa* strain T 359 produced pyocyanine, oxychlororaphine, and phenazine-ω-carboxylic acid simultaneously. During fractionation of the fermentation products performed to determine if any fraction of *P. aeruginosa* Mac 436 could induce the apyocyanogenic strain of *P. aeruginosa* (Azuma and Witter, 1964) to produce pyocyanine, a yellow acidic compound was obtained that was identified as phenazine-ω-carboxylic acid. In further trials, *P. aeruginosa* Mac 436 was shown to produce pyocyanine, phenazine-α-carboxylic acid, and oxychlororaphine in the media of Frank and DeMoss (1959), Ingram and Blackwood (1962), and in media containing glycerol, acetate, or glucose as sole source of carbon in modified Kurachi's salts. An interesting phenomenon observed with *P. aeruginosa* Mac 436 was that it did not produce oxychlororaphine nor phenazine-α-carboxylic acid in medium containing peptone, e.g., Haynes et al.'s (1956) and Pseudomonas agar P (Difco, from which agar was omitted). Takeda (1959b) reported that *P. aeruginosa* T359 did not produce any phenazine pigment in medium containing peptone. Attempts to devise a synthetic medium for *P. aureofaciens* for the production of phenazine-α-carboxylic acid were unsuccessful (Kluyver, 1956). In this respect, *P. aeruginosa* has an advantage over *P. aureofaciens* in that the synthetic media (Takeda, 1959b; Korth, 1962; Frank and DeMoss, 1959; Ingram and Blackwood, 1962) and the media containing glycerol, acetate, or glucose in modified Kurachi's salts were available for production of
phenazine-6-carboxylic acid. Frank and DeMoss' medium was chosen for radioactive experiments, due to more even production of the three phenazine pigments. In retrospect, a medium which would support the production of the three phenazines in similar amounts should be devised prior to radioactive experiments. This medium should contain a single carbon source and inorganic nitrogen source.

When urea is used as a source of nitrogen, the carbon atom is expected to be released as CO₂ supplying ammonia as the nitrogen source. The fact that urea-C¹⁴ was incorporated into the methyl carbon of pyocyanine as well as into the phenazine nucleus, together with the fact that acetate-1-C¹⁴ labeled the same carbons suggests that in the process of biogenesis of pyocyanine, CO₂ was fixed. Na₂CO₃ was reported to be incorporated into the phenazine nucleus of oxychlororaphine by Carter and Richards (1961). Arora (1965) reported that Na₂CO₃ was incorporated into pyocyanine to a small extent, but data were not given. The addition of CaCO₃ promotes pyocyanine production as originally suggested by Hellinger (1951). At first glance, the role of CaCO₃ is to neutralize any acid formed during the fermentation, and this favours the formation of pyocyanine. However, CaCO₃ could supply carbon for biosynthesis of the phenazine ring and from the known mechanism of heterotrophic CO₂ assimilation (Wood and Stjernholm, 1962), an organic acid should be formed, and this acid should be one of the intermediates in the biosynthetic
pathway leading to the formation of the phenazine ring.

The ratio for the carbon atoms entering 1-hydroxyphenazine from carbons 1 plus 3 of glycerol as compared to carbon 2 of glycerol showed that neither the shikimic acid pathway nor the acetate pathway were involved in the formation of benzene rings of the phenazine nucleus.

When three phenazine pigments were biosynthesized simultaneously, from various specifically labeled acetate, glycerol, succinate, glucose, and shikimic acid, all radioactive substrates were metabolized to a great extent as evidenced by the recovery of high radioactivity in the respiratory CO₂, and in the cells. Incorporation of the radioactivity from the substrates into the pigments was low but labeled glycerol was incorporated better than other substrates tried. Incorporation of the radioactivity into pyocyanine was higher than the radioactivity incorporated into phenazine-α-carboxylic acid or oxychlororaphine. The methyl carbon of acetate was incorporated much more than the carboxyl carbon of acetate, and suggests that decarboxylation of acetate occurs during the processes of biogenesis of phenazines. Likewise, the carboxyl carbons of succinate were incorporated to a lesser extent than the ethylene carbons of the succinate, but the difference between those two carbon groups was not as great as the difference observed between the carboxyl carbon and the methyl carbon of acetate. Of variously labeled glucose, glucose-1-C¹⁴ was metabolized to a larger extent,
and consequently, was incorporated less into the phenazines. Shikimic-1,6-C\textsuperscript{14} was incorporated second to glycerol when calculated on a formula or radioactive carbon basis and this incorporation was also much higher than acetate, succinate or glucose. However, shikimic acid was metabolized and radioactivity was recovered in the cells and in the respiratory CO\textsubscript{2}. The ratio for the carboxyl carbon of acetate entering the phenazine rings of pyocyanine, phenazine-\(\alpha\)-carboxylic acid and oxychlororaphine as compared to the methyl carbon of acetate showed that the acetate pathway was not involved in the synthesis of these phenazines. The ratio for the carbon atoms 1 plus 3 of glycerol entering the phenazine ring of pyocyanine, phenazine-\(\alpha\)-carboxylic acid, and oxychlororaphine as compared to the carbon 2 of glycerol also showed that shikimic acid was not the sole intermediate from which the phenazine nucleus was synthesized. Similar ratio for the carbons entering phenazine-\(\alpha\)-carboxylic acid, and oxychlororaphine from the carbons 1 plus 3 of glycerol as compared to carbon 2 of glycerol showed that the carboxylic carbons of phenazine-\(\alpha\)-carboxylic acid and of oxychlororaphine did not originate from the carboxylic carbon of shikimic acid. The fact, that glucose-1-C\textsuperscript{14} and shikimic acid-1,6-C\textsuperscript{14} labeled both the carboxyl carbons of phenazine-\(\alpha\)-carboxylic acid and oxychlororaphine, also showed that the carboxyl carbons of phenazine-\(\alpha\)-carboxylic acid and oxychlororaphine did not originate from the carboxylic carbon of shikimic acid.
Degradation of the phenazine rings of pyocyanine and oxychlororaphine formed from various labeled substrates reveals that the shikimic acid pathway was not involved. As an example, if the shikimic acid pathway were involved, glycerol-1,3-C\textsuperscript{14} should give radioactive pyocyanine labeled in the C\textsubscript{13}, C\textsubscript{2+3}, C\textsubscript{1+4} and C\textsubscript{7+8}, and this was not found in this study or by Ingram and Blackwood (1962).

The conclusion is that shikimic acid and acetic acid were not the sole intermediates through which the carbocyclic ring of phenazine nucleus was synthesized. However, this does not exclude the possibility that one of the benzene rings of phenazine can originate through the shikimic acid pathway or the acetate pathway.

Degradation of phenazines also reveals that dilution of radioactivities of each carbon or pairs of carbons are not the same. This could mean that an isotopic equilibrium was not established, and there was competition among radioactive substrates added and the carbon sources in the medium for supplying the carbon skeleton of phenazine. Similar results were also given by Ingram and Blackwood (1962), and by Arora (1965). This phenomenon could be avoided, if a labeled substrate were used as the sole source of carbon.

Ratios of specific activities of the degradation products of the phenazine nuclei of pyocyanine formed from various labeled substrates differ from the corresponding ratios of specific activities of the degradation products of the phenazine nuclei of oxychlororaphine, for an example,
when glycerol-1,3-$^{14}$C was used as a labeled substrate, a ratio of specific activity of phenazinol to specific activity of quinoxaline dicarboxylic acid was 1.18 which differed from 0.78, ratio of specific activity of phenazine-1-amine to that of quinoxaline dicarboxylic acid. If the phenazine nuclei of pyocyanine and oxychlororaphine were synthesized via the same pathway, those ratios should be the same. Therefore, it is unlikely that the phenazine nucleus of pyocyanine and the phenazine nucleus of oxychlororaphine are biosynthesized by the same pathway. The similar conclusion was drawn by Arora (1965).

Kögl et al. (1932) reported that phenazine-$\alpha$-carboxylic acid stimulates oxychlororaphine production by P. chlororaphis. It is found that oxychlororaphine is synthesized via phenazine-$\alpha$-carboxylic acid both by P. chlororaphis and P. aeruginosa.
Elucidation of biosynthetic pathways depend on three general methods (Neish, 1964):

1. Studies with cell-free system:— Glycolysis is an example where the whole scheme is elucidated by chemical studies on multi-enzyme systems using suitable inhibitors. Cell-free extracts, in addition, offer the advantage that permeability effects are nullified. Usually, information is gained with the complex living system first, and then attempts to duplicate the postulated reaction with cell-free preparations are made.

2. Use of isotopically labeled compounds:— The degradation of products produced from specifically labeled compounds give an insight into the possible mechanism provided that the degradation scheme is established.

3. Studies on biochemical mutants:— This can be a powerful tool in establishing metabolic pathways, for example, the shikimic acid pathway for aromatic amino acids. For practical reasons, studies are restricted to establishing the origin of primary metabolites.

Unfortunately, no active cell-free extracts were reported for production of pyocyanine, phenazine-\(\text{-N-\text{carboxylic acid}}\) or oxychlororaphine. The question whether "resting cells" actively synthesize pyocyanine has been controversial (Grosswicz et al., 1957; Frank and DeMoss,
1959; Halpern et al., 1962). According to Quastel (1928), resting cell incubations should be carried out in a medium devoid of either nitrogen or carbon sources in a short time - usually under 30 minutes. Since phenazines contain nitrogen and carbon, the so-called "resting cell" incubation is impossible in the 'traditional' way. The incubation time used for pyocyanine production by the above mentioned authors was 12-24 hours, which is too long. The same is true for phenazine-1-carboxylic acid synthesis by Levitch et al. (1964) where the incubation time lasted for 12 to 24 hours.

The phenazine nucleus contains C, H and N; all of these have isotopes. For phenazine derivatives oxygen is added which also has isotopes. Therefore, isotopic experiments in various isotopic combinations can be used to study the mechanism of biogenesis. However, the usage of isotopes, so far, has been limited to the radioactive isotopes $^{14}C$ and $^3H$, and double labeled glycerol-2-$^{14}C$ and glycerol-2-$^3H$ was used only by Arora (1965). The radioactivity experiments are hampered by low incorporation of added precursor, usually in the order of a few per cent and sometimes much less. This leads to difficulties in further degradation to elucidate possible mechanisms of precursor incorporation. The radioactive substrates used were usually amino acids, carbohydrates or organic acids that would normally be actively metabolized and this dual use tended to confuse the results.
Moreover, the added substrates are generally incubated in the flasks for many days which is necessary to produce higher yields. Finally, the chemical degradation schemes for phenazine-α-carboxylic acid, oxychlororaphine and pyocyanine are not complete. Pyocyanine was degraded to the extent that 7 carbons out of 13 carbons can be assessed in one single carbon and in three pairs of carbons (Ingram and Blackwood, 1962). Phenazine-1-carboxylic acid and oxychlororaphine were degraded only to phenazine (Arora, 1965; Carter and Richards, 1961). According to the literature, the degradation scheme of Ingram and Blackwood (1962) could be extended to account for all the carbons of pyocyanine in one single carbon and six pairs of carbons as depicted in the accompanying Figure 18. However, the yield of each reaction is low. In the key degradation, from quinoxaline via quinoxaline di-N-oxide to phenylenediamine (MacIlwain, 1943), the yield was not given. This degradation scheme would prove the incorporation of the preformed ring in toto, if experiments were well designed.

Phenazine-1-carboxylic acid and phenazine-1-carboxamide could be degraded according to the above scheme via phenazine-κ-amine. Oxychlororaphine was degraded to the extent that 7 out of 13 carbons were assessed in one single carbon and three pairs of carbons in these studies. Trials to convert phenazine-κ-carboxylic acid to phenazine-κ-amine by the Schmidt reaction (Wolff, 1946) were not successful. To minimize dilution of radioactivity during the degradation
Figure 18 The degradation scheme for pyocyanine, phenazine-1-carboxylic acid, and oxychlororaphine.
of phenazine-ω-carboxylic acid, this reaction needs further study. In using this degradation scheme, however, one has to calculate the specific activity of one single carbon or one carbon-pair from the difference in the specific activities of two compounds, and this gives rise to a greater error, as pointed out by Sheikh & MacDonald (1964). The error would be the greatest, if a uniformly labeled substrate was used as the sole substrate, such as L-leucine-U-Cl\textsubscript{14} used as the sole substrate by Arora (1965).

One may conveniently distinguish between two aspects in the process of phenazine biogenesis: formation of the basic phenazine, and secondary conversions to the derived phenazines. In this respect, a derived phenazine, iodinin, was proven to be synthesized from 1,6-phenazinediol via 1,6-phenazinediol-N-oxide (Lechevalier, 1965); and griseolutein A synthesized from griseoluteic acid (Yagishita, 1960). In this study, it was proven in a radioactive experiment that oxychlororaphine is synthesized from phenazine-ω-carboxylic acid.

Possible methods of phenazine biogenesis have been suggested with oxidative coupling of anilines and phenolic anilines (Hollstein et al., 1966), and dimerization of substituted aniline (Morgan and Aubert, 1962). However, in vivo experiments (Hollstein et al., 1966; Carter and Richards, 1961) indicated that the incorporation of radioactive substrates were low. Moreover, radioactive phenazine was not degraded to prove the incorporation of the
radioactive benzene nuclei in toto.

The fact that, in riboflavin synthesis, ring A was shown to be produced by the coupling of a four carbon fragment with compound G (6,7-dimethyl-8-ribityl-lumazine) indicates that, biogenesis of either one or both benzene rings of phenazine could originate from a route distinct from the established shikimic acid or acetate pathway. In this aspect, the reports of natural occurring pyrazine derivatives (Kosuge & Kamiya, 1962) and quinoxalines (Otzuka & Shoji, 1963) deserve attention.

Of the phenazines, pyocyanine was studied more extensively than others, followed by phenazine-\(\text{N}\)-carboxylic acid and oxychlororaphine. Due to the versatility of the organisms, nutritional studies have not succeeded in finding "an obligate intermediate" (Davis, 1955). However, the importance of glycerol as a precursor (Gessard, 1891) was confirmed by radioisotopic experiments (Blackwood & Neish, 1957; Ingram & Blackwood, 1962; Arora, 1965; and this study). Biogenesis of \(\text{N}\)-methyl group also was first demonstrated with the growing culture (Kurachi, 1959a) and later demonstrated radioactively (Sheikh & MacDonald, 1964), however, the experiment was not designed to demonstrate the transfer of the methyl group in the intact form; the isotope ratio of the methyl group such as \(\text{C}^{14}\text{DH}_2\), \(\text{C}^{14}\text{TH}_2\) or in other isotopic combinations should remain constant, if the methyl group is transferred intact (Ramstad & Agurell, 1964), thus in the experiment where
glycerol-2-c<sup>14</sup> and glycerol-2-H<sup>3</sup> was fed, there was a discrepancy in the incorporation of radioactivity into the N-methyl group (Arora, 1965).

For phenazine-α-carboxylic acid, glycerol was shown also to increase the yield (Kluyver, 1956) and radioactive incorporation studies (Levitch et al., 1964; Arora, 1965; and this experiment) confirmed this finding. For oxychlororaphine too, the same was found to hold (Lasseur, 1911; Sierra et al., 1958; Arora, 1965; and this study). Kögl et al. (1932) reported that phenazine-α-carboxylic acid stimulates oxychlororaphine production by <i>P. chlororaphis</i>. The radioactive experiments in this study showed that oxychlororaphine is produced via phenazine-α-carboxylic acid both by <i>P. chlororaphis</i> and <i>P. aeruginosa</i>.

The other phenomenon, which was observed in all three pigments, was that phosphate ion played an important role in phenazine biosynthesis. Higher phosphate ion concentrations are inhibitory and Levitch et al. (1964) actually used phosphate-free medium to produce phenazine-l-carboxylic acid.

More than half a century has elapsed since the first study on pyocyanine was made by Gessard. In the passage of time more phenazine derivatives have been isolated and more understanding concerning their chemical nature has been obtained. To some extent, formation of certain derived phenazines from the basic phenazines is understood. Yet, these advances have failed to reveal the
mystery of the processes whereby the basic phenazine is biosynthesized. The break-through could occur either through the finding of an active cell-free system, acetone dried cells included, or by studies on biochemical mutants. Basic chemical studies of phenazine especially for its degradation to assess individual carbon atoms and individual nitrogen atoms deserve more effort. Thus, the problems of phenazine biogenesis pose challenging questions for several branches of science. The key to the secret is reserved for an inquisitive mind.
SUMMARY

Nutritional studies on the biogenesis of pyocyanine by washed cell suspensions of *Pseudomonas aeruginosa* Mac 436 revealed that the yield of pyocyanine depends not only on the carbon source, but also on the inoculum, and the composition of the salts in the medium. Some substrates with preformed benzene rings, such as tryptophane, phenylalanine and catechol supported pyocyanine formation whereas o-aminophenol, indole, and anthranilic acid did not act as precursors under the conditions tested. Since catechol, succinate, acetate and DL-alanine supported pigmentation as the sole carbon source, it remains unknown whether the preformed benzene ring was incorporated into pyocyanine *in toto* or after degradation. No essential inorganic ions were required for the washed cell suspensions to produce pyocyanine from D-quinic acid, but the optimum concentration of $\text{HPO}_4^{2-}$ was found to be 0.0002M, and that of $\text{SO}_4^{2-}$ to be 0.08M.

A basal salts solution containing 0.41% MgCl$_2$·6H$_2$O, 0.71% Na$_2$SO$_4$, 0.0005% FeSO$_4$·7H$_2$O, 0.2% urea, 0.010% K$_2$HPO$_4$ was devised. The pH of the medium was adjusted to 7.0-7.2 after the addition of 2% acetate, glucose or glycerol either of which was used as a single carbon source. The media supported not only the formation of pyocyanine, but also the production of phenazine-$\alpha$-carboxylic acid, and oxychlororaphine.
The radioactive experiments were conducted with glycerol-1,3-C$^{14}$, glycerol-2-C$^{14}$, acetate-1-C$^{14}$ as the single substrate for pyocyanine formation. Urea-C$^{14}$ was tested with added unlabeled glycerol and was incorporated into the methyl carbon and the phenazine nucleus of pyocyanine. The ratio of carbon atoms entering α-hydroxyphenazine from carbons 1 plus 3 of glycerol compared to carbon 2 of glycerol showed that neither the shikimic acid pathway nor the acetate pathway was the sole route in the biogenesis of the aromatic rings of the phenazine nucleus of pyocyanine.

Radioactive pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine were biosynthesized simultaneously in Frank and DeMoss’ medium by adding to a heavy suspension of _P. aeruginosa_ Mac 436 acetate-1-C$^{14}$, acetate-2-C$^{14}$, glycerol-1,3-C$^{14}$, glycerol-2-C$^{14}$, succinate-1,4-C$^{14}$, succinate-2,3-C$^{14}$, glucose-1-C$^{14}$, glucose-2-C$^{14}$, glucose-6-C$^{14}$, and shikimate-1,6-C$^{14}$. The ratio for carboxyl carbon of acetate entering the phenazine rings of pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine as compared to the methyl carbon of acetate showed that the acetate pathway was not involved in the biosynthesis of these phenazines. The ratio for the carbon atoms 1 plus 3 of glycerol entering the phenazine rings of pyocyanine, phenazine-α-carboxylic acid and oxychlororaphine as compared to the carbon 2 of glycerol showed that shikimic acid was not the sole intermediate from which the phenazine
nucleus was synthesized. A similar ratio for the carbons entering phenazine-α-carboxylic acid and oxychlororaphine from the carbons 1 plus 3 of glycerol as compared to the carbon 2 of glycerol showed that the carboxylic carbons of phenazine-α-carboxylic acid and oxychlororaphine did not originate from the carboxylic group of shikimic acid. The fact that glucose-1-C\textsubscript{14} and shikimic acid-1,6-C\textsubscript{14} labeled the carboxyl carbon of phenazine-α-carboxylic acid and oxychlororaphine showed also that the carboxyl carbons of phenazine-α-carboxylic acid and oxychlororaphine did not originate from the carboxyl carbon of shikimic acid.

Pyocyanine and oxychlororaphine were degraded chemically to the extent that 7 carbons out of 13 carbons were assessed as one single carbon and three pairs of carbons. The distribution of radioactivities among the degradation products could not be explained by biogenesis of the pigments through the shikimic acid pathway.

In all radioactive experiments, the incorporation of radioactive substrates was low, although all substrates tested were actively metabolized as evidenced by the recovery of high radioactivity in the respiratory CO\textsubscript{2} and in the cells.

Using radioactive phenazine-α-carboxylic acid, _P. chlororaphis_ and _P. aeruginosa_ were found to synthesize oxychlororaphine from phenazine-α-carboxylic acid.
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APPENDIX I

ISOLATION OF 2-HYDROXYPHENAZINE-1-CARBOXYLIC ACID FROM P. AUREOFACIENS MAC 371

INTRODUCTION

During the preparation of carrier phenazine-α-carboxylic acid by the biosynthetic method described by Haynes et al. (1956), it was found that there was always an accompanying orange pigment aside from phenazine-1-carboxylic acid, detectable on TLC (silica gel G, developed with MeOH:CHCl₃, 1:9) even after purification by sublimation as described by Arora (1965). This accompanying pigment was isolated. At that time, Toohey et al. (1965) reported that P. aureofaciens produces 2-hydroxyphenazine-1-carboxylic acid. Then Levitch et al. (1966) reported that P. aureofaciens produces 2-hydroxyphenazine. Therefore, the sample was characterized by infrared spectroscopy and precision mass spectroscopy to determine whether it was 2-hydroxyphenazine-1-carboxylic acid or 2-hydroxyphenazine.

ISOLATION

P. aureofaciens, Mac 371, NRRL B-1543-c, was grown in the medium of Haynes et al. After five days' incubation the broth was acidified to pH 2 and extracted with chloroform. The chloroform extract was washed with 2.5% NaOH, and the aqueous layer was re-acidified and re-extracted
with chloroform. The chloroform extract was dried over 
\( \text{Na}_2\text{SO}_4 \) overnight, then filtered to remove \( \text{Na}_2\text{SO}_4 \). The 
dried chloroform extract was evaporated to reduce its vol­
ume, and streaked on a thin-layer plate of silica gel G. 
The plate was developed in \( \text{MeOH}:\text{CHCl}_3 \) (1:9). The orange 
band \((R_f=0.35)\) was scraped off, and eluted with ammoni­
cal methanol \((\text{MeOH}:\text{conc. NH}_4\text{OH, 100:1})\). The eluate was 
evaporated to dryness, acidified and extracted with chloro­
form. The chloroform extract was evaporated to dryness 
and the residue of evaporation was sublimed under vacuum 
at \(180^\circ\text{C}\). The sublimate was homogeneous on TLC, but did 
not crystallize well from a \(\text{MeOH}:\text{H}_2\text{O}\) pairs, or from a 
chloroform:ether pairs or from dimethylformamide.

PRELIMINARY CHARACTERIZATION

Infrared Spectra

The infrared spectra were recorded in solid state 
in potassium bromide, using a Perkin-Elmer Grating Infra­
ed Spectrophotometer model 337, by Mr. Y. C. Lin, Depart­
ment of Chemistry, McGill University, Montreal.

Interpretation:- There is strong absorption at 760 
\(\text{cm}^{-1}\) corresponding to the absorption of four adjacent 
aromatic hydrogens (Stammer and Taurins, 1963), indicating 
that one benzene ring has no substituents. There is also 
strong absorption at 850 \(\text{cm}^{-1}\) corresponding to the 2-
substitution. The absence of any significant absorption 
in the 860-900 \(\text{cm}^{-1}\) indicates 1,2-substitution. Moreover,
Figure. Infrared spectrum of the unknown compound.
Figure. Infrared spectrum of the phenazine-1-carboxylic acid.
Figure: Mass spectrum of the unknown compound.
the carbonyl stretching band which occurs as a broad band between 1700-1750 cm$^{-1}$ in the spectrum of phenazine-1-carboxylic acid is displaced to 1670 cm$^{-1}$. This is a characteristic shift observed when an aromatic carboxyl group forms a hydrogen-bond chelate with an adjacent hydroxyl group (Bellamy, 1958).

**Mass spectrum**

The mass spectrum of the sample and the interpretation was provided by Dr. G. P. Arsenault, Atlantic Regional Laboratory, National Research Council of Canada, Halifax.

Interpretation:- The molecular ion is clearly at m/e 240. The loss of CO$_2$ from m/e 240 followed by the loss of CO from m/e 196 is established by the presence of the corresponding metastable peak at m/e 160 and 144.

**CONCLUSION**

From the spectra, the sample was tentatively identified as 2-hydroxyphenazine-1-carboxylic acid.

**NOTE**

Attempts were made in vain to synthesize authentic 2-hydroxyphenazine-1-carboxylic acid from 5-amino-salicylic acid and nitrobenzene, following the method of Kögl and Postowsky (1930) for the synthesis of phenazine-1-carboxylic acid.
DISCUSSION

Levitch et al. (1966) reported that \textit{P. aureofaciens}, NRRL-B1543 produces 2-hydroxyphenazine in Haynes et al.'s medium and the results reported here indicate that the same organism produces 2-hydroxyphenazine-1-carboxylic acid. Toohey et al. (1965) reported that certain soil bacteria can convert 2-hydroxyphenazine-1-carboxylic acid to phenazine-1-carboxylic acid. This indicates that 2-hydroxyphenazine-1-carboxylic acid can be dehydroxylated, and may be the obligatory intermediate step prior to the formation of phenazine-1-carboxylic acid. However, Levitch et al. showed that washed cell suspensions of \textit{P. aureofaciens} produces about three times more 2-hydroxyphenazine than phenazine-1-carboxylic acid during 12 hours of incubation. This indicates that 2-hydroxyphenazine may be a precursor of phenazine-1-carboxylic acid, i.e., possibly carboxylated and dehydroxylated before the formation of phenazine-1-carboxylic acid.

The biological interconversion of these three phenazine derivatives produced by \textit{P. aureofaciens} would make an interesting study. If 2-hydroxyphenazine-1-carboxylic acid could be proven as a precursor of phenazine-1-carboxylic acid, it would provide a clue to the formation of both or one carbocyclic ring of the phenazine.
APPENDIX II*
COMPUTING PROGRAMME

DIMENSION C(99), CR(99), D(99), T(99)
READ 1, NOS, NOST, NOC, NOB, W
1 FORMAT (4(2,F8.1))
PRINT 1000, NOS, NOST, NOC, NOB, W
1000 FORMAT (1H, 4(3,F8.1))

M = 0
BG = 0.
ST = 0.
XNOST = NOST
XNOB = NOB
20 DO 21 J = 1, NOC
READ 2, T(J), C(J)
2 FORMAT (3X, F5.2, F7.0)
21 CR(J) = C(J) / T(J)
3 M = M + 1
PRINT 500, M
500 FORMAT (1H, 4H M = , I3)

NO = NOC
KIK = 3
KAN = 0
4 S = 0.
SS = 0.
DO 5 J = 1, NO
5 S = S + CR(J)
XNO = NO
AV = S / XNO
NO = XNO
DO 6 J = 1, NO
D(J) = CR(J) - AV
6 SS = SS + D(J)**2
XNO = NO
STD = SQRTF(SS / (XNO - 1.))
CHISQ = SS / AV
PSTD = STD * 100. / AV
KIK = KIK - 1
NO = XNO
CF = 1.96
TEST = CF * STD
DO 7 J = 1, NO
JJ = NO + 1 - J
IF (TEST - ABSF(D(JJ))) 100, 7, 7
100 TEMP = CR(NO)
CR(NO) = CR(JJ)
CR(JJ) = TEMP
KAN = KAN + 1
7 CONTINUE
NO = NO - KAN
IF (KAN) 8, 8, 200
200 IF (KIK) 8, 8, 4
8 PRINT 9, (CR(J), J = 1, 10)
9 FORMAT (1H, 3HNO , 10F8.1)
COMPUTING PROGRAMME (continued)

PRINT 10, AV, STD, PSTD, CHISQ, KAN
10 FORMAT(1H, 5HA = , F8.1, 3X, 9HSTD DEV = , F6.1, 3X,
14HPERCENT STD = , 1F5.1, 3X, 8HCHISQ = , F5.1,
16HCANCELLED DATA =, 12)
IF (M-NOB) 12, 12, 300
300 IF (M-NOB=NOST) 15, 15, 400
400 IF (M-NOS) 800, 16, 800
800 AV=AV/F

PRINT11, AV
11 FORMAT(1H, 20X, 6HDPM = , F15.6)
GO TO 13
12 BG=BG+AV/XNOB
PRINT 30, BG
30 FORMAT(1H, 8HAV BG = , F15.6)
13 DO 14J=1, NOC
   READ22, T(J), C(J)
22 FORMAT(3X, F5.2, F7.0)
14 CR(J)=C(J)/T(J)-BG
GO TO 3
15 ST=ST+AV/XNOST
PRINT 17, ST
17 FORMAT(1H, 5HST = , F15.6)
   F=ST/W
PRINT 18, F
18 FORMAT(1H, 15HCOUNTING EFF = , F7.4)
GO TO 13
16 AV=AV/F
PRINT 111, AV
111 FORMAT(1H, 20X, 6HDPM = , F15.6)
   CALL EXIT
END

*This programme was deposited in the Packard Computer Program Library as program NO. 01671.
FLOW DIAGRAM

\[ CR(j) = \frac{C(j)}{T(j)} \]

- \( M = 0 \)
- \( READ NOS, NOST, NOC, NOB, W \)
- \( READ NOC CARDS T(j), C(j) \)
- \( CR(j) = \frac{C(j)}{T(j)} \)
- \( M = M + 1 \)
- \( KIK = 3 \)
- \( COMPUTE CR, \sigma, \%\sigma, \chi^2, DEV(j) \)
- \( KIK = KIK - 1 \)
- \( TEST - |DEV(j)| \)
- \( PRINT M, CR(j), CR, \sigma, \%\sigma, KAN \)
- \( M = NOB \)
- \( G : E \)
- \( L : E \)
- \( BG = BG - \frac{CR}{XNOB} \)
- \( ST = ST - \frac{CR}{XNOST} \)
- \( AV = \frac{CR}{F} \)
- \( PRINT AV \)
- \( PRINT AV \)
- \( STOP \)
APPENDIX III

An example of a radioactive experiment described in detail

Medium: Frank and DeMoss' (1959) pH 7.05, 500 ml. in 2L Erlenmeyer's flask, total volume, 1L medium.

Inoculum: Washed cell suspension of _P. aeruginosa_ Mac 436 from nutrient broth which was incubated at 30°C. on rotary shaker for 24 hours. Five ml. of washed cell suspension was used per flask.

Radioactive substrate:

DL-shikimic acid-1,6-Cl^4, 100 μc; sp. activity 10.15 mc/mM (Schwarz BioResearch Inc. Catalog No. 6423-76F, Lot No. 6601P). The substrate was dissolved in water and made to 100 ml., 50 ml. was added per flask.

Incubation:

30°C. on rotary shaker for 5 days.
SEPARATION OF PRODUCT

Broth → H₂SO₄ (1:1) to pH 1.9

Ether ext'n (continuous)

H₂O layer

Centrifuge

Supernatant

H₂O → Centrifuge → Cells

H₂O → Centrifuge → H₂O

H₂O → Centrifuge → Cells

H₂O → Cells

Made to 100 ml. (vol.) cell

Pyocyanine fraction

Phenazine-α-carboxylic acid fraction

Oxychlororaphine fraction

1N NaOH → Ether → H₂O → Dry over Na₂SO₄

Filtration

Filtrate

evaporate to dryness

NaOH → Ether → H₂O

H₂SO₄ (1:1) to pH 2.0

CHCl₃ ext'n (continuous)

CHCl₃

Flash evaporate to dryness
**AMIDE FRACTION**

Sublimate (2X), 170-180°C, under vac.

TLC (silica gel G) CHCl₃:MeOH (1:1)

Cut & elute (with CHCl₃ & acetone)

- eluant
  - flash evaporate to dryness
  - refluxing acetone
  - filtration
  - filtrate
  - conc'n & crystallization acetone
  - filtration ether
  - filtrate crystal

- dry at 80°C.

Yield 41.6 mgm

m.p. 185-237°C (uncorrected)

Rechromatog. TLC (silica gel G), acetone
cut & elute (with CHCl₃)

- eluant
  - evaporate to dryness on steam bath acetone
  - filtration
  - filtrate
  - conc'n & crystallization (acetone:H₂O pair) ether
  - filtration acetone
  - filtrate H₂O crystal
  - Mother liquid dry at 80°C.

Use 0.4 ml. for radioactivity measurement

made to 10 ml. CHCl₃ (redistilled)

yield 11.2 mgm

mp. 244°C. sharp (uncorr.)

TLC one spot
ACID FRACTION

TLC on silica-gel G. CHCl₃:MeOH (9:1)
Cut & eluted with MeOH:conc. NH₄OH (100:1)
filtration
eluant
evaporate to dryness
\[ \text{1N HCl} \]
CHCl₃ ext’n
CHCl₃
evaporate to dryness
\[ \text{MeOH} \]
reflux
filtration
filtrate
conc’n & crystallization
filtration
\[ \text{ether} \]
\[ \text{acetone} \]
\[ \text{H₂O} \]
Mother liquor
Crystalline product
conc’n & crystallization
filtration
crystalline product (2nd crop)
dry at 80°C.
crystalline product
\[ \text{2.5 ml. conc. H₂SO₄} \]
\[ \text{H₂O} \]
filtration
yield 47.5 mgm
m.p. 238-241°C (uncorr.)
TLC one yellow
fluorescent
impurity
ACID FRACTION (continued)

waste

residue

H₂O (wash until free of acid)

MeOH

reflux

filtration

filtrate

crystallization

filtration

mother liquor    crystalline product

dry at 80°C. oven

Yield 7.2 mgm

m.p. 238-241°C. (uncorr.)

TLC one yellow fluorescent impurity
ACID FRACTION (continued)

All crystals (47.5 + 7.2 = 54.7 mgm)
sublimate at 170-180°C., under vac.

TLC. one yellow fluorescent impurity

CHCl₃ ext'n

H₂O layer

H₂SO₄ (1:1), to pH 2

CHCl₃ ext'n

H₂O

CHCl₃

dry over Na₂SO₄

filtration

filtrate

crystallization

filtration

ether

H₂O

crystalline product

use 0.3 ml. made to dry at 80°C. oven
for combustion 10 ml.

Yield 12.9 mgm
m.p. 242-243°C. (uncorr.)

TLC pure
Flash evaporate to dryness

Ether ext'n

H₂O layer

← NaHCO₃

CHCl₃ ext'n

CHCl₃

← 0.1N HCl

H₂O layer

← NaHCO₃

CHCl₃ ext'n

CHCl₃

Dry over Na₂SO₄

Filtration

Filtrate

Flash evaporate to dryness

CHCl₃ redistilled

Petroleum ether b.p. 37.1-54.3°C.

Kept in refrigerator

Filtration under suction

Crystalline product

Dry over P₂O₅ under vac., at room temp.

Yield 323.4 mgm

weigh out 9.9 mgm, dissolve in 10 ml. 0.2N HCl

use 0.5 ml. for radioactivity measurement
### Per cent distribution of radioactivity

<table>
<thead>
<tr>
<th>Fractions</th>
<th>dpm</th>
<th>Hyamine correction factor</th>
<th>dpm corrected</th>
<th>$\mu$C</th>
<th>Amount used for combustion $\mu$M</th>
<th>Specific activity $\mu$C/$\mu$M</th>
<th>Total activity dpm</th>
<th>Dist. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyocyanine</td>
<td>1,005.6</td>
<td>10</td>
<td>10,056</td>
<td>4.530</td>
<td>$9.9 \times \frac{0.5}{10}$</td>
<td>2.346</td>
<td>1.930</td>
<td>4.93x10^8 2.22</td>
</tr>
<tr>
<td>Phennazine</td>
<td>723.3</td>
<td>10</td>
<td>7,233</td>
<td>3.258</td>
<td>$12.9 \times \frac{0.3}{10}$</td>
<td>1.728</td>
<td>1.885</td>
<td>2.41x10^5 0.11</td>
</tr>
<tr>
<td>Phenazine</td>
<td>723.3</td>
<td>10</td>
<td>7,233</td>
<td>3.258</td>
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<td>1.728</td>
<td>1.885</td>
<td>2.41x10^5 0.11</td>
</tr>
<tr>
<td>Oxychloro-raphine</td>
<td>502.9</td>
<td>10</td>
<td>5,029</td>
<td>2.265</td>
<td>$11.4 \times \frac{0.4}{10}$</td>
<td>2.045</td>
<td>1.108</td>
<td>1.26x10^5 0.06</td>
</tr>
<tr>
<td>CO2</td>
<td>329.9</td>
<td>10</td>
<td>3,299</td>
<td>1.486</td>
<td>0.1 $\frac{498}{100}$</td>
<td>-</td>
<td>-</td>
<td>1.64x10^7 7.39</td>
</tr>
<tr>
<td>Cells</td>
<td>1,985.9</td>
<td>10</td>
<td>19,859</td>
<td>8.945</td>
<td>0.1 $\frac{100}{100}$</td>
<td>-</td>
<td>-</td>
<td>1.99x10^7 8.96</td>
</tr>
</tbody>
</table>

*"dpm" is an output of computer (see Appendix II)*

Sample calculation:

Total radioactivity of pyocyanine = $10,056 \times \frac{323.4}{0.495} = 4.93 \times 10^8$
DEGRADATION OF PYOCYANINE

Hemipyocyamine Formation:

Pyocyanine \((106.8 + 9.9 \times 9.5 = 116.1 \text{ mgm})\)

\[ \xrightarrow{50 \text{ ml. } 1\text{N} \text{NaOH}} \]

Bubbling \(O_2\) in a \(50^\circ\text{C}\). water bath for 50 min.

\[ \xrightarrow{4\text{N} \text{HCl to pH 2}} \]

\(\text{CHCl}_3\) ext'n

\[ \xrightarrow{\text{Evaporate to dryness}} \]

Sublimation under vac. 120-130\(^\circ\text{C}\)., twice

Yield 84.9 mgm

m.p. 157-158\(^\circ\text{C}\). (uncorrected)

weigh out 10.5 mgm dissolve in 10 ml.

redistilled \(\text{CHCl}_3\), use 0.5 ml. for combustion

Oxidation of Hemipyocyamine:

Hemipyocyamine (99.9 mgm)

\[ \xrightarrow{10 \text{ ml. } 1\text{N HCl}} \]

Heat to dissolve

\[ \xrightarrow{\text{Cool in ice bath}} \]

\[ \xrightarrow{10 \text{ ml. } 1\text{N NaOH}} \xrightarrow{\text{stir in } 672 \text{ mgm } \text{KMnO}_4} \]

Left at room temp. overnight

\[ \xrightarrow{1\text{N NaOH to pH 11}} \]

Filtration through a sintered glass filter

\[ \xrightarrow{\text{Filtrate}} \]

\[ \xrightarrow{10 \text{ ml. } \text{MeOH}} \xrightarrow{4\text{N HCl to pH 2.8}} \]

Flash evaporate to small vol.

\[ \xrightarrow{\text{Column chromatog. (Dowex 50w-X } H^+ \text{ form)}} \]

elute with \(H_2O\)
Oxidation of Hemipyocyanine (continued)

\[ \text{collect } \lambda_{max} \text{ fractions 19-29} \]
\[ \text{pyrazine-tetra-carboxylic acid fraction} \]
\[ \text{collect } \lambda_{max} \text{ fractions 30-45} \]

Quinoxaline-dicarboxylic acid fraction

Quinoxaline-dicarboxylic acid:

\[ \text{Flash evaporate to dryness} \]
\[ \text{Heat to boil} \]
\[ \text{Filtrate} \]
\[ \text{Flash evaporate to dryness} \]
\[ \text{To 5 ml. vol., use 1 ml. for combustion} \]
\[ \text{yield 5 mgm} \]

Pyrazine-tetra-carboxylic acid:

\[ \text{Flash evaporate to dryness} \]
\[ \text{To 5 ml. vol., use 0.1 ml. for combustion} \]
\[ \text{yield 55.9 mgm} \]
Decarboxylation of quinoxaline-dicarboxylic acid:

Quinoxaline-dicarboxylic acid (7.4 mgm)
----- Diluent quinoxaline-dicarboxylic acid (25.6 mgm)
----- Absolute EtOH
----- 0.3 ml. conc. NH₄OH

Dry in a long tube submerged into boiling water-bath by passing N₂

Pyrolysis (230-260°C.)

Quinoxaline collected at the water-cooled end of tube

Cut the end containing quinoxaline

Ether

HgCl₂ (saturated aqueous sol'n) 2 ml.

Filtration

Residue

Air drying

Sublimation under vac. 110-125°C.

Sublimate (mercuric chlorid salt of quinoxaline)

yield 3.9 mgm (quinoxaline)

Dissolve in 5 ml. 1N HCl, use 2 ml. for combustion
DEGRADATION OF OXYCHLORORAPHINE

Phenazine-α-amino formation:

Oxychlororaphine (10.8 mgm)

\[ \text{Diluent oxychlororaphine (89.4 mgm)} \]

\[ \text{MeOH} \]

Reflux

\[ \text{Cool in refrigerator} \]

\[ \text{5 ml. NaOMe} \]

\[ \text{2.5 ml. Br}_2 \text{ (1:99 in chilled CHCl}_3 \text{)} \]

Evaporate to dryness in a steam-bath

\[ \text{200 ml. of 20% NaOH} \]

Reflux with an air condenser attached

\[ \text{Cool at room temp.} \]

\[ \text{CHCl}_3 \text{ ext'n} \]

\[ \text{CHCl}_3 \]

\[ \text{1N HCl} \]

\[ \text{H}_2\text{O layer} \]

\[ \text{NaOH to alkaline} \]

\[ \text{Ether ext'n} \]

\[ \text{Ether} \]

\[ \text{Dry over Na}_2\text{SO}_4 \]

\[ \text{Filtration} \]

\[ \text{Filtrate} \]

\[ \text{Evaporate to dryness} \]

\[ \text{Sublimation under vac., 160-170°C., twice} \]

Sublimate

yield 34.6 mgm

m.p. 179-182°C. (uncorrected)

Dissolve in 10 ml. redistilled CHCl₃, use 0.4 ml. for combustion
Oxidation of phenazine-α-amine:

Phenazine-α-amine (32.9 mgm)

← Diluent phenazine-α-amine (68.5 mgm)
← 10 ml. 1N HCl
← 10 ml. 1N NaOH
← Stir in KMnO₄ (545 mgm)

Left at room temp., overnight
← NaOH to pH 11

Filtration through a sintered glass filter

Filtrate
← 4N HCl to pH 2.5

Flash evaporate to small vol.

Column chromatog. (Dowex 50w-x8 H⁺ form, elute with H₂O)

collect λmax 282 mμ, fraction 17-20

pyrazine-tetra-carboxylic acid fraction

collect λmax 325 mμ, fraction 21-61

quinoxaline-dicarboxylic acid fraction

Quinoxaline-dicarboxylic acid fraction:
treated as outlined for quinoxaline-dicarboxylic acid fraction from degradation of pyocyanine

yield 70.0 mgm
dissolved in 5 ml. 0.2N Na₂CO₃, use 0.3 ml. for combustion

Pyrazine-tetra-carboxylic acid fraction:
treated as outlined for pyrazine-tetra-carboxylic acid fraction from degradation of pyocyanine

yield 26.2 mgm
dissolved in 5 ml. 1N HCl, use 0.1 ml. for combustion

Decarboxylation of quinoxaline-dicarboxylic acid:

proceeded as outlined for pyrolysis of quinoxaline-dicarboxylic acid in degradation of pyocyanine

yield 7.8 mgm
dissolved in 10 ml. 1N HCl, use 7 ml. for combustion
DEGRADATION OF PHENAZINE-κ-CARBOXYLIC ACID

Phenazine-κ-carboxamide formation:

Phenazine-κ-carboxylic acid (12 mgm) ← Diluent phenazine-κ-carboxylic acid (92.6 mgm) ↓ 2 ml. Thionylchloride
Reflux in a steam-bath for 75 min.

Suction to dryness ← 4 ml. conc. NH₄OH

Left at room temp. for 3 hours ↓
Decantation

H₂O layer to acid ↓ Residue
4N HCl to acid ↓ Air drying

CHCl₃ ext'n

CHCl₃ ← 1N NaOH

CHCl₃
Dry over Na₂SO₄ ↓
Filtration

Filtrate Evaporate to dryness

Reflux with MeOH ↓ Filtration

Filtrate Crystallization ↓ Ether

Filtration Crystallization product ↓ H₂O

Dry at 80°C. oven

Crude oxychlororaphine
yield 58.8 mgm

Phenazine-κ-amine:
Phenazine-κ-carboxamide was transformed to phenazine-κ-amine as outlined at the degradation of oxychlororaphine

yield 7.2 mgm
m.p. 179-181°C.
dissolved in 10 ml. redistilled CHCl₃, use 0.5 ml. for combustion
Degradation of Pyocyanine

<table>
<thead>
<tr>
<th>Products</th>
<th>dpm</th>
<th>Hyamine correction factor</th>
<th>dpm corrected</th>
<th>Amount used for combustion mgm</th>
<th>µM</th>
<th>Dilution factor</th>
<th>µM corrected</th>
<th>Specific activity dpm/µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyocyanine</td>
<td>1,005.6</td>
<td>10</td>
<td>10,056</td>
<td>9.9x0.5</td>
<td>2.346</td>
<td>1</td>
<td>2.346</td>
<td>4,286</td>
</tr>
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<tr>
<td>Phenazinol</td>
<td>1,006.4</td>
<td>10</td>
<td>10,064</td>
<td>10.5x0.5</td>
<td>2.917</td>
<td>1</td>
<td>2.917</td>
<td>3,450</td>
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</tr>
<tr>
<td>Quinoxaline</td>
<td>3,719.5</td>
<td>5</td>
<td>18,598</td>
<td>5x1</td>
<td>4.587</td>
<td>1</td>
<td>4.587</td>
<td>4,055</td>
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<tr>
<td>dicarboxylic acid</td>
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</tr>
<tr>
<td>Pyrazine</td>
<td>3,906.6</td>
<td>5</td>
<td>19,533</td>
<td>55.9x0.1</td>
<td>4.367</td>
<td>1</td>
<td>4.367</td>
<td>4,473</td>
</tr>
<tr>
<td>tetra-carboxylic acid</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>684.9</td>
<td>5</td>
<td>3,425</td>
<td>3.9x2</td>
<td>12.000</td>
<td>29.6</td>
<td>1.622</td>
<td>2,112</td>
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<td></td>
</tr>
</tbody>
</table>
Degradation of oxychlororaphine

<table>
<thead>
<tr>
<th>Products</th>
<th>dpm</th>
<th>Hyamine corr. factor</th>
<th>dpm corrected for Hyamine</th>
<th>Amount used for combustion mgm</th>
<th>μM</th>
<th>Dilution factor</th>
<th>μM corr. for dilution</th>
<th>Spec. act. dpm/μM on oxychlororaphine basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxychlororaphine</td>
<td>502.9</td>
<td>10</td>
<td>5,029</td>
<td>11.4x0.4 = 0.456</td>
<td>2.045</td>
<td>1</td>
<td>2.045</td>
<td>2,459</td>
</tr>
<tr>
<td>Phenazine-κ-amine</td>
<td>197.3</td>
<td>10</td>
<td>1,973</td>
<td>34.6x0.5 = 1.73</td>
<td>8.357</td>
<td>100.2</td>
<td>0.901</td>
<td>2,190</td>
</tr>
<tr>
<td>Quinoxaline dicarboxylic acid</td>
<td>229.3</td>
<td>10</td>
<td>1,147</td>
<td>70x0.3 = 4.2</td>
<td>19.266</td>
<td>101.4</td>
<td>6.251</td>
<td>184</td>
</tr>
<tr>
<td>Pyrazine-tetra-carboxylic acid</td>
<td>33.5</td>
<td>10</td>
<td>168</td>
<td>26.21x0.1 = 0.52</td>
<td>2.048</td>
<td>101.4</td>
<td>0.664</td>
<td>253</td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>234.9</td>
<td>10</td>
<td>1,175</td>
<td>7.8x7 = 5.46</td>
<td>42.000</td>
<td>1</td>
<td>42.000</td>
<td>28</td>
</tr>
</tbody>
</table>

*Sample calculation:  
\[
28 \times \frac{101.4}{32.9} \times \frac{100.2}{10.8} = 801
\]

\[
\frac{101.4}{32.9} \quad \text{dilution factor for phenazine-κ-amine}
\]

\[
\frac{100.2}{10.8} \quad \text{dilution factor for oxychlororaphine}
\]
# Degradation of Phenazine-α-carboxylic Acid

<table>
<thead>
<tr>
<th>Products</th>
<th>dpm</th>
<th>Hyamine correction factor</th>
<th>dpm corrected for Hyamine</th>
<th>Amount used for combustion mgm</th>
<th>µM</th>
<th>Dilution factor</th>
<th>µM corrected for dilution</th>
<th>Specific activity dpm/µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenazine</td>
<td>723.3</td>
<td>10</td>
<td>7,233</td>
<td>12.9 x 0.3</td>
<td>1.728</td>
<td>1</td>
<td>1.728</td>
<td>4,186</td>
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<tr>
<td>-α-carboxylic acid</td>
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<tr>
<td>Phenazine</td>
<td>40.5</td>
<td>10</td>
<td>405</td>
<td>7.2 x 0.5</td>
<td>1.739</td>
<td>104.6</td>
<td>0.212</td>
<td>1,910</td>
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<tr>
<td>-α-amine</td>
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1.728 x 10 = 0.337
7.2 x 0.5 = 0.36