OXIDATIVE ASSIMILATION OF GLUCOSE BY PSEUDOMONAS AERUGINOSA

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ABSTRACT

DUNCAN, MARGARET G. (The University of British Columbia, Vancouver, British Columbia, Canada) AND J. J. R. CAMPBELL. Oxidative assimilation of glucose by Pseudomonas aeruginosa. J. Bacteriol. 84:784-792. 1962—Oxidative assimilation of glucose by washed-cell suspensions of Pseudomonas aeruginosa was studied using C¹⁴-labeled substrate. At the time of glucose disappearance, only small amounts of radioactive were present in the cells, and α-ketoglutaric acid accumulated in the supernatant fluid. Most of the material synthesized by the cells during oxidative assimilation was nitrogenous, the ammonia being supplied by the endogenous respiration. The cold trichloroacetic acid-soluble fraction and the lipid fraction appeared to be important during the early stages of oxidative assimilation, and the largest percentage of the incorporated radioactivity was found in the protein fraction. In the presence of added ammonia, assimilation was greatly increased and no α-ketoglutaric acid was found in the supernatant fluid. Sodium azide partially inhibited incorporation into all major cell fractions, and at higher concentrations depressed the rate of glucose oxidation. During oxidative assimilation, chloramphenicol specifically inhibited the synthesis of protein. Oxidative assimilation of glucose by this organism did not appear to involve the synthesis of a primary product such as is found in the majority of bacteria.

The studies of Barker (1936) on oxidative assimilation in the colorless alga, Prototheca zopfii, led him to conclude that the product of assimilation in this organism had the empirical composition (CH₅O). Since the organism was known to synthesize and store glycogen, Barker concluded that glycogen was the "primary product" of oxidative assimilation of organic substrates, and that this compound subsequently might be used as the raw material for all secondary syntheses within the cells during prolonged incubation or under conditions where growth was possible.

Studies by Giesberger (1936) on various Spirillum sp., by Clifton (1937) on Pseudomonas calco-aceatica and Escherichia coli, and by Doudoroff (1940) on P. saccharophila lent further support to the theories advanced by Barker. More recently, radioactive substrates have been used to determine the products of oxidative assimilation by bacteria. Doudoroff and Stanier (1959) found that a major fraction of the carbon assimilated from glucose, acetate, or butyrate by P. saccharophila was found in poly-β-hydroxybutyric acid. This polymer was also formed during photosynthetic assimilation of acetate and butyrate by Rhodospirillum rubrum. Bacillus species also synthesize poly-β-hydroxybutyric acid from various carbon sources (Macrae and Wilkinson, 1958). Binnie, Dawes, and Holms (1959) found that assimilation of glucose by Sarcina lutea gave rise to a polysaccharide composed essentially of glucose units, and this was utilized when the external supply of glucose was withdrawn. The accumulation of a glycogenlike material by E. coli cells during nitrogen starvation in the presence of glucose was studied by Holme and Palmstierna (1956). A quite different situation was recently reported by Clifton (1962). He found that C¹⁴-labeled glucose, added to resting cells of B. cereus, was assimilated via the cold 5% trichloroacetic acid (TCA)-soluble pool, primarily into the hot 5% TCA-soluble and -insoluble materials, fractions which are normally composed of nitrogenous constituents. Since nitrogenous substances form part of the endogenous reserves of this organism (Clifton and Sobek, 1961), Clifton suggested that oxidative assimilation was, at least in part, at the expense of endogenous reserves which appear to be replenished or replaced.

Warren, Ells, and Campbell (1960) showed that the endogenous respiration of P. aeruginosa and of several other organisms (Gronlund and
Campbell, 1961) resulted in the production of ammonia, and that the ammonia was rapidly reincorporated into cellular material when glucose was added. No increase in intracellular carbohydrate could be detected in P. aeruginosa after the addition of glucose, indicating that nitrogenous compounds undoubtedly accounted for much of the substrate shunted into oxidative assimilation. In the present study, oxidative assimilation by P. aeruginosa was followed in greater detail by using C\textsuperscript{14}-labeled glucose as substrate.

**MATERIALS AND METHODS**

*Preparation of resting-cell suspensions. P. aeruginosa* (ATCC 9027) was grown in a glucose, ammonium phosphate-salts medium as previously described (Warren et al., 1960). After 20 hr at 30 C, the cells were harvested by centrifugation in the cold, washed twice with cold 0.05 M tris-(hydroxymethyl)aminomethane (tris) buffer (pH 7.2) and resuspended to ten times the growth concentration. Dry weights were established by drying 5 ml of the cell suspension to constant weight at 100 C.

*Assimilation experiments.* Manometric measurements were carried out in a Warburg apparatus at 30 C, using conventional procedures for establishing oxygen consumption and CO\textsubscript{2} production. Each cup contained 1 ml of cell suspension (usually 5 mg dry wt of cells), 0.05 M tris buffer (pH 7.2), and substrate, ammonia or inhibitors when required, to a total volume of 3 ml. In experiments on the influence of inhibitors, the cells were in contact with the inhibitor for 30 min prior to the addition of substrate.

To stop reactions at the stated time intervals, the cup contents were pipetted into ice-cold centrifuge tubes containing 1 ml of buffer, and were immediately centrifuged in the cold. The supernatant fluid was decanted and the cell pellet resuspended to 2 ml with cold distilled water. (Washing the cell pellet did not remove any significant amount of materials known to be present in the supernatant, so this was not routinely carried out.) A sample of the cell suspension was removed for radioactive measurement, and then an equal volume of cold 10% TCA was added to give a final concentration of 5% (w/v) TCA, after which the tubes were held at 5 C for 30 min. After centrifugation, the pellet was resuspended in 75% (v/v) ethanol, which had been adjusted to pH 2.5 as suggested by Clifton and Sobek (1961). After 30 min at 45 C, the mixtures were centrifuged and the fractionation continued according to the procedure of Roberts et al. (1955).

*Analytical methods.* The ammonia in the supernatant fluid was determined by the Conway microdiffusion technique (Conway, 1950), glucose by the "glucostat" method of Worthington Biochemicals, Freehold N.J., and \(\alpha\)-ketoglutarate and pyruvate by the method of Friedemann (1957), modified by mechanical shaking in place of nitrogen-bubbling.

The oxidation of reduced diphosphopyridine nucleotide by glutamic dehydrogenase, as measured in the spectrophotometer at 340 m\textmu, in the presence of excess ammonia and limiting \(\alpha\)-ketoglutarate was also used for the quantitative estimation of \(\alpha\)-ketoglutarate and was found to be a specific, sensitive, and rapid method. The results corresponded to those obtained by the chemical method.

*Chromatographic methods.* Routine chromatography of the supernatants and various cell fractions was carried out on Whatman no. 1 paper using secondary butanol-formic acid-water (70:10:20, v/v; Roberts et al., 1955). The protein residue was hydrolyzed and chromatographed according to the methods of Roberts et al. (1955) using the solvent system n-butanol-acetic acid-water (63:10:27). For chromatography of 2,4-dinitrophenylhydrazine derivatives of keto acids in the supernatants and of keto acid standards, Whatman no. 4 paper was used with the solvent n-butanol-ethanol-ammonia (70:10:20, v/v) using the method of El Hawari and Thompson (1953).

*Isotopic methods.* Uniformly labeled glucose-C\textsuperscript{14} was obtained from Merck and Co., Ltd., and diluted with nonradioactive glucose solution so that 5 \(\mu\)M of glucose with a specific activity of approximately 3.5 \(\mu\)C per 5 \(\mu\)M was added to each reaction vessel. The count/min added per cup were verified by dilution and plating of the glucose solutions. Samples of the supernatants, cell suspensions, and cell fractions were plated in duplicate and counted at infinite thinness using a Nuclear-Chicago scaler, model 181 A, equipped with a gas-flow counter having a thin end-window Geiger tube. Corrections were made for coincidence and background.

Radioactive areas on paper chromatographs
were determined by running strips through a Nuclear-Chicago Model C 100 B Actigraph II with the gas-flow counter, a Model 1620 B Analytical Count Ratemeter and Chart Recorder.

RESULTS

Oxidation of glucose-\( ^{14} \text{C} \) by resting cells. Typical curves for the uptake of oxygen during dissimilation of glucose by \( P. \text{aeruginosa} \) are shown in Fig. 1. The endogenous values have been subtracted as previous work (Norris, Campbell, and Ney, 1949) showed that the endogenous respiration continued at approximately the same rate in the presence and absence of glucose. The initial rapid rate of oxidation had a \( Q_02 \) of approximately 110 and a respiratory quotient (RQ) of 0.9. The break in the curve corresponded to 45 to 50% of the theoretical value for complete oxidation. This was followed by the second stage, characterized by an RQ of 1.0 to 1.05 and a low \( Q_02 \) that varied with the ratio of substrate to cells. This stage continued until 65 to 70% of the theoretical oxygen uptake was achieved.

From the oxygen-uptake values, one might expect that about 50% of the substrate had been assimilated during the initial stage. However, when glucose-\( ^{14} \text{C} \) was used as substrate, in an effort to determine the distribution of \( ^{14} \text{C} \) in the supernatant and cells, it was found that only 10 to 15% of the carbon from the glucose was assimilated into the cells (Fig. 2a). The glucose in the reaction vessel had disappeared by 30 min, and, since oxidation and assimilation at the rate referred to as "stage two" continued from this point, it was apparent that there must have been a transient accumulation of some intermediate compound or compounds. On chromatographic analysis of the supernatants at 30 and 60 min, the \( ^{14} \text{C} \) was found to be present in a single spot, which was identified as \( \alpha \)-ketoglutarate by chemical assays and by chromatography of the 2,4-dinitrophenylhydrazones. No significant amounts of pyruvic acid or other compounds were detected. The concentration of \( \alpha \)-ketoglutaric acid in the supernatant fluid continued to rise as long as glucose was being oxidized and fell as soon as the glucose had disappeared (Fig. 2b). As the \( \alpha \)-ketoglutaric acid disappeared, small amounts of glutamic acid, other amino acids, ultraviolet-absorbing materials, and larger molecules accounted for the radioactivity in the supernatant fluid.

Since all the evidence indicates that the endogenous respiration of this organism continues unabated in the presence of an oxidizable substrate such as glucose (Norris et al., 1949), and since ammonia is a major product of endogenous respiration (Warren et al., 1961), it is assumed that ammonia formation by resting cells will be the same in the presence or absence of substrate. However, it is known that this endogenous ammonia is rapidly reincorporated into the cell on the addition of glucose so that, within 5 min of the time that 5 \( \mu \text{M} \) of glucose is added to the cell suspension until 60 min, no ammonia was detectable (Table 1). The amount of ammonia reincorporated into the cells was calculated as the amount released by endogenously respiring cells minus that present in the reaction vessel containing added glucose (Table 1; Fig. 2a). The similarity in shapes of the curves representing incorporation of \( ^{14} \text{C} \) into the cells and the disappearance of ammonia suggested that ammonia was the factor which determines the rate of assimilation of carbon into the cell.

When the cells were fractionated, the radioactivity was found to be present in all major fractions (Table 2). The percentage of the total \( ^{14} \text{C} \) found in each fraction was compared to the percentage composition of 20-hr cells grown in a
complete medium containing glucose-U-C\textsuperscript{14}. The cold 5\% TCA fraction and also the lipid fractions appeared to be important during the early stages of assimilation, and most of the substrate appeared to be assimilated into nitrogenous compounds. Chromatography, or hydrolysis followed by chromatography, verified that the composition of each fraction was as expected, and no evidence for the synthesis of poly-\(\beta\)-hydroxybutyrate or any significant amounts of a polysaccharide material was obtained. Subsequent specific assays for the poly-\(\beta\)-hydroxybutyrate confirmed the conclusion that this organism does not synthesize this polymer (Hogg, unpublished data).

Stimulation of oxidative assimilation of glucose by ammonia. Since assimilation occurred primarily into nitrogenous compounds and appeared to depend on the ammonia available from endogenous respiration, corresponding experiments were carried out in the presence of 5 \(\mu\)M of ammonia (Fig. 3a and 3b). There was a very marked stimulation of assimilation; by 30 min, 32\% of the added glucose was converted to cellular material. The incorporation of ammonia was compatible with this assimilation and no \(\alpha\)-ketoglutarate appeared in the supernatant fluid. This organism contains a strong glutamic dehydrogenase, and the coincident removal of ammonia and \(\alpha\)-ketoglutarate indicated that carbon was being assimilated by way of glutamic acid. The calculated rate of CO\textsubscript{2} evolution was increased during the early stages, which was in agreement with the increased RQ values in the

\begin{table}
\centering
\caption{Production and reincorporation of NH\textsubscript{3}}
\begin{tabular}{c|c|c|c|c}
\hline
Time & \multicolumn{3}{c}{NH\textsubscript{3} present per 3-ml vessel} & Calculated NH\textsubscript{3} uptake \\
  & Endogenous & Glucose & & \\
\hline
min & \(\mu\)M & \(\mu\)M & \(\mu\)M & \(\mu\)M \\
\hline
0 & 0.27 & 0.27 & 0 & \\
15 & 0.50 & 0 & 0.50 & \\
30 & 0.73 & 0 & 0.73 & \\
60 & 1.20 & 0.14 & 1.06 & \\
120 & 1.72 & 0.42 & 1.30 & \\
\hline
\end{tabular}
\end{table}
TABLE 2. Incorporation of C\textsuperscript{14} from 5 \, \mu M of glucose-U-C\textsuperscript{14} into washed-cell suspensions of Pseudomonas aeruginosa

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cold TCA-soluble</th>
<th>Lipid, phospholipid</th>
<th>Alcohol-soluble protein</th>
<th>Hot TCA-soluble</th>
<th>Residual protein</th>
<th>Total in fractions</th>
<th>Unfractionated cells</th>
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<td>2.2</td>
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<td>2.9</td>
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<td>10.8</td>
</tr>
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<td>2.8</td>
<td>0.5</td>
<td>1.4</td>
<td>5.5</td>
<td>12.7</td>
<td>12.8</td>
</tr>
<tr>
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<td>0.6</td>
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<td>7.7</td>
<td>15.9</td>
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</table>

% of total C\textsuperscript{14} added to vessel

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% of total C\textsuperscript{14} incorporated into cell fractions</th>
<th>% of total C\textsuperscript{14} added to vessel</th>
</tr>
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<tr>
<td>15</td>
<td>26</td>
<td>26</td>
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</tr>
<tr>
<td>120</td>
<td>15</td>
<td>21</td>
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Growth composition

- Determined by growing cells in a complete medium containing glucose-U-C\textsuperscript{14}, harvesting and washing the cells, and fractionating the washed-cell suspensions.

**FIG. 3a.** (left) Time course of the distribution of C\textsuperscript{14} added to washed-cell suspensions of Pseudomonas aeruginosa as 5 \, \mu M of uniformly labeled glucose-C\textsuperscript{14} in the presence of 6 \, \mu M of added NH\textsubscript{3}. The figure shows the per cent of C\textsuperscript{14} found: in the supernatant (Δ—Δ); in the cells (O—O); and as calculated C\textsuperscript{14}O\textsubscript{2}, initial C\textsuperscript{14} of cells and supernatant (●—●). The percentage of the theoretical oxygen uptake is shown by the broken line.

**FIG. 3b.** (right) Time course of the disappearance of glucose from the supernatant (Δ—Δ); the concentration of \(\alpha\)-ketoglutaric acid in the supernatant (○—○); and the calculated NH\textsubscript{3} uptake (X—X), during oxidation of 5 \, \mu M of glucose by washed-cell suspensions of Pseudomonas aeruginosa in the presence of 5 \, \mu M added NH\textsubscript{3}.
TABLE 3. Incorporation of C\textsuperscript{14} from 5 μM of glucose-U-C\textsuperscript{14} into washed-cell suspension of Pseudomonas aeruginosa in the presence of 5 μM NH\textsubscript{3} added as (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cold TCA-soluble</th>
<th>Lipid, phospholipid</th>
<th>Alcohol-soluble protein</th>
<th>Hot TCA-soluble % of total C\textsuperscript{14} added to vessels</th>
<th>Residual protein % of total C\textsuperscript{14} incorporated into cell fractions</th>
<th>Total in fractions % of total C\textsuperscript{14} added to vessels</th>
<th>Unfractionated cells % of total C\textsuperscript{14} added to vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3.5</td>
<td>4.4</td>
<td>0.4</td>
<td>1.6</td>
<td>8.6</td>
<td>18.5</td>
<td>17.1</td>
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<td>0.6</td>
<td>2.8</td>
<td>17.5</td>
<td>28.6</td>
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Growth composition

6 16 3 16 59 100

In the early diversion of glucose into the pentose phosphate pathway of oxidation, the pattern of assimilation into cell fractions was close to that found during growth (Table 3). An exception was the hot TCA-soluble fraction. This may result from the lack of phosphate or be due to a slow rate of turnover or resynthesis during the oxidation of glucose by washed-cell suspensions of P. aeruginosa. Maximal incorporation was observed at 30 to 60 min, after which protein and the compounds of the cold 5% TCA-soluble fraction were oxidized.

Influence of chloramphenicol on oxidative assimilation. The influence of chloramphenicol on oxidative assimilation was studied because much of the material synthesized during oxidative assimilation of glucose appeared to be protein. The presence of chloramphenicol decreased the final $O_2$ uptake during oxidation of glucose and appeared to inhibit the second stage known to be due to the oxidation of α-ketoglutaric acid. There was a slight increase in the C\textsuperscript{14} incorporated into the cells and released as C\textsuperscript{14}O\textsubscript{2} at 30 min. After this time, the radioactivity in the cells decreased and the radioactivity of the supernatant fluid remained higher than in the control (Fig. 4). Chromatography of the supernatant fluid showed that α-ketoglutaric acid was still present in considerable quantity at 150 min.

The incorporation of radioactivity into cellular fractions in the presence of chloramphenicol is shown in Table 4. Incorporation into the cold TCA-soluble, the lipid, and the hot TCA-soluble fractions was increased, whereas incorporation into the residual protein was greatly decreased,
TABLE 4. Incorporation of C\textsuperscript{14} from 5 \(\mu\)M of glucose-U-C\textsuperscript{14} into washed-cell suspension of *Pseudomonas aeruginosa* in the presence of 0.8 mg of chloramphenicol

<table>
<thead>
<tr>
<th>Time (min)</th>
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<th>Lipid, phospholipid</th>
<th>Alcohol-soluble protein</th>
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% of total C\textsuperscript{14} added to vessel

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especially in the later stages. These results are in agreement with the observations of other workers that chloramphenicol specifically inhibits protein synthesis by bacteria.

Influence of azide on oxidative assimilation. Uncoupling agents such as sodium azide and 2,4-dinitrophenol have been found to inhibit oxidative assimilation and in some instances increase the O\textsubscript{2} uptake to the theoretical value for complete combustion of the substrate (Clifton, 1951). It was of interest, therefore, to determine the influence of azide on incorporation of C\textsuperscript{14} from glucose-U-C\textsuperscript{14} by *P. aeruginosa*. Concentrations of 0.001 to 0.0033 M increased the final oxygen uptake to approximately 80% of the theoretical value. At the higher concentrations, this was accompanied by a pronounced decrease in the rate of O\textsubscript{2} consumption (Fig. 5). If 0.001 M azide was used, the increase in the O\textsubscript{2} uptake at 60 min was found to be largely due to disappearance of C\textsuperscript{14} from the supernatant fluid rather than to a decrease in assimilation. Much less \(\alpha\)-ketoglutaric acid accumulated in the supernatant fluid, none was present at 30 min, and very small amounts were present at 60 min. The increased O\textsubscript{2} consumption at 150 min could be accounted for by a decrease in the C\textsuperscript{14} of the cells and supernatant fluid. At 0.0033 M azide, assimilation of C\textsuperscript{14} into the cells was decreased even further (Fig. 6). It was found on fractionation of the cells that azide did not selectively affect incorporation into any particular fraction. This inhibitor undoubtedly acts by decreasing the energy available for synthesis.

At a concentration of 0.001 M azide, the addition of ammonia caused a 6% increase in assimilation but, at a concentration of 0.0033 M azide, assimilation was the same regardless of the ammonia available.
P. AERUGINOSA OXIDATIVE GLUCOSE ASSIMILATION

![Graph showing the distribution of C14 added to washed-cell suspensions of Pseudomonas aeruginosa with time.](http://jb.asm.org/)

**DISCUSSION**

The manometric results for the oxidation of glucose by resting cells of *P. aeruginosa* suggested that approximately 50% of the substrate was assimilated during the initial stage of oxidation, and that the slow secondary rate of oxidation which followed might be due to the oxidation of assimilated material. This would be similar to the extent of oxidative assimilation of glucose by resting cells of other bacterial species (Binnie et al., 1959; Clifton, 1962; Doudoroff and Stanier, 1959). However, using C14-labeled glucose as substrate, it was found that during the period of rapid glucose oxidation only a small percentage of the C14 was assimilated into the cells, whereas a large quantity of α-ketoglutarate accumulated in the supernatant fluid. The slow secondary rate of oxygen uptake corresponded to the gradual oxidation of some of the α-ketoglutarate, and the remaining α-ketoglutarate was incorporated as ammonia became available from the breakdown of endogenous reserves. Oxidative assimilation of glucose by *P. aeruginosa* does not appear to involve the synthesis of a "primary product" of assimilation such as found in bacteria which form a polysaccharide or poly-β-hydroxybutyric acid during oxidative assimilation. Since most of the material synthesized by *P. aeruginosa* from the glucose was nitrogenous, and since the endogenous respiration supplies the ammonia for this synthesis, oxidative assimilation may serve, at least in part, to replenish the endogenous reserves. Clifton (1962) reached a similar conclusion in a study of the oxidative assimilation of *B. cereus*. The addition of ammonia greatly increased the amount of C14 assimilated and prevented the accumulation of α-ketoglutarate.

The accumulation of α-ketoglutarate during the growth of *Pseudomonas* in a medium with a low nitrogen to carbon ratio was observed by Lockwood and Stodola (1946) and studied by Koepsell, Stodola, and Sharpe (1952) and Koepsell (1950). Kogut and Podoski (1953) found that α-ketoglutarate and pyruvate were present in the supernatant fluid during the oxidation of succinate by resting cells of *P. fluorescens*. The accumulation of α-ketoglutarate by growing or resting cells of many other genera has been reported (Asai et al., 1955; Perry and Evans, 1960). In a soil and water organism, such as *P. aeruginosa*, in which assimilation appears to rely almost exclusively on the available ammonia, the presence of a partial metabolic block at the α-ketoglutarate stage of carbohydrate oxidation is indeed advantageous, conserving, as it does, the carbon compound which will act as the point of entry to protein and nucleic acid synthesis when ammonia becomes available by diffusion, leaching, or from endogenous storage products.

The increased oxygen value obtained when glucose was oxidized in the presence of azide was accompanied by a significant decrease in the amount of radioactivity incorporated into the cells. The specific action of azide is most probably the uncoupling of oxidative phosphorylation, for in the presence of high concentrations of azide there was little change in the relative amounts of the various products of assimilation but the rate of formation was slower and the amounts synthesized smaller. In contrast, chloramphenicol specifically inhibited the synthesis of protein, although incorporation into the nucleic acid, lipid, and cold 5% TCA fractions was increased.
The role of the lipid fraction of \textit{P. aeruginosa} during the early stages of oxidative assimilation is of interest, since Silberman and Gaby (1961) showed that the phospholipids of this organism may be important in the uptake of amino acids. Lipo-amino acid complexes have been isolated from a variety of cell types and their possible role in protein synthesis has been discussed (Hunter and Goodsal, 1961).

\textbf{ACKNOWLEDGMENT}

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\textbf{LITERATURE CITED}


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