A STUDY OF CARBOHYDRATE METABOLISM AND PIGMENT
FORMATION IN PSEUDOMONAS AERUGINOSA
STRAIN NUMBER 20094

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A STUDY OF CARBOHYDRATE METABOLISM
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This thesis is approved as a creditable independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.
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INTRODUCTION

This study of Pseudomonas aeruginosa has been conducted to obtain information of carbohydrate metabolism in respect to pigment formation. It is a known fact that microorganisms, like higher forms of life, require specific substrates in the never ending process of metabolism. Various phases of dissimilation and assimilation shall be discussed in this paper.

The organism used in this study is a strain of P. aeruginosa isolated from the intestinal tract of a carp caught in the Sioux River near Brookings, South Dakota. The organism differs from Pseudomonas aeruginosa (Schroeter) Migula in a number of characteristics:

1. this organism is non-motile and non-flagellated,
2. growth occurs with equal abundance in a range from 25 to 37 degrees centigrade,
3. the pigment produced does not fluoresce,
4. this strain always produces indole, and
5. glucose is always utilized as a source of energy.

The organism was compared culturally with Pseudomonas aeruginosa (Schroeter) Migula 10197, L. H. Schwartz and J. A. Lazarus N.Y.C. strain P7, 1947, from the American Type Culture Collection. The organism P. aeruginosa strain 20094 differs in the characters given from the American Type Culture Collection and is not identical with any described in Bergey's Manual of Determinative Bacteriology. It should perhaps be considered as a new species of the genus Pseudomonas. This culture is carried
in the South Dakota State College Bacteriological Laboratory under the name *Pseudomonas aeruginosa* strain 20094. This particular strain of *P. aeruginosa* has been seeded over anaerobic cultures in this laboratory as a means of producing an anaerobic condition.

A review of the literature shows that a water soluble pigment, pyocyanin or a pyocyanin derivative, seems to be present in many of the Pseudomonads. Wrede and Strack\(^2\) isolated pyocyanin from *Bacillus pyocyaneus*. Kuhn and Schon\(^3\) first synthesized pyocyanin according to the following reaction:

\[
\begin{align*}
\text{Radical Salt} & \quad +2 \text{OH}^- & \rightarrow & \quad \text{Pyocyanin} & \quad +2\text{H}_2\text{O} \\
\text{ClO}_4^- & \quad & & \quad \text{Leuco} & \quad +2\text{CO}_4^- \\
\end{align*}
\]

Steeman\(^4\) postulated that decolorization and regeneration of the color in the presence of oxygen is a biological process which does not take place when the pigment is separated from living cells. The change of pyocyanin takes place only under such conditions as to indicate that the pigment functions as a hydrogen carrier.

Friedheim and Michaelis\(^5\) studied the blue pigment and noted it is reversibly oxidizable and reducible. They were
able to show in the range of pH greater than 6 it behaves entirely as a reversible dye of quinoid structure. At pH less than 6 the titration curves show two hydrogen atoms are accepted in two separate steps. On reduction in acid reaction, the color changes from red to green to colorless. In alkaline reaction the color change takes place in one step, blue to colorless. For this reason it has been used in some experiments as an accessory respiratory ferment. 6

Great difficulty has been encountered in attempting to isolate intermediate compounds of microbial metabolism because of the extremely small amounts of the respective intermediate products. Norris and Campbell 7 report that P. aeruginosa oxidizes its substrate rapidly, and because of this strong oxidative power, few of the intermediate compounds can be isolated for study under normal physiological conditions. By means of paper chromatography and an early assay of the culture (sixteen to twenty-four hours) they were able to show the presence of some of the intermediates in glucose dissimilation:

METHODS

Stock cultures of P. aeruginosa strain 20094 were maintained on nutrient agar (Difco) slants and incubated at 37 degrees centigrade. A bluish-green pigment appeared after a period of 48 hours. This pigment darkened intensively upon further standing. At room temperature, little difference in
intensity was noted. The organism, therefore, seems to be quite thermal adaptive, at least within the range of 25 degrees to 37 degrees centigrade.

The pigment soon became dispersed throughout the nutrient agar medium, indicating it was water soluble or partially water soluble. The actively growing slant cultures were washed with distilled water to remove the pigment. Water washing the slants proved to be of little benefit because of the tenacious adherence of the pigment to the agar. Maceration of the agar with a spatula and suspending the pigmented agar particles in distilled water overnight, proved to be of little value, the pigment failed to wholly enter the solvent. Another portion of a prepared slant culture was macerated, placed in a Seitz filter, and continually extracted with warm water. This was no more effective than the previous method.

A series of organic compounds were tested in an attempt to establish a better solvent. The pigment was found to be very soluble in chloroform, but limited amounts of cultural growths restricted attempts to harvest the pigment from slant cultures and this method was discontinued.

Pseudomonas aeruginosa strain 20094 was found to thrive exceedingly well on nutrient broth (Difco). Maximum pigmentation appeared between the 48 to 72 hour growth period. The nutrient broth was centrifuged in an International Equipment Company centrifuge at 3000 r.p.m. for ten minutes, the supernatant liquid was filtered through a Seitz filter to obtain a rela-
tively bacteria free liquid. This supernatant liquid was treated with a saturated solution of ammonium sulfate to precipitate the suspended protein material of the original medium. The supernatant liquid was decanted and filtered to give the water soluble pigment.

The supernatant liquid, containing the pigment, was extracted in a separatory funnel with chloroform. Chloroform was added to give a fifty per cent chloroform mixture by volume. During the extraction of the pigment, a fat or fat-like substance was noted at the interface of the two liquids. This fat-like substance was not soluble in the chloroform fraction and evaporated upon heating on a steam bath. This substance was not ether soluble and had a sweet, aromatic odor. The colored solution was decanted and evaporated on a steam bath leaving a green waxy residue. Further heating caused the deposit to evaporate, indicating volatility of the pigment.

Several attempts were made to concentrate the pigment for qualitative and quantitative analysis. However, this proved to be futile in that the end product was not pure and was recovered in such small quantities (less than one milligram) that such determinations were not possible.

Another method, employing basic and acidic solvents, was used and found to be more successful in obtaining the final product. The pigment, in the liquid culture, was extracted with chloroform. The latter portion was decanted and added to distilled water, previously acidified with sulfuric acid. The
pigment was dispersed throughout the acidic solvent and immediately turned from a Prussian blue to a rosy red color. The acidic fraction was decanted and treated with a strong potassium hydroxide solution. This immediately caused a color change, red to blue. This latter fraction was used for obtaining spectrophotometric data (Figure 1, page 10).

A quantitative analysis of pigment production in relationship to time, was attempted by using colorimetric procedures. The previously described process of media clarification was employed. Cultures were analyzed every 24 hours for a change in pigment intensity. The pigment was visually observed at the 60 hour growth period with greater intensities of color being noted at subsequent intervals. This procedure was of little value in that the Coleman colorimeter (Model 8) was not sensitive enough to record the visible change. This portion of the problem was consequently abandoned.

Chemically defined media were required to study the metabolic processes. Various carbon sources affiliated with the tricarboxylic acid cycle were used as the sole carbon source. Inorganic salts, as previously determined for essential growth, were added.

The chemically defined media consisted of:

\[(NH_4)_2SO_4 \quad \text{--------- 0.10%}\]
\[MgSO_4 \quad \text{--------- 0.025%}\]
\[K_2HPO_4 \quad \text{--------- 0.025%}\]
[Carbon source \quad \text{--------- 1.0%}\]
The carbon sources were: glucose, sodium acetate, sodium pyruvate, and sodium citrate. A chemically defined medium similar to the above, but substituting KNO$_3$ for (NH$_4$)$_2$ SO$_4$ and adding only glucose as a carbon source, was used to observe the ability of the organism to utilize the nitrate. All of the above media were adjusted to approximately pH 7.0.

One hundred milliliters of the respective substrates were dispensed into each of the series of five Erlenmeyer flasks. All cultures were incubated at 31 degrees centigrade and the pH determined with a Beckman glass electrode pH meter at 24 hour intervals.

**Residual Sugar Test**

The test for residual sugar was performed on a 24 hour sample according to the Folin-Malmros method:

The appropriate sample was diluted to 1.60 milliliters in a colorimeter tube. To this dilution was added 0.80 milliliter of a 0.40% K$_3$ Fe (CN)$_6$ and 0.40 milliliter of the cyanide-carbonate reagent. The solutions were heated in a boiling water bath for eight minutes, cooled for two minutes, and 2.0 milliliters ferric iron reagent added. The volume was made to 10.0 milliliters with distilled water and the final color read against a reagent blank at 575 mu.

**Manometric Procedures**

Twenty-four hour nutrient broth cultures were centrifuged in an IEC centrifuge at 4500 r.p.m. for twenty minutes.
The supernatant liquid was discarded and the bacterial precipitate was washed twice with physiological saline. A portion of the final washed cells was used for cell counts employing the dilution plate method.

The four respective substrates were inoculated with 0.5 milliliter of the washed bacterial suspension. All center wells of the Warburg flasks contained 0.2 milliliter of a 15 per cent potassium hydroxide solution. All substrates were run in a series of triplicates and an average reading of the three flasks was used in compiling the data. The water bath temperature was held constant at 34 degrees centigrade.

Chromatography

The descending method of paper chromatography was used to isolate the possible intermediate compounds. One drop from each of the four uninoculated media was used as a control and transferred to Whatman #1 filter paper sheets. The filter paper sheets were then placed in a Chromatocab (Berkeley) and developed in the chamber, saturated with the appropriate solvent, for 24, 48, and 72 hour intervals. The same procedure was followed for the inoculated flasks.

Various organic compounds were used in order to satisfactorily resolve the intermediate compounds. The following solvents were used:

1. Ethyl alcohol (45%); methyl alcohol (45%); water (10%).

*All percentages are expressed as volume/volume.
2. Butanol (90%); water (10%).
3. Water saturated butanol (95%); formic acid (5%).

Developing agents used:
1. 5N NH₄OH; 0.1N AgNO₃.
2. Phenol red.
4. Methyl red (3 parts) and Bromcresol Green (1 part).

RESULTS

Pyocyanin is produced only in media containing acetate, citrate and pyruvate, and pigment production is very limited in the latter. Glucose does not support any pigment formation.

Spectrophotometric analysis of the pigment, in a basic solution, shows a broad maximum absorption band extending from 650 μm to 770 μm. In an acidic solution the absorption maximum shifts toward the lower wave lengths, 360 μm to 400 μm. (Figure I, page 10). The pigment, responding as a hydrogen ion indicator, changes from a Prussian blue to a rosy red color at pH 5.5 - 5.1. In a basic solution the pigment may be reduced to a colorless state by the addition of a few crystals of sodium hyposulfite; however, constant shaking (permitting oxygen to be absorbed by the solution) brings about a change from a leuco state to the Prussian blue. Acidification results in a change of color from blue to rosy red. The latter color state also may be reduced to a leuco condition upon the addition
Figure I

Beckman spectrophotometric analysis of pyocyanin in basic and acidic solutions.
of sodium hyposulfite. Aeration then results in a change from the leuco state to a yellow color. Further acidification causes no change in color. Addition of sufficient base again changes the color to Prussian blue. The yellow color cannot be attained without first passing through the acidic red state; the red color cannot be obtained from the yellow without passing through the blue color step, which would require making the solution alkaline.

Reducing Sugar Test

Twenty-four hour samples of the cultures were tested according to the Folin-Malmros method. Greater amounts of reducing substances were present in the 24 hour sample than the control (uninoculated), indicating reducing substances were being rapidly formed. It was, therefore, impossible to accept the colorimetric data as being valid for the residual sugar test.

Manometric Data

Unwashed cell suspension

An unwashed cell suspension, 0.3 milliliter, from a nutrient broth culture was transferred to one milliliter of the respective substrates, i.e., citrate, acetate, glucose, and pyruvate. Rate of oxygen uptake was measured and plotted (Figure II, page 12). The medium containing the acetate was immediately utilized at a very rapid rate by the organism, approximating 198 microliters of oxygen in 90 minutes. Citrate and
Warburg analysis of unwashed cell suspensions from nutrient broth.

One milliliter substrate containing:

\[
\begin{align*}
\text{MgSO}_4 & \quad -0.025\% \\
(NH_4)_2 \text{SO}_4 & \quad -0.10\% \\
K_2\text{HPO}_4 & \quad -0.025\% \\
\text{Carbon source} & \quad -1.0\% \\
\end{align*}
\]

0.5 milliliter inoculum (550,000 cells per milliliter).
pyruvate were consumed at nearly parallel rates of 135 microliters of oxygen in 90 minutes. Oxidation of the two latter substrates did not approximate acetate utilization. Glucose was oxidized at a very low rate, compared to the other three media with 45 microliters of oxygen taken up in 90 minutes.

Washed cell suspension

One milliliter of the four respective carbon substrates was inoculated with 0.5 milliliter of a washed cell suspension. Results (Figure III, page 14) are quite different than those of unwashed cells (Figure II, page 12). Oxygen uptake was at a much lower rate than unwashed suspensions. Nanometric readings were recorded in hours rather than minutes because of the lower oxygen consumption.

Glucose was consumed at a greater rate than the other three substrates. Oxygen uptake being approximately 135 microliters at maximum during a four hour period. After fourteen hours of incubation in the Warburg flasks, all oxygen uptake had reached a minimum. Citrate, following two hour recordings, produced a systematic type of oxygen consumption resulting in a straight line. Acetate dissimilation continued in a straight line very similar to citrate except that the oxygen uptake was much less than that of citrate or glucose. Pyruvate metabolism showed no activity during the first eight and one half hours. Oxygen uptake then proceeded at the lowest oxidative rate of all media.
Warburg analysis of washed cell suspension.

One milliliter substrate containing:

- MgSO$_4$--0.025%
- (NH$_4$)$_2$SO$_4$--0.10%
- K$_2$HPO$_4$--0.025%
- Carbon source--1.0%

0.5 milliliter inoculum (270 million cells per milliliter).
Chromatography

Twenty-four hour cultures

Twenty-four hour growth periods showed only a slight change in media composition with the ammonia-silver nitrate developing agent (Figure IV, page 16). More reducing substances were present in the inoculated glucose than in the glucose control (darker spot in Figure IV). This substantiated similar uncalculable colorimetric determinations (page 11). The inoculated acetate showed a smaller spot than the acetate control indicating rapid utilization of this medium by the organism. The citrate control showed no reducing powers while the inoculated portion displayed a faint spot. Pyruvate showed little change, both the inoculated and control samples displaying identical resolving factor values.

Forty-eight hour cultures

Forty-eight hour growth periods (Figure V, page 18) showed reactions similar to the twenty-four hour inoculum. Citrate was the only medium showing any change. The inoculated citrate spot was much smaller than that of the control. The presence of pigment was detected at this time.

Phenol Red Developer

After 48 hour growth periods, both citrate media responded to an acid-base indicator. Both substances displayed the presence of basic substances. The RF values of these substances showed such slight differences they could not be
Figure IV

Chromatographic results of twenty-four hour cultures.

Left to right: (1) inoculated glucose; (2) glucose control; (3) inoculated acetate; (4) acetate control; (5) inoculated citrate; (6) citrate control; (7) inoculated pyruvate; (8) pyruvate control.

Solvents: Ethyl alcohol (45%); methyl alcohol (45%); water (10%).

Developing agents: 0.1N AgNO₃ dissolved in 5N NH₄OH.
calculated (Figure VI, page 19). The other media failed to respond to this indicator.

**Effects of Other Solvents**

A butanol-formic acid solution (9 parts butanol: 1 part formic acid) was of no value. The various substrates failed to be resolved and consequently, measurements were not possible.

A water saturated butanol-formic acid solution (9.5 parts water saturated butanol: 0.5 part formic acid) was no more effective than the preceding solution.

**Effects of Other Developing Agents**

Bromthymol blue failed to define any of the spots, and similarly the use of methyl red-bromoresol green solution (3 parts methyl red: 1 part bromoresol green) was of no value.

**DISCUSSION**

Turfitt\(^9\) has found that the green pigment, fluorescein, has a maximum absorption at 410 mu in a basic solution while displaying a maximum peak at 370 mu in an acid. Turfitt\(^10\) has attempted to justify the classification of the *pyocyanus-fluorescens* group of bacteria on the basis of their distinctive property of producing a diffusible, green, fluorescent pigment in the culture medium. This hypothesis may well be contradicted by supporting evidence in this paper.

According to spectrophotometric data, the pigment of *P. aeruginosa* strain 20094 differs greatly from that of
Figure V

Chromatographic results of forty-eight hour cultures.

Left to right: (1) inoculated glucose; (2) glucose control; (3) inoculated acetate; (4) acetate control; (5) inoculated citrate; (6) citrate control; (7) inoculated pyruvate; (8) pyruvate control.

Solvents: Ethyl alcohol (45%); methyl alcohol (45%); water (10%).

Developing agents: 0.1N AgNO₃ dissolved in 5N NH₄OH.
Figure VI

Chromatographic results of forty-eight hour cultures.

Encircled spots are left to right: (1) inoculated acetate; (2) acetate control.

Solvents: Ethyl alcohol (45%); methyl alcohol (45%); water (10%).

Developing agent: Phenol red.
Ps. aeruginosa. Since fluorescein is not soluble in chloroform, this pigment could not enter into the analysis. The maximum absorption of pyocyanin in an acid solution does compare quite favorably to Turfitt's acidified form of fluorescein since the substances may have identical absorption spectra; however, in a basic solution pyocyanin of Ps. aeruginosa strain 20094 shows a broad absorption band extending from 660 μm to 770 μm (Figure I, page 10).

The results of oxidation and reduction of pyocyanin in acidic and basic solutions may be diagrammatically represented as:

The ability of pyocyanin to respond as a hydrogen acceptor may be represented as follows:
The degree of pigment formation seems to have a direct relationship to pH and the type of medium. In an acetate medium pigmentation is first visible after 24 hours of incubation, while in citrate at least 60 hours are required. It is difficult to observe pigment formation in pyruvate because of the natural brown color of the medium. It has, however, been observed to be present after a period of about ten days.

Acetate, being the most favorable for pigment production, attains a maximum pH of about 8.9 on the fourth day of growth (Figure VII, page 22). At this time the pigment is also at its maximum intensity. The short carbon chain of acetate (CH₃COONa) seems quite ideal for pigment formation. It seems likely that a condensation product may be formed in such a manner as to produce a closed ring structure. Complete oxidation of acetate is most unlikely since this would not result in a ring formation.

Complete oxidation of acetic acid:

\[ \text{CH}_3\text{COOH} + 2 \text{O}_2 \rightarrow 2 \text{H}_2\text{O} + 2 \text{CO}_2 \]

Partial oxidation may be represented as:

\[ \text{CH}_3\text{COOH} + \text{O}_2 \rightarrow \text{CH}_2\text{O} + \text{H}_2\text{O} + \text{CO}_2 \]
pH curves of various carbon sources inoculated with *Ps. aeruginosa* strain 20094.

Media consisted of:

\[(\text{NH}_4)_2\text{SO}_4--0.10\%\]
\[\text{MgSO}_4--0.025\%\]
\[\text{K}_2\text{HPO}_4--0.025\%\]

Carbon sources*--1.0%  

*Carbon sources: glucose, acetate, pyruvate, or citrate.
Sodium citrate \((\text{COOH.CH}_2\text{COH.COOH.CH}_2\text{COONa})\) could be acted upon by a decarboxylase and the resultant five carbon chain broken down into acetate according to the Kreb's cycle.

Pigment is not formed in a glucose medium containing \((\text{NH}_4)_2\text{SO}_4\). This inability to form a pigment may be due to the rapid oxidation of glucose to gluconic acid and shorter chained acids which lower the pH to such an extent that pigment will not be present.

Pigmentation occurs in a glucose medium containing \(\text{KNO}_3\). The pH becomes alkaline as opposed to an acidic pH in the glucose-ammonium sulfate medium (Figure VIII, page 25). The change in alkaline pH can be attributed to the following reaction:

\[
\text{KNO}_3 \rightarrow \text{K}^+ + \text{NO}_3^- \\
\text{K}^+ + \text{OH}^- \rightarrow \text{KOH} \\
\text{NO}_3^- \text{Reduction} \rightarrow \text{NH}_2 \text{ or NH}_3
\]

The \(\text{NH}_3\) or \(\text{NH}_2\) would be used in the synthesis of pyocyanin.

In the acidic condition the accumulative acids resulting from glucose degradation would bind the basic \(\text{NH}_3\) and pigment would fail to be produced. Extraction by chloroform and alkalizing the supernatant liquid of the glucose medium, fails to show the presence of the pigment. It does not appear in a leuco state or a colored state. Growth in glucose is in no apparent way impeded due to pigment absence. It appears that metabolism can be achieved in all media although the pigment may be absent.
Pigmentation will not occur unless a sufficient nitrogen source is present. Wassersugll showed that KNO$_3$ will inhibit pigment formation in *Pse. aeruginosa*. This, however, does not hold true for *Pse. aeruginosa* strain 20094. Pigment formation in a medium containing KNO$_3$ is more retarded, but not completely absent. A bluish-green pigment occurs in such a medium in about four days.

*Pse. aeruginosa* strain 20094 has the ability to reduce the nitrate to a more utilizable nitrogen intermediate. Nitrogen, as an imide, appears in the pyocyanin ring structure and was reduced to this form by the necessary enzymatic functions.

Media containing citrate, acetate and pyruvate show an intense alkaline pH after a period of at least four days (Figure VII, page 22). Glucose on the other hand, displays a marked acidic pH. This variation in the metabolic process can be attributed to at least two items:

1. Presence of certain salts.

2. Media proper.

In the glucose medium the carbohydrate is rapidly oxidized to a number of acids which greatly influence the pH proper as previously explained on page 23. However, media containing citrate, acetate, and pyruvate may be oxidized to short chained acids and aldehydes, and the effects of these compounds are masked by the more alkaline substances, such as $\text{NH}_4\text{OH}$, Mg(OH)$_2$, KOH, and pyocyanin.
Figure VIII

pH of media containing KNO$_3$ or (NH$_4$)$_2$SO$_4$.

Composition of media:

- glucose--1.0%
- MgSO$_4$--0.025%
- K$_2$HPO$_4$--0.025%
- KNO$_3$--0.10% OR
- (NH$_4$)$_2$SO$_4$--0.10%
**Manometric Data**

Thoroughly washing bacterial cells has a most noticeable effect on the metabolism of organisms. Unwashed bacterial cells (Figure II, page 12) permits the utilization of the four mentioned substrates at a rapid rate. Necessary exoenzymes have been freed from the bacteria proper by washing with physiological saline. This washing results in reduction in the ability to utilize certain given substrates.

According to the theory of "Simultaneous Adaptation"\(^{12}\) cells harvested from a medium containing an energy source will attack a new substrate or compounds that act as intermediates without first requiring adaptation to that compound or compounds. Although nutrient broth is not chemically defined, the application of this theory would indicate that acetate, citrate, and pyruvate had been formed in the nutrient broth culture because of the ability of the organism to become adapted so readily to the three substrates. Pyruvate and citrate are utilized at nearly the same rates. Chemically undefined substances as added in the nutrient broth may act as beneficial growth factors which ordinarily may be lacking in the chemically defined media.

Washed cells gave an entirely different picture since glucose, which is only slightly oxidized by unwashed cells, is rapidly oxidized by washed preparations. The ability of the washed organism to adapt itself to glucose was greater than in the unwashed state.
The tricarboxylic acid cycle has been proposed as a possible pathway for bacterial metabolism by some investigators. In *P. aeruginosa* strain 20094 it appears that this pathway is not adhered to exclusively.

The following theoretical breakdown of glucose is based upon pigment production by the organism from short-chained carbon elements.

![Chemical structures](image)

The latter two acids have been isolated from *P. mucidolens* and may be the resultant end products of *P. aeruginosa* strain 20094. This would account for a decrease in pH and the inability of the organism to form a pigment.

Citrate may be oxidized according to two definite schemes:

1. Direct or complete oxidation of citrate to acetate.

![Chemical structures](image)
2. Oxidation of citrate by tricarboxylic acid route:

\[
\begin{align*}
\text{Citric acid} & \rightarrow \text{Oxalosuccinic acid} & \text{Alpha keto-glutaric acid} & \rightarrow \text{Succinic acid} \\
\text{Fumaric acid} & \rightarrow \text{Malic acid} & \text{Oxaloacetic acid} & \rightarrow \text{Pyruvic acid} & \rightarrow \text{Acetic acid}
\end{align*}
\]

It appears from the experimental data that the above scheme is rather doubtful.

P. aeruginosa strain 20094 utilizes pyruvate at a very slow rate as evidenced by poor growth and little pigment prod-
Pyruvate can be metabolized only according to the following reaction:

\[
\begin{align*}
\text{H} & - \text{C} - \text{H} \\
\text{C}=\text{O} & \quad \rightarrow \quad \text{H} - \text{C} - \text{H} \\
\text{HO} & \\
\end{align*}
\]

Pyruvic acid \quad \text{Acetic acid}

Acetate could be broken down into simpler substances as proposed under the section on pigment synthesis, page 21. Partial utilization of an acetate molecule would result in pigment formation and in the liberation of carbon dioxide and water. The carbon sources, glucose, citrate, and acetate are undoubtedly oxidized rapidly and vigorously into shorter chained compounds and products of mineralization. However, it would appear that the tricarboxylic acid cycle does not function in \textit{P. aeruginosa} strain 20094 and that pyruvate is a doubtful intermediate. The length of time required for pyruvate adaptation would seem to rule out this possibility.
SUMMARY.

Pyocyanin or a pyocyanin derivative is formed only when a utilizable nitrogen source is available and the pH is in the alkaline range. Short chain carbon elements of carbohydrate degradation are the basis for the ring type structure of pyocyanin. The pigment probably behaves in such a manner as to act as a hydrogen carrier although it apparently is unessential for growth, when other physiological conditions are favorable.

*P. aeruginosa* strain 20094 appears to produce pyocyanin only. The organism does not produce the green pigment, fluorescein. It is, therefore, not possible to classify all Pseudomonads on the sole basis of fluorescein production.

The pigment displays a broad maximum absorption band extending from 650 μm to 770 μm in a basic solution. In an acidic solution the absorption maximum shifts toward the lower wave lengths, 360 μm to 400 μm.

In acidic and basic solutions the pigment behaves as a hydrogen ion indicator. It is blue in the basic range and red in acid. Under certain conditions a yellow state in acid solution may be produced, but this requires that the pyocyanin first pass through the red state, be reduced and subsequently oxidized to the yellow color. The red to yellow step is irreversible, but the addition of base to the solution will restore the original blue color.

Unwashed cell suspensions metabolized citrate, and acetate at a vigorous rate, while washed cells require a
period of adaptation. This may be attributed to the decrease in the amount of the enzymes present, particularly exo-enzymes.

The metabolism of *P. aeruginosa* strain 20094 indicates that the organism does not follow the tricarboxylic acid cycle, at least in media containing glucose, acetate, or pyruvate. The tricarboxylic acid cycle or a modification of such, may be possible with citrate as the carbon source. It appears that citrate is probably metabolized directly to acetate.

It was possible to observe the degradation of the original glucose content by means of paper chromatography. Resolving factors were of little significance due to the minute differences in distances covered on the filter paper.

Phenol red indicator was the only chemical to display the presence of a basic substance in the citrate medium. Though chromatographic analysis would seem to be a likely means for differentiating the possible intermediates, other solvents and developing agents must be employed in order to obtain the desired results.
REFERENCES CITED


