1962

Assimilation of CO2 by bacteria

Daryl Stokesbury Bates
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ASSIMILATION OF CO₂ BY BACTERIA

by

Daryl Stokesbury Bates

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>Stimulation of the Growth of Heterotrophs by CO₂</td>
<td>2</td>
</tr>
<tr>
<td>Carboxylation of Organic Compounds</td>
<td>3</td>
</tr>
<tr>
<td>Other Mechanisms of CO₂ Fixation</td>
<td>32</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>33</td>
</tr>
<tr>
<td>Enzyme and Whole-cell Preparations</td>
<td>33</td>
</tr>
<tr>
<td>Chemicals</td>
<td>37</td>
</tr>
<tr>
<td>Microbiological Methods</td>
<td>38</td>
</tr>
<tr>
<td>Paper Chromatographic Methods</td>
<td>39</td>
</tr>
<tr>
<td>Analytical Methods</td>
<td>43</td>
</tr>
<tr>
<td>EXPERIMENTARY</td>
<td>50</td>
</tr>
<tr>
<td>Carboxylation of Phosphoenolpyruvate</td>
<td>50</td>
</tr>
<tr>
<td>Carboxylation of Carboxylic Acids</td>
<td>62</td>
</tr>
<tr>
<td>Nutrition and Oxygen Assimilation Studies</td>
<td>77</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>83</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>96</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>98</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>119b</td>
</tr>
<tr>
<td>APPENDIX A</td>
<td></td>
</tr>
<tr>
<td>Growth of <em>Nocardia corallina</em> in the Fermentor</td>
<td>120b</td>
</tr>
<tr>
<td>APPENDIX B</td>
<td></td>
</tr>
<tr>
<td>Fractionation of <em>Nocardia corallina</em> Extracts on DEAE-Cellulose</td>
<td>124</td>
</tr>
<tr>
<td>APPENDIX C</td>
<td></td>
</tr>
<tr>
<td>Calibration of the Fowler-Rhinehart Scanner</td>
<td>126</td>
</tr>
<tr>
<td>APPENDIX D</td>
<td></td>
</tr>
<tr>
<td><em>R_f</em> Values of Known Compounds in the Solvents Used</td>
<td>130a</td>
</tr>
</tbody>
</table>

INTRODUCTION

A growing interest in the carboxylation of organic acids by heterotrophic cells, initiated by the demonstration of heterotrophic carbon dioxide assimilation by Wood and Werkman in 1935, has prompted a number of investigations. As a result of these studies, several organic acids or their active forms have been found to serve as substrates in carboxylation systems other than the pyruvate system studied by Werkman and associates.

One explanation for this widespread attention is the fact that nearly every phase of metabolism concerns some type of carbon dioxide assimilation.

Although various bacteria have been studied in this connection, it was considered of interest to examine the scope of the various carboxylation reactions, to investigate additional compounds as possible carbon dioxide acceptors, and to obtain more knowledge concerning the characteristics required of a molecule to serve in this capacity.
Prior to 1935, the incorporation of CO$_2$ into C-C bonds of organic compounds by living organisms was known to occur only in higher plants, algae, photosynthetic bacteria, and chemosynthetic bacteria. However, the latter was the only non-photosynthetic group and was usually considered special in this regard.

Stimulation of the Growth of Heterotrophs by CO$_2$

Although no mechanism had been suggested for the assimilation of CO$_2$ into organic compounds by heterotrophs, the stimulation of growth by CO$_2$ had been reported. Among the first observations leading to this conclusion was the inhibition of the tubercle bacillus when CO$_2$ was absorbed by alkali (Wherry and Ervin, 1918). Cohen and Fleming (1918) found that 10% CO$_2$ had the same stimulatory effect on meningococci as the addition of an aerobe, and Chapin (1918) reported that CO$_2$ facilitated the growth of gonococci. Although growth stimulation by an associated aerobe was considered due to the CO$_2$ supplied (Novy et al., 1925), it was later concluded that CO$_2$ was not essential for growth. Inhibition of growth by alkali was thought to be due to desiccation and not CO$_2$. 
removal (Novy and Soule, 1925). Rockwell and Hightberger (1926) questioned this conclusion when no inhibition occurred with dehydrating agents that did not absorb CO₂. It was suggested that all bacteria, yeasts, and molds require CO₂ as a carbon source (Rockwell and Hightberger, 1927). Valley and Rettger (1927), Gladstone et al. (1935), and Rahn (1941) confirmed the fact that bacteria require CO₂ for growth, but the only evidence at this point was that growth occurred only in the presence of CO₂.

Carboxylation of Organic Compounds

The first mechanism for the incorporation of CO₂ into C-C bonds of organic compounds by heterotrophs was proposed by Wood and Werkman (1935, 1936) as an explanation for the production of acetic, propionic, and succinic acids from the assimilated CO₂. Since this suggestion was contrary to the concepts of thermodynamics, it was not readily accepted.

The significance of this mechanism of biological CO₂ assimilation was later recognized, as exemplified by Ochoa's statement (Ochoa, 1951): "The discovery of CO₂ fixation in heterotrophic organisms by Wood and Werkman marked a milestone because it focused attention on the primary reactions by which
CO₂ is incorporated into organic compounds."

One of the first confirmations was the report (Elsden, 1938) that succinic acid production was enhanced in cultures of *Bact. coli commune* by increasing the CO₂ concentration.

**Pyruvate**

In 1938 Wood and Werkman confirmed and extended the previous experiments which prompted the proposal of CO₂ fixation. Since pyruvic acid had been isolated from glycerol fermentations (Wood and Werkman, 1934), pyruvate was suggested as the CO₂ acceptor and succinate was proposed as the subsequent product. Carbon dioxide utilization in the synthesis of citric acid by avian tissue was also suggested. Citrate synthesis was thought to proceed by the condensation of oxalacetate and "triose" derived from carbohydrate (Krebs and Johnson, 1937).

Direct confirmation of the heterotrophic assimilation of CO₂ appeared in a report by Phelps *et al.* (1939). Included in the introductory remarks was a statement concerning the reluctance of Barker (1936) and van Niel (1937) in accepting this unusual concept. They also stated that the follow-up article by Wood and Werkman (1938) should remove all doubts.

**Wood-Werkman reaction** Although the general concept
of CO₂ assimilation had been established, the first details were revealed following a study of various inhibitors and substrates (Wood and Werkman, 1940a). The widespread nature of this phenomenon was suggested and the following mechanism was proposed:

\[ \text{CO}_2 + \text{pyruvate} \rightarrow \text{oxalacetate} \rightarrow \text{malate} \rightarrow \text{fumarate} \rightarrow \text{succinate}. \]  

(1)

The inhibition of CO₂ fixation by NaF (Wood and Werkman, 1940b) indicated that the reaction involved a fluoride-sensitive phosphorylating mechanism, and the presence of phosphopyruvate was suggested.

When various isotopes of carbon became available, more intricate details were obtained. Investigators with access to a cyclotron used C¹¹ as a metabolic tracer. Carson and Ruben (1940) detected radioactivity in succinate and propionate synthesized by Propionibacterium pentosaceum in the presence of glycerol and C¹¹O₂. Since pyruvate was inactive, a C₃-acid intermediate between glycerol and propionate was proposed as the CO₂ acceptor. Simultaneously, Wood et al. (1940) used the heavy isotope of carbon, C¹³, which required the use of a mass spectrometer for analysis. Both Escherichia coli and Propionibacterium pentosaceum were found to fix CO₂ in the carboxyl
groups of succinic acid. These findings supported the previously proposed conversion of pyruvate and CO$_2$ to succinate. Carson et al. (1940) concluded that all the carbons of propionic acid were labeled and suggested that CO$_2$ was reduced to propionic acid by glycerol. Wood et al. (1941) confirmed their original proposal that only the carboxyl group of propionate contained C$^{13}$, and the Wood-Werkman reaction was restated:

\[ \text{CO}_2 + \text{pyruvate} \rightarrow \text{oxalacetate} \]  

Propionic acid was thought to arise from the C$_4$ compounds, and a recent report (Siu et al., 1961) proved the validity of this suggestion (see "Carboxytransphosphorylase").

The scope of CO$_2$ fixation was widened (Evans and Slotin, 1940) by the demonstration of the incorporation of C$^{13}$O$_2$ into α-ketoglutarate in pigeon liver tissue. Reaction 2 was thought to produce oxalacetate which was converted to α-ketoglutarate (Evans, 1940).

The possibility of cofactors in the CO$_2$ fixing reaction was introduced by Krebs and Eggleston (1940), based on experiments with Bacterium coli and pigeon liver. Reaction 2 was supported and it was concluded that vitamin B "catalyzed" the carboxylation of pyruvate. Smyth (1940) supported this role
of vitamin B in the synthesis of oxalacetate in Staphylococcus aureus and Staphylococcus albus.

Another step toward the final elucidation of the mechanism of CO₂ assimilation was the utilization of cell-free enzyme systems. Krampitz and Werkman (1941) prepared an enzyme system from Micrococcus lysodeikticus which catalyzed the production of pyruvate and CO₂ from oxalacetate. Cocarboxylase (thiamine pyrophosphate) or aneurin (thiamin) had no effect, but Mg⁺⁺ was a necessary cofactor. When C¹³O₂ and oxalacetate were added to the enzyme preparation, labeled oxalacetate was formed by an exchange reaction (Werkman et al., 1942; Krampitz et al., 1943), indicating that this could be the enzyme involved in the carboxylation of pyruvate. Cell-free extracts of pigeon liver behaved in a similar manner (Evans et al., 1942), and the enzyme (referred to as oxalacetic carboxylase) was thought to catalyze the Wood-Werkman reaction.

The widespread nature of CO₂ assimilation by heterotrophic bacteria was established (Slade et al., 1941) in a study of several different bacteria.

The role of CO₂ fixation in glycogen synthesis was revealed by the results of several investigators. Solcmon et al. (1941) explained the incorporation of C¹¹-bicarbonate into
glycogen in rats by the reversal of glycolysis with the exception of pyruvate phosphorylation. The entry of CO₂ was thought to occur at this step. Lorber et al. (1943) demonstrated the incorporation of CO₂ into glycogen in both heart muscle and liver tissues; Wood et al. (1945) found carbons 3 and 4 labeled in glucose after rats were injected with C¹³. These results disclosed the importance of Reaction 2 as a possible means of by-passing the irreversible phosphorylation of pyruvate in glycogen synthesis.

Direct evidence for the carboxylation of pyruvate was provided by Kalnitsky et al. (1943), based on the observation that labeled succinate was formed from pyruvate and C¹³O₂ by Escherichia coli extracts. However, a phosphorylated intermediate was again suggested. The possibility of the carboxylation of phosphopyruvate by liver enzymes was also proposed by Leloir and Muñoz (1944). Kaltenbach and Kalnitsky (1951a) later reported the direct carboxylation of pyruvate in extracts of Escherichia coli and Proteus morganii. Inorganic phosphate and Mg⁺⁺ were required for the reaction in the latter organism, and under certain conditions, biotin had a stimulatory effect (Kaltenbach and Kalnitsky, 1951b).

Direct evidence for the Wood-Werkman reaction in animal
tissues was lacking until Utter and Wood (1945) reported the presence of $^{13}C$ in the carboxyl group of oxalacetate formed by pigeon liver extracts in the presence of $^{13}CO_2$, pyruvate, and ATP. ATP was considered necessary either for the formation of a phosphorylated cofactor such as diphosphothiamine, or an intermediate such as a phosphate derivative of pyruvate (Utter and Wood, 1946). In comparison, ATP was not required for CO$_2$ fixation by *Micrococcus lysodeikticus* (McManus, 1951).

Non-photosynthetic CO$_2$ fixation was also studied in parsley root and other higher plant tissues (Vennesland and Felsher, 1946; Gollub and Vennesland, 1947). These investigators concluded that the synthesis of plant dicarboxylic acids resulted from the Wood-Werkman reaction.

Various investigators have implicated biotin in CO$_2$ fixation, but its function has been difficult to determine. Shive and Rogers (1947) suggested a biotin function in the carboxylation of pyruvate in yeast, and the inability of biotin-deficient organisms to synthesize aspartate was attributed to the failure to form oxalacetate from pyruvate and CO$_2$ (Lardy et al., 1947). Williams and Fieger (1947) inferred that biotin was not a component of an enzyme system, and its action on tissue was assigned to an effect on cell permeability or some
type of surface alteration.

Later studies with biotin-deficient rats (MacLeod and Lardy, 1949) indicated that biotin was a coenzyme in CO₂ fixation. Wessman and Werkman (1950) provided direct evidence for this function of biotin when they demonstrated the inhibition of Reaction 2 by avidin in extracts of *Micrococcus lysodeikticus*.

More recently, the ATP-dependent carboxylation of pyruvate was demonstrated in extracts of *Aspergillus niger* (Woronick and Johnson, 1960), and some evidence was presented for a similar reaction in barley roots (Graham and Young, 1959). Utter and Keech (1960) also reported a similar reaction in avian and beef liver but in addition to Mg²⁺ and ATP, acetyl-CoA was required in catalytic amounts.

\[
\text{ATP + } ^{12}\text{CO}_2 + \text{pyruvate} \xrightarrow{\text{acetyl-CoA}} \text{ADP} + P_i \\
\text{+ oxalacetate} \quad (3)
\]

**Malic enzyme** Until 1947, the product of pyruvate carboxylation was generally considered to be oxalacetate, but Ochoa et al. (1947b) obtained an enzyme from pigeon liver which catalyzed the following reaction:

\[
\text{L-malate} + \text{TPN} \leftrightarrow \text{pyruvate} + \text{CO}_2 + \text{TPNH} \quad (4)
\]

The enzyme catalyzing this reaction has become known as the
"malic enzyme". Reaction 4 did not occur when malic dehydrogenase, oxalacetic carboxylase (from *Micrococcus lysodeikticus*), Mn++, and DPN or TPN were combined. Although biotin was apparently involved, none was detected in the purified enzyme, and it was suggested that biotin participated in enzyme synthesis. Blanchard *et al.* (1950) proposed that biotin was involved in the synthesis of other prosthetic groups or apoenzymes. Plaut (1961) suggested that the dependence of biotin nutrition on the level of malic enzyme in *Lactobacillus arabinosus* may be due to an unrecognized cofactor that is formed under the influence of biotin.

Before it was realized that several types of CO₂ fixation reactions exist and that separate enzymes catalyze Reactions 2 and 4, attempts were made to explain one by the other. Thus the decarboxylation of oxalacetate to pyruvate and CO₂ was attributed to malic enzyme (Ochoa *et al.*, 1947a).

On the other hand, Vennesland *et al.* (1947) supported the ATP-dependent exchange reaction in liver preparations. These preparations, however, were later considered to be "enzyme systems" (Plaut and Lardy, 1949), and therefore CO₂ fixation was due to a coupling of enzymes. Little if any CO₂ fixation in oxalacetate was produced by oxalacetate decarboxylase from
Azotobacter vinelandii.

In support of the Wood-Werkman reaction, oxalacetic carboxylase from parsley root (Vennesland et al., 1949) was unaffected by phosphate, TPN, DPN, diphosphothiamine, ATP or biotin, while malate inhibited the reaction. Parsley root preparations also contained malic dehydrogenase which, coupled with Reaction 2, results in Reaction 4. No malic enzyme could be demonstrated in Micrococcus lysodeikticus (McManus, 1951), and Herbert (1950) suggested that the CO₂ fixation in malate was due to the oxalacetic decarboxylase and malic dehydrogenase sequence.

In support of malic enzyme, evidence including the pH optima of the enzymes involved was presented which indicated that the Wood-Werkman reaction was the sum of the reactions catalyzed by malic enzyme and malic dehydrogenase (Veiga Salles et al., 1950).

Finally, Utter (1951) demonstrated that malate was not the precursor of oxalacetate in Reaction 2 and oxalacetate was not the precursor of malate in Reaction 4. One explanation was that CO₂ was fixed in oxalacetate and malate by two separate reactions requiring ATP and TPN, respectively, and that the two products were inter-convertible by malic dehydro-
genase.

**Phosphoenolpyruvate (PEP)**

**PEP carboxykinase** That two separate mechanisms occur in CO₂ fixation was further substantiated when Utter and Kurahashi (1953) partially purified an enzyme from chicken liver which catalyzed the following nucleotide-dependent reversible reaction:

\[
\text{PEP} + \text{HCO}_3^- + \text{ADP(IDP)} \rightleftharpoons \text{oxalacetate} + \text{ATP(ITP)}. \quad (5)
\]

No evidence was obtained for an interaction between PEP carboxykinase and malic enzyme (Utter and Kurahashi, 1954a). The purified enzyme was specific for guanosine and inosine phosphates (Kurahashi et al., 1957) and the utilization of adenosine phosphates was explained by the action of nucleoside diphosphokinase (Utter and Kurahashi, 1954b). This enzyme catalyzes the following reactions (Berg and Joklik, 1954):

\[
\begin{align*}
\text{ATP} + \text{IDP} & \rightleftharpoons \text{ADP} + \text{ITP} \quad (6) \\
\text{ATP} + \text{UDP} & \rightleftharpoons \text{ADP} + \text{UTP}. \quad (7)
\end{align*}
\]

PEP carboxykinase from lamb liver mitochondria was dependent upon inosine phosphate (Bandurski and Lipmann, 1956). The mechanism of Reaction 5 was found to involve the production of the keto form of oxalacetate (not the enol form) (Graves et al., 1956) and possibly included biotin since a purified
enzyme preparation contained bound biotin (Lichstein, 1955).

The demonstration of PEP carboxykinase provided an explanation for several earlier observations. Krebs (1954) explained the conversion of malate to phosphopyruvate (Kalckar 1939) and the alternate path of pyruvate phosphorylation on this basis. The latter consisted of the sequence of malic enzyme, malic dehydrogenase, and PEP carboxykinase.


**PEP carboxylase** Almost simultaneously with the first report of PEP carboxykinase, Bandurski and Greiner (1953) isolated an enzyme from spinach which catalyzed the irreversible nucleotide-independent carboxylation of PEP according to Reaction 8.

$$\text{PEP} + \text{CO}_2 \rightarrow \text{oxalacetate} + \text{inorganic phosphate} \quad (8)$$
This enzyme required a relatively low CO₂ tension and was distinct from malic enzyme.

Apparently PEP carboxylase from wheat germ functioned like PEP carboxykinase since the keto form of oxalacetate (rather than the enol form) was produced (Tchen et al., 1955). Reaction 8 has been demonstrated in Bryophyllum calycinum leaves (Saltman et al., 1956), Crassulacean plants (Walker, 1957), Thiobacillus thiooxidans (Suzuki and Werkman, 1957), various tissues of higher plants (Jackson and Coleman, 1959), etiolated barley leaves (Hall, et al., 1959), bush bean roots (Huffaker and Wallace, 1961), and barley roots (Graham and Young, 1959). In the last case, AMP stimulated the reaction.

From these reports it would seem that PEP carboxylase is limited to plants. However, reference was made recently to the presence of this enzyme in lobster nerve (Cheng and Waelsch, 1962). If this is actually the nucleotide-independent enzyme, the presence of Reaction 8 may be more widespread than supposed. Regardless of the mechanism, the report implied a role of CO₂ in nerve metabolism.

Carboxytransphosphorylase This enzyme has recently been discovered in Propionibacterium shermanii by Wood and coworkers (Siu et al., 1961) and catalyzes the following reaction:
Glycerol $\rightarrow$ glycerol-P $\rightarrow$ triose-P $\rightarrow$ PEP $\rightarrow$ OAA $\rightarrow$ succinate (10)

Labeled propionate was assumed to originate from succinate via methylmalonic isomerase, methylmalonic-oxalacetic transcarboxylase, and propionyl-CoA transferase. (These enzymes are discussed with propionyl-CoA.)

With the discovery of PEP and propionyl-CoA carboxylation, the mechanism for the Wood-Werkman reaction has been explained by the coupling of some of the various carboxylation reactions. Krebs and Lowenstein (1960) considered the Wood-Werkman reaction due to at least three separate enzymes: malic enzyme, PEP carboxykinase, and propionyl carboxylase. However, in the light of the various recent reports concerning the direct carboxylation of pyruvate, a simpler explanation may be discovered.

**Acetyl-CoA**

During the study of the incorporation of acetate into long-chain fatty acids in avian liver (Wakil et al., 1958), acetyl carboxylase was discovered which catalyzed Reaction 11:

$$\text{ATP} + "\text{CO}_2" + \text{acetyl-CoA} \rightarrow \text{ADP} + \text{Pi} + \text{malonyl-CoA}. \quad (11)$$

Lynen (1959) first suggested that malonyl-CoA might be formed from acetyl-CoA, CO$_2$, and ATP. This suggestion was based on
thermodynamic considerations and the presence of biotin in the fatty acid synthesizing enzyme preparations (Wakil et al., 1958). The CO₂ requirement for fatty acid synthesis (Gibson et al., 1958) provided further evidence of a carboxylation reaction. Brady (1958) repeated the suggestion and Wakil (1958) demonstrated a malonyl derivative formed from the reactants in Reaction 11. Since biotin was involved, Lynen considered the mechanism similar to that for the carboxylation of β-methylcrotonyl-CoA (Lynen, 1961). Hatch and Stumpf (1961) found that acetyl-CoA carboxylase from wheat germ catalyzed both carboxylation and transcarboxylation reactions. Although some quantitative differences occurred, it was suggested that acetyl carboxylase as well as other acyl-CoA carboxylases may be bifunctional. It is interesting to note (Numa et al., 1961) that Reaction 11 was the rate limiting step in the synthesis of fatty acids in fasting rats.

Recently Martin and Vagelos (1962) found that citrate stimulated Reaction 11 in adipose tissue of rats. The action of citrate was considered to be either the alteration of the enzyme, the removal of some inhibitor, or the formation of an intermediate in catalytic amounts which remained attached to the enzyme. The stimulation of fatty acid synthesis by
citrate or isocitrate had previously been explained on the basis of TPNH or CO₂ production, although additional effects had been suspected (see Martin and Vagelos, 1962, for literature review). Abraham et al. (1961) suggested the transcarboxylation of the tertiary carboxyl group of citrate to acetyl-CoA, forming malonyl-CoA. Hulsmann (1962) implied that the stimulation of fatty acid synthesis by oxalacetate, isocitrate, or oxalosuccinate is due to malonyl-CoA formation in transcarboxylation reactions (Wood and Stjernholm, 1961).

Thus:

\[
\text{oxalacetate} + \text{acetyl-CoA} \leftrightarrow \text{pyruvate} + \text{malonyl-CoA} \quad (12)
\]

\[
\text{oxalosuccinate} + \text{acetyl-CoA} \leftrightarrow \alpha\text{-ketoglutarate} + \text{malonyl-CoA} \quad (13)
\]

The conversion of citric acid carbon into fatty acids has been demonstrated in pigeon liver preparations (Formica, 1962). Srere and Bhaduri (1962) also demonstrated the presence of labeled carbon in fatty acids from (1,5-\text{C}^{14}) citrate in pigeon liver extracts. A possible explanation was the production of acetyl-CoA and oxalacetate by the action of citrate-cleavage enzyme which catalyzes the following reaction:

\[
\text{citrate} + \text{ATP} + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{oxalacetate} + \text{ADP} + \text{Pi} \quad (14)
\]
On the other hand, citrate did not stimulate fatty acid synthesis from acetate in extracts of lactating-rat mammary gland under optimal TPNH-producing conditions (Dils and Popjak, 1962). It would appear, therefore, that the action of citrate is still open to question.

The sum of Reactions 12 and 15 has been suggested (Hulsmann, 1962) as an explanation for the ATP-dependent carboxylation of pyruvate which required catalytic amounts of acetyl-CoA (Reaction 3).

\[
\text{malonyl-CoA} \longrightarrow \text{acetyl-CoA + CO}_2
\]

(15)

Acetyl-CoA carboxylation has also been demonstrated in pig heart (Formica and Brady, 1959), Mycobacterium avium, Mycobacterium smegmatis, and Nocardia asteroides (Kusunose et al., 1959), dog muscle, dog heart, chicken and rat liver (Stern et al., 1959), bovine liver mitochondria (Halenz and Lane, 1960), Nocardia corallina (Baugh et al., 1961) and yeast (Den and Klein, 1961).

**Acetyl-phosphate and other C₂ compounds**

The exchange of \(\text{C}^{14}\text{O}_2\) with the carboxyl group of pyruvate has been observed in various organisms and several different explanations have been presented. Utter et al. (1945) suggested that this reaction in *Escherichia coli* was due to a
reversal of the "phosphoroclastic reaction", and thus a carboxylation of acetyl-phosphate:

\[ \text{CH}_3\text{-CO-COOH} + \text{H}_3\text{PO}_4 \rightleftharpoons\text{CH}_3\text{-COOPO}_3\text{H}_2 + \text{HCOOH(CO}_2) \]. (16)

Acetaldehyde in its active form has also been suggested as the CO\textsubscript{2} acceptor in CO\textsubscript{2}-pyruvate exchange reactions. Carson et al. (1941) explained the presence of C\textsuperscript{11}O\textsubscript{2} in pyruvate by the reversal of the reaction catalyzed by yeast carboxylase. The following modified reaction was suggested (Strecker and Ochoa, 1954) for the exchange in the presence of diphosphothiamine:

\[ \text{pyruvate + diphosphothiamine} \rightleftharpoons \text{"acetaldehyde"} + \text{CO}_2 \]. (17)

"Acetaldehyde" could be interpreted as (CH\textsubscript{2}CHO•thiamine pyrophosphate).

Several species of clostridia also contain CO\textsubscript{2}-pyruvate exchange systems (Wilson et al., 1948; Rabinowitz and Allen, 1961). The C\textsubscript{2} fragment that is carboxylated in Clostridium acidi-urici remains unknown (Rabinowitz and Allen, 1961), but certain derivatives of vitamin B\textsubscript{12} were found to function as cofactors. However, recent evidence (Peel, 1962) indicates that the cofactor functions in the oxidation of the sulfhydryl compounds necessary for the exchange reaction. Shuster and Lynen (1960) suggested that the CO\textsubscript{2}-pyruvate exchange reaction proceeds by the transfer of CO\textsubscript{2} from CO\textsubscript{2}-biotin-enzyme to
"α-hydroxyethyl-thiamine pyrophosphate" with the formation of "α-pyrophosphoryl-thiaminyl-lactic acid.

**Propionyl-CoA**

The overall reaction for the carboxylation of propionyl-CoA in pig heart preparations was presented by Flavin *et al.* (1957) and occurred as follows:

\[
\text{ATP} + \text{"CO}_2" + \text{propionyl-CoA} \xrightleftharpoons{Mg^{++}} \text{ADP} + P_i + \text{methylmalonyl-CoA}.
\] (18)

Propionyl carboxylase from different sources has been studied extensively and various physical and catalytic constants have been determined for the purified and crystallized enzyme (Halenz *et al.*, 1962; Kaziro *et al.*, 1961).

Observations contributing to the elucidation of Reaction 18 include the carboxylation of propionate with the formation of succinate (Barban and Ajl, 1951), the carboxylation of propionyl-CoA (Lardy and Adler, 1956), and the detection of methylmalonate during propionate carboxylation (Katz and Chaikoff, 1955).

Reaction 18 is currently believed to consist of the following steps (Lane *et al.*, 1960; Ochoa and Kaziro, 1961):

\[
\text{ATP} + \text{"CO}_2" + \text{enzyme} \xrightleftharpoons{Mg^{++}} \text{ADP} + P_i + \text{CO}_2-\text{enzyme}
\] (19)
**CO\textsubscript{2}-enzyme + propionyl-\text{CoA}$\leftrightarrow$enzyme + methylmalonyl-\text{CoA} (20)**

Although the carboxylation of biotin was not demonstrated, CO\textsubscript{2}-enzyme was considered equivalent to enzyme-biotin-CO\textsubscript{2} (see \(\beta\)-methylcrotonyl-CoA) and a concerted mechanism was suggested (Ochoa and Kaziro, 1961) for Reaction 19. It was suggested that all ATP-dependent carboxylases are "double-headed" enzymes which first activate CO\textsubscript{2} and then transfer the activated CO\textsubscript{2} to the specific CO\textsubscript{2} acceptor.

Prior to the acceptance of the concerted mechanism for the formation of active CO\textsubscript{2}, Reaction 19 was thought to proceed by two steps (Halenz and Lane, 1960; Kaziro et al., 1960); the first was the formation of enzyme-P by ATP, followed by a reaction with CO\textsubscript{2}, producing enzyme-CO\textsubscript{2}. Carbonylphosphate had also been considered to be the active species of CO\textsubscript{2} formed by a reaction between CO\textsubscript{2} and ATP (Flavin et al., 1957). On the other hand, Calvin and Pon (1959) suggested that in carboxylation reactions requiring ATP, the CO\textsubscript{2} acceptor initially underwent activation with the formation of the enol phosphate of the thiol esters.

**Transcarboxylation** Another method of methylmalonyl-CoA formation from propionyl-CoA is shown by Reaction 21 and catalyzed by oxalacetic transcarboxylase (Swick and Wood, 1960;
Wood and Stjernholm, 1961):

\[
\text{propionyl-CoA} + \text{oxalacetate} \leftrightarrow \text{methylmalonyl-CoA} + \text{pyruvate}.
\]

In this case an intermolecular transfer of a carboxyl group produces the product rather than a direct carboxylation. Reaction 21 has been explained (Ochoa and Kaziro, 1961) by the coupling of Reaction 20 and Reaction 22.

\[
\text{oxalacetate} + \text{enzyme} \leftrightarrow \text{pyruvate} + \text{CO}_2\text{-enzyme}
\]

The sum of Reactions 21 (reversed) and 18 was suggested as an explanation for Reaction 3, since propionyl-CoA could substitute for acetyl-CoA. It is interesting to note that Reaction 22 can be considered the sum of Reaction 19 and the reverse of Reaction 3.

Another transcarboxylation reaction was reported to proceed according to Reaction 23 and is catalyzed by purified mitochondrial propionyl carboxylase (Halenz and Lane, 1961).

\[
\text{methylmalonyl-CoA} + \text{butyryl-CoA} \leftrightarrow \text{propionyl-CoA} + \text{ethylmalonyl-CoA}
\]

However, since propionyl carboxylase produces ethylmalonyl-CoA from butyryl-CoA, Reaction 23 has been explained by the coupling of Reaction 24 with Reaction 20 (Ochoa and Kaziro, 1961).
ethylmalonyl-CoA + enzyme $\rightleftharpoons$ butyryl-CoA + CO$_2$-enzyme (24)

Evidence has also been presented for the carboxylation of propionyl-CoA in dog muscle, dog heart, chicken liver, and rat liver (Stern et al., 1959), dog skeletal muscle and ox liver (Stern et al., 1961), Nocardia corallina (Baugh et al., 1961), wheat germ (Hatch and Stumpf, 1961), Rhodospirillum rubrum (and probably Rhodopseudomonas capsulata; Knight, 1962), and Mycobacterium smegmatis (Noble et al., 1962).

Acetone

Plaut and Lardy (1950) detected the carbons of formate and carbonate in the carboxyl group of acetoacetate in the presence of rat liver slices and suggested the following explanation:

acetone + CO$_2$ $\rightleftharpoons$ acetoacetic acid (25)

This reaction was used as the last step in the mechanism for the conversion of leucine to acetoacetate (Coon, 1950) and as the first step in the photoassimilation of acetone by Rhodopseudomonas gelatinosa (Siegel and Smith, 1955).

Butyryl-CoA

Stern et al. (1959) reported the carboxylation of butyryl-CoA in extracts of dog muscle, dog heart, chicken liver, and
rat liver according to Reaction 26:

\[
\text{butyryl-CoA} + \text{ATP} + \text{CO}_2 \leftrightarrow 2\text{-ethylmalonyl-CoA} + (\text{ADP} + \text{P}_i). \tag{26}
\]

This reaction was also shown in dog skeletal muscle and ox liver (Stern et al., 1961). Other thiol esters were carboxylated and the relative rate was 1:25:3 for acetyl-CoA, propionyl-CoA, and butyryl-CoA, respectively. Butyryl-CoA was carboxylated by propionyl carboxylase from pig heart (Kaziro et al., 1961) and from bovine liver mitochondria (Halenz and Lane, 1960), but this behavior was later ascribed to the above-mentioned transcarboxylation reaction (Reaction 23). Extracts of Nocardia corallina (Baußh et al., 1961) and acetyl carboxylase from wheat germ (Hatch and Stumpf, 1961) also carboxylated butyryl-CoA.

**α-Aminobutyrate**

An alternative mechanism for photosynthetic CO₂ assimilation is the carboxylation of α-aminobutyrate. Warburg (1958) suggested the following stationary state between the decomposition and synthesis of glutamate in Chlorella:

\[
\text{L-glutamate} \leftrightarrow \alpha\text{-aminobutyrate} + \text{CO}_2. \tag{27}
\]

Glutamate was also identified as the CO₂ fixation product in *Euglena* (Vishniac and Fuller, 1958).
Crotonyl-CoA

The carboxylation of crotonyl-CoA was catalyzed by both purified (Lane et al., 1960) and crystalline propionyl carboxylase (Kaziro et al., 1961), but the product was not identified. It was stated, however, that the product was not glutaconic, mesaconic, citraconic or citramalic acid (Lane et al., 1960).

Glutaconyl-CoA was the product of crotonyl-CoA carboxylation in rat liver enzyme fractions (Tustanoff and Stern, 1960).

Acetoacetyl-CoA

Acetoacetyl-CoA was a poor substitute for propionyl-CoA as the CO2 acceptor in the transcarboxylation reaction (21) (Wood and Stjernholm, 1961). The product was not identified but was suggested to be either acetomalonyl-CoA or β-ketoglutararyl-CoA.

Isobutyryl-CoA

Purified propionyl carboxylase from bovine liver mitochondria was found to carboxylate isobutyryl-CoA with the formation of dimethylmalonyl-CoA (Lane et al., 1960). 2,2-Dimethylmalonyl-CoA was the suggested product of isobutyryl-CoA carboxylation in dog skeletal muscle, dog heart, and ox, chicken and
rat liver (Stern et al., 1961).

**β-Methylcrotonyl-CoA**

The discovery of the carboxylation of β-methylcrotonyl-CoA resulted from the study of leucine degradation. β-Hydroxy-β-methylglutaryl-CoA was suggested to arise from the carboxylation of β-hydroxyisovaleryl-CoA in heart extracts (Bachhawat et al., 1954). However, the true CO₂ acceptor was later found to be β-methylcrotonyl-CoA in extracts of *Mycobacterium* spp. (Lynen, 1959). The overall reaction was presented as follows:

\[
\text{ATP} + \text{CO}_2 + \beta\text{-methylcrotonyl-CoA} \rightarrow \text{ADP} + \text{P}_i + \beta\text{-methylglutaconyl-CoA. (28)}
\]

Subsequently, β-hydroxyisovaleryl-CoA (del Campillo-Cambell et al., 1959) and β-methylvinylacetyl-CoA (Rilling and Coon, 1960) were eliminated as possible CO₂ acceptors in favor of β-methylcrotonyl-CoA. Apparently, sufficient crotonase was present in the earlier preparations to cause the conversion of β-hydroxyisovaleryl-CoA to β-methylcrotonyl-CoA (Stumpf, 1960).

β-Methylcrotonyl carboxylase, like the other acyl-CoA carboxylating enzymes, is a biotin enzyme and catalyzes a more involved reaction than indicated by Reaction 28. Various references have been made to the function of biotin in...
carboxylation reactions, but a discussion of biotin was deferred until this section in order to treat it with the enzyme in which the currently accepted formulation of active CO₂ was discovered.

Burk and Winzler (1943) suggested that biotin was the coenzyme of CO₂ transfer and functioned by opening and closing of the urea ring with a subsequent gain or loss of CO₂. However, Melville et al. (1949) observed no transfer of labeled ureido-carbonyl groups during CO₂ fixation. Delwiche et al. (1954) provided evidence for an active species of CO₂ which was later thought to be carbonyl-phosphate (Flavin et al., 1957) or CO₂-adenylate (Bachhawat et al., 1956). Lynen (1959) reported that a carboxylated derivative of biotin had been detected in the 3-methylcrotonyl carboxylase reaction and proposed that "active CO₂" is N-carboxybiotin (Lynen, 1961).

The following reactions were proposed as comprising Reaction 28 (Lynen, 1959):

\[
\begin{align*}
\text{ATP} + \text{biotin-enzyme} & \xrightleftharpoons{\text{Mg}^{++}} \text{ADP-biotin-enzyme} + \text{P} \quad (29) \\
\text{ADP-biotin-enzyme} + \text{CO₂} & \xrightleftharpoons{} \text{CO₂-biotin-enzyme} + \text{ADP} \quad (30) \\
\text{CO₂-biotin-enzyme} + \beta\text{-methylcrotonyl-CoA} & \xrightleftharpoons{} \text{biotin-enzyme} + \beta\text{-methylglutaconyl-CoA} . \quad (31)
\end{align*}
\]

The structure of CO₂-biotin-enzyme was given by Lynen et al.
Valeryl-CoA

Lane et al. (1960) reported that propionyl carboxylase also catalyzed the carboxylation of valeryl-CoA, and although the product was not identified, it was not the anticipated α-carboxylation product, n-propylmalonic acid.

Coenzyme A esters of long chain β-keto acids

The incorporation of HC\textsubscript{14}O\textsubscript{3} into malonyl-CoA has been reported in Clostridium kluyveri extracts (Vagelos and Alberts, 1960) by the reversal of the following condensation-decarboxylation reaction:

\[
*\text{COOH}-\text{CH}_2-\text{COSCoA} + \text{R-COSCoA} \leftrightarrow *\text{CO}_2 + \text{R-CO-CH}_2-\text{COSCoA} + \text{CoASH}
\]  

(32)

Succinate

Cell-free extracts of Escherichia coli catalyzed the following reversible reaction (Aj1 and Werkman, 1948):

\[
\text{COOHCH}_2\text{CH}_2\text{COCOOH} + 0 \leftrightarrow \text{COOHCH}_2\text{CH}_2\text{COOH} + \text{CO}_2
\]  

(33)

The incorporation of labeled CO\textsubscript{2} in α-ketoglutarate indicated the carboxylation of succinate. ATP enhanced the reversibility of the reaction, and a phosphorylated intermediate was suggested.
α-Ketoglutarate

Crude preparations of isocitric dehydrogenase from pig heart contained oxalosuccinic carboxylase which catalyzed the carboxylation of α-ketoglutarate (Ochoa, 1945). The reaction was also demonstrated in pigeon liver extracts (Grisolia and Vennesland, 1947), pigeon breast muscle, and pig kidney (Ochoa and Weisz-Tabori, 1948). Both isocitric dehydrogenase and oxalosuccinic carboxylase were associated with the same purified protein from pig heart extracts (Moyle and Dixon, 1956).

Ribulose-5-phosphate

Horecker and Smyrniotis (1952) reported the carboxylation of ribulose-5-phosphate in an enzyme preparation from yeast by the reversal of the following reaction:

$$6\text{-phosphogluconate} + TPN \rightarrow \text{ribulose-5-phosphate} + \text{CO}_2 + \text{TPNH} + \text{H}^+ \quad (34)$$

Ribulose-1,5-diphosphate

The carboxylation of ribulose-1,5-diphosphate is catalyzed by carboxydismutase and proceeds according to Reaction 35:

$$\text{ribulose-1,5-diphosphate} + \text{CO}_2 \rightarrow 2\text{ phosphoglyceraldehyde} \quad (35)$$

This reaction is the photosynthetic mechanism for CO₂ fixation.
and was first observed in extracts of *Chlorella* (Quayle *et al.*, 1954) and spinach leaves (Weissbach *et al.*, 1954). A more detailed mechanism for Reaction 35 was later presented (Calvin, 1954, 1962). The carboxylation of ribulose-1,5-diphosphate has also been demonstrated in the photosynthetic bacterium, *Rhodospirillum rubrum* (Glover *et al.*, 1952), in the autotrophic bacteria, *Thiobacillus thioparus* (Santer and Vishniac, 1955), *Thiobacillus denitrificans* (Trudinger, 1956), and *Thiobacillus thiooxidans* (Suzuki and Werkman, 1958b), and in the heterotrophic bacterium, *Escherichia coli* (Fuller, 1956). The proposed carboxylation of α-aminobutyrate (Warburg, 1958) and the reduction of CO₂ to glycolate (Tanner *et al.*, 1960) indicate that Reaction 35 is not the only method of photosynthetic CO₂ assimilation.

**5-Aminoimidazole ribotide**

One of the steps in the synthesis of nucleotides is the carboxylation of 5-aminimidazole ribotide. This conversion is shown in Reaction 36 and was demonstrated by Lukens and Buchanan (1957) in extracts of avian liver.

\[
5\text{-aminoimidazole ribotide } + \text{ CO}_2 \leftrightarrow 5\text{-amino-4-}\text{-imidazolecarboxylic acid ribotide}
\] (36)
Biotin was implicated in this reaction in *Saccharomyces cerevisiae* (Moat et al., 1956) but since apparently no added energy sources are necessary for the reaction, a direct carboxylation occurs (Calvin and Pon, 1959) and, therefore, no activation of CO$_2$ with the formation of CO$_2$-biotin would be necessary. The indirect function in the synthesis of the enzyme would be one explanation.

**Other Mechanisms of CO$_2$ Fixation**

An attempt has been made to limit this review to mechanisms involved in the addition of CO$_2$ to the carbon chain of organic compounds. Thus the following types of CO$_2$ fixation have been purposely omitted: reduction to formic acid (Woods, 1936), acetic acid (Wieringa, 1940), and methane (Barker, 1936); function as a respiratory hydrogen acceptor (Hes, 1938); and carbamate formation in urea synthesis (Jones and Lipmann, 1960).

For more information concerning CO$_2$ fixation and Cl metabolism, one should see: Werkman and Wood (1942a, 1942b), Wood (1946), Carson (1948), Werkman (1949, 1951), Ochoa (1951), Utter and Wood (1951), Vennesland and Conn (1952), and Quayle (1961).
MATERIALS AND METHODS

Enzyme and Whole-cell Preparations

The two organisms used in this study were *Noccardia corallina* ATCC 4273 and *Rhodospirillum rubrum* ATCC 11170.

Large amounts of *Noccardia corallina* were grown for 48 hr at 30 C in six liter flasks containing two liter quantities of a medium consisting of 0.3% each of peptone and yeast extract. The inoculum was a 48 hr culture in the same medium.

Aerated cells were grown in ten liters of the above medium in a sparger-equipped twelve liter flask for 20 hr at 28 C or in a New Brunswick Fermentor (Model FS614) for 36 hr at 30 C. The medium in the sparger flask contained 1.4 ml of Antifoam 60 (General Electric); that in the fermentor contained 5.5 ml of Antifoam A (Dow Corning). Inocula for aerated cultures were incubated 48 hr on a Burrell wrist-action shaker (Model DD) at 30 C. A description of the growth of *Noccardia corallina* is presented in Appendix A.

The cells were harvested in a steam driven Sharples Super-Centrifuge (Type TIP), washed with distilled water, and resuspended (with the aid of a syringe or a Waring Blender) in 10 volumes of 0.1 M Tris buffer, pH 7.8. Cell-free extracts
were prepared by treating the cells three minutes in a Raytheon 200 watt, 10Kc Magnetostrictive Oscillator, Model DF-101, and centrifuging for 15 min at 24,500 x g in an International Refrigerated Centrifuge (Model PR-1) at 4 C.

The crude extracts were partially purified by various procedures, one of which consisted of dialysis for 8 hr against 4 liters of 0.001 M Tris buffer, pH 7.8 (containing 2 x 10^{-5} M GSH), in an Oxford Multiple Dialyzer (Model B). The buffer was changed at two hr intervals. Dialyzed extracts were also obtained by treatment in an electroconvection apparatus (Model E-C-25, E. C. Apparatus Company) containing 4 liters of the same buffer without the GSH for 1.5 hrs at 40 volts.

An ammonium sulfate precipitated preparation was obtained by treating the dialyzed extract with increasing amounts of saturated ammonium sulfate. The precipitates recovered by centrifugation were suspended in a volume of 0.001 Tris buffer, pH 7.8 (containing 2 x 10^{-5} M GSH), equal to the volume of the initial extract and were dialyzed in the Oxford Dialyzer as previously described against 12 liters of the same buffer.

DEAE-cellulose was also used to purify the enzymes
according to the following procedure: One g of DEAE-cellulose was washed with 40 ml of 0.05 M Tris buffer, pH 7.5, resuspended in 20 ml of 0.05 M Tris, pH 7.5, and introduced into a 42.5 cm length of glass tubing (9.8 mm I.D.) stoppered with glass wool. The resulting column was washed again with the same buffer and then 10 ml of crude extract was added to the column. The following amounts of 1.0 M KC1 were made up to 10 ml with 0.05 M Tris, pH 7.5, and added in succession after two 10 ml additions of Tris: 0.25 ml, 0.5 ml, 1.0 ml. Finally two successive 10 ml volumes of 1.0 M KCl were added. All operations were carried out at 4 C and the collected fractions were also stored at 4 C (See Appendix B).

*Rhodospirillum rubrum* was grown for 5 days at 30 C in 500 ml screw-top prescription bottles completely filled with a lactate medium described by Woody and Lindstrom (1954). The bottles were illuminated with two 75 watt incandescent light bulbs at a distance of 50 cm. The inoculum was grown under the same conditions in screw-top test tubes. Dark-grown cells were grown with sparging in 6 L of a medium prepared according to Hug (1956) in a light-tight box for 3 days at 30 C. The cells were harvested in a compressed
air driven Sharples Super-Centrifuge (Type T-41-24 IHY), washed three times with distilled water, and suspended in 5 volumes of 0.1 M Tris buffer, pH 7.4. The cell suspension was treated in a Raytheon 50 watt, 9 Kc. Magnetostriction Oscillator (Model S-102) for 5 min, and the debris was removed by the same procedure as described for Nocardia corallina.

Dialyzed extracts were obtained by continuous treatment for about 30 hr against 10 L 0.005 M phosphate buffer (pH 6.0) on a rocker dialyzer. Partially purified extracts were prepared by fractionation with ammonium sulfate. The precipitated fractions resulting from increasing amounts of saturated ammonium sulfate solution were centrifuged 10 min at 24,500 x g at 4 °C in the refrigerated centrifuge, suspended in 0.9% KCl, and dialyzed one hr each in the electroconvection apparatus against distilled water and against 0.005 M potassium phosphate buffer, pH 5.9. Crude extracts which had been frozen two months at -20 °C were made 10% volume with saturated ammonium sulfate solution. After centrifugation as described above, the precipitate was discarded and the supernatant solution was dialyzed as before.
Chemicals

NaHCO\textsubscript{14}O\textsubscript{3} was prepared according to Hug (1956) from BaC\textsubscript{14}O\textsubscript{3} obtained from Oak Ridge National Laboratory. Propylmalonic acid was prepared by hydrolyzing diethyl-n-propylmalonate (Sapon Laboratories), extracting with ether, evaporating to dryness, and recrystallizing twice from benzene. The observed melting point was 90 C; the published melting point of propylmalonic acid is 96 C (Hodgman, 1953).

Other chemicals were commercial preparations: 3-methylvaleric acid, DL-β-hydroxybutyric acid, α-hydroxybutyric acid, tiglic acid, α-ketoisovaleric acid, caproic acid, heptanoic acid, capric acid, caprylic acid, β-hydroxy-β-methylglutaric acid, phosphoenolpyruvic acid (tricyclohexylamine salt), tartronic acid, glyoxylic acid and β-hydroxypropionic acid were obtained from the California Foundation for Biochemical Research; isobutyric acid, α-hydroxyisobutyric acid, DL-2-methylbutyric acid, acrylic acid, isovaleric acid, nonanoic acid, diethylethylmalonate, DEAE-cellulose, hydroxylamine hydrochloride, 2,2′-iminodiethanol and thiourea from Eastman Kodak; 2-hexenoic acid, 4-phenylbutyric acid, 5-methylcaproic acid, 2-pentenoic acid and vinylacetic acid from Sapon Laboratories; γ-methylvaleric acid, crotonic acid,
β-methylvaleric acid, methacrylic acid, methylmalonic acid, ethylmalonic acid, mesaconic acid and iodoacetamide from K and K Laboratories, Inc.; 3,3-dimethylacrylic acid, dimethylmalonic acid and glutaconic acid from Aldrich Chemical Co., Inc.; nucleoside polyphosphates (sodium salts) and coenzyme A (CoA) from Pabst Laboratories; reduced glutathione (GSH), oxidized glutathione (GSSG), bovine albumin (crystalline), avidin, oxalacetic acid (OAA) and L-cysteine (free base) from Nutritional Biochemicals Corporation; sodium pyruvate from Mann Research Laboratories, Inc.; tris (hydroxymethyl) aminomethane (Tris) from Sigma Chemical Company; potassium propionate-1-C^{14} from Texas Foundation.

Microbiological Methods

The Barcroft-Warburg apparatus was used in the manometric studies according to the standard techniques of respirometry as described by Umbreit et al. (1957). Conventional 18 ml flasks with two side arms were used; 0.2 ml of 10% KOH and accordian folded pieces of filter paper were placed in the center well. The reactions were carried out at 30 C for lengths of time specified in the discussion of the results.

The effect of CO_{2} on O_{2} utilization was determined
according to Pardee (1949).

Enzymatic reactions were also conducted in the Warburg apparatus at 30 C after flushing the flasks for 10 min with oxygen-free nitrogen. Appropriate amounts of the compounds to be added to the reaction were dissolved in deionized water and neutralized with saturated NaOH or KOH.

In the nutrition experiments with *Nocardia corallina*, 0.1 ml of yeast extract-peptone culture was added to each of several tubes containing 5 ml of the following synthetic medium: 

\[
\begin{align*}
(NH_4)_2SO_4, & \quad 4.72 \text{ g; NaCl, 5.00 g; KH}_2\text{PO}_4, \quad 0.6 \text{ g; K}_2\text{HPO}_4\cdot3\text{H}_2\text{O, } \quad 16.18 \text{ g; traces of MgCl}_2, \quad \text{FeCl}_3, \quad \text{and CaCl}_2; \quad \text{and 1000 ml of distilled water. Each tube contained 10 mg of the acid to be tested and the pH was adjusted to 8.0 with saturated NaOH. The tubes were incubated at 30 C and growth was estimated by observing the characteristic pellicle and pink color.}
\end{align*}
\]

**Paper Chromatographic Methods**

**Chambers**

In the ascending technique, cylinders of Whatman No. 1 or Whatman No. 3 filter paper were developed in one gallon wide-mouth jars. Large test tubes were also employed as chambers when it was desirable to use only small amounts of
of solvent or single strips of filter paper. The strip was supported and lowered into the solvent by a glass hook protruding through a rubber stopper. In the descending technique, 22-inch strips of Whatman No. 1 filter paper were developed either in a Chromatocab (Model A, Research Equipment Corporation), a 12" square Pyrex Chromatography jar containing a CALAB Pyrex Brand Glass Rack, or an 8" x 12" rectangular chromatography jar in which the solvent troughs were supported by a RSCo Model 102-03 Band Spring Insert Rack. In certain cases, the papers were washed with citric acid or acetic acid and rinsed with distilled water prior to use.

**Solvent systems**

The following solvents were used to separate and identify the various compounds, and the figures in parentheses indicate the proportions by volume. (See Appendix D for Rf values). CoA esters were developed in EA (ethanol:0.1M sodium acetate buffer, pH 4.5, 1:1) and PIW (pyridine:isopropanol:water, 1:1:1) (Stadtman, 1957). The liberated acids following alkaline hydrolysis of the CoA esters and the hydroxamic acid derivatives were developed in the following solvents: EaFW (ethylacetate:formic acid:water, 30:10:5), EAW-I (ethanol: concentrated NH₄OH:water, 8:5:20), EAW-II (ethanol:concen-
trated NH₄OH:water, 40:5:5) ifF (isopentanol:formic acid) (Flavin and Ochoa, 1957), PFW (n-pentanol:formic acid:water, 3:1:3), and OFW (octanol:formic acid:water, 3:1:3), BA-I (butanol:1.5N NH₄OH, 1:1), PA (propanol:concentrated NH₄OH, 6:4). The 2,4-dinitrophenylhydrazones were separated by BA-II (butanol:3% NH₄OH, 1:1). The above mixtures were agitated vigorously and in the cases where two phases appeared, the water phase was separated from the organic phase and placed in the chamber in a small beaker. In the one-dimensional technique, the compounds to be analyzed were placed along one side of the paper, and the solvent was allowed to flow perpendicular to the line of spots. In the two-dimensional technique, one spot was made near the corner of a square piece of paper. The chromatogram was developed first as described for the one-dimensional technique but after drying, it was turned 90° and developed again. Better resolution is achieved with this technique especially if two different solvents are used.

**Location of Spots**

The location of the various compounds on the dried chromatograms was determined by spraying with the appropriate reagents or by observing the absorption of ultraviolet light under a Mineralight (Model SL, Ultra-Violet Products, Inc.).
This is a simple and sensitive method of detecting compounds containing the electronic configuration capable of absorbing radiation of this wave length. Organic acids developed in acidic solvent systems were detected by a mixed acid-base indicator (Aronoff, 1956). A stock solution was prepared by dissolving 0.5 g of methyl yellow (dimethylaminobenzene) and 1.5 g of brom phenol blue in 200 ml of 95% ethanol and adjusting the pH to 6.3. The spray reagent was prepared by diluting one part of the stock solution with twenty parts of ethanol. Free acids appeared as yellow to red spots on a blue-green background. Organic acids on chromatograms developed in ammonia solvents were detected by spraying with the indicator described by Kennedy and Barker (1951). This indicator consisted of 50 mg of brom phenol blue and 200 mg of citric acid in 100 ml of water. The ammonium salts of the acids appeared as blue spots on a yellow background. Hydroxamic acids were located by spraying with a reagent prepared by diluting 5.0 g FeCl₃·6H₂O and 0.83 ml concentrated HCl to 100 ml with 95% ethanol (Stadtman and Barker, 1950). The ferric complex appeared as a purple spot on a yellow background.

Frequently it was desirable to transfer a compound from one chromatogram to another. This was accomplished by cut-
ting the area surrounding the spot in the shape of a wedge, attaching the rectangular end to a filter paper cylinder, and placing it in an eluting solvent. The pointed end of the wedge touched the origin of the new chromatogram and transfer was effected by upward movement of the solvent. A plastic cylinder surrounding the wedge decreased evaporation and a stream of air directed on the new chromatogram controlled the size of the spot. Ethyl acetate, benzene, or 95% ethanol were not satisfactory as eluting solvents. Water was used in some cases but one part of 95% ethanol diluted with three parts of water was most satisfactory.

Analytical Methods

Assay of Radioactivity

The total nonvolatile $^{14}C\text{O}_2$ fixed in the enzymatic reactions was determined by counting the radioactivity with a lead-shielded end-window (mica) Geiger-Muller tube (Tracerlab type TGC2) with a window thickness of 1.8 mg per cm$^2$. A Nuclear Instrument and Chemical Corporation, Model 163, scaling unit was used. The counts per min were corrected for background; the error was about 5%. Since comparison between samples rather than absolute cpm was desired, the uniformity
in samples and geometry made other corrections unnecessary. The reaction mixtures were acidified to precipitate the protein and to remove unreacted $^{14}\text{O}_2$. Reaction mixtures containing extracts of *Rhodospirillum rubrum* were acidified with 0.2 ml of 50% trichloroacetic acid and those containing extracts of *Nocardia corallina* were acidified with 0.1 ml of concentrated HCl. Aliquots of the supernatant solutions following centrifugation were plated uniformly on ground glass planchets. The material was transferred by $\lambda$ pipettes and deposited gradually while the planchets rotated on a small turntable (Autonomous Instruments) and was dried under a stream of air. The planchets were then counted with the GM tube described above. The 2,4-dinitrophenylhydrazone of radioactive oxalacetic acid was collected on Whatman No. 42 filter paper, dried, and counted.

The total radioactivity in the reaction mixtures was usually due to several substances and the amount contained in each compound was determined by chromatography followed by use of the Fowler-Rhinehart Chromatogram Scanner (U. S. Patent Nos. 3,027,458 and 3,033,986). The individual radioactive compounds produced peaks on the automatic recording chart paper. The area under the peaks was proportional to the
amount of radioactivity, and was determined with a planimeter (Keuffel and Esser Co., Model 4236M). The operating conditions for the scanner were: 1600 v, 4 cm Q gas (The Matheson Co.) and a distance of 16 mm between the anode and the resolving slit (See Appendix C).

Identification of Radioactive Compounds

Analysis of the radioactive compounds in the reaction mixtures was accomplished by chromatographing directly or by first converting to some other form. CoA esters or organic acids were chromatographed without previous treatment; however, the free acids liberated by alkaline hydrolysis were usually studied. Portions of the reaction mixtures were made basic with KOH, heated 15 min in a 60 C water bath, and acidified with HCl. In certain cases the hydroxamic acid derivatives of the organic acids were prepared from the CoA esters. Excess neutral hydroxylamine was added to neutralized aliquots of the reaction mixtures and heated and acidified as described above for alkaline hydrolysis. Both the free acids and the hydroxamic acid derivatives were separated from substances in the mixtures which would interfere in the chromatographic analysis, by evaporation to dryness and extraction with ether or absolute ethanol. Interfering ions were also removed in
some experiments with an RSCo Electric Desalter (Model A-1930) or by Dowex-50 cation-exchange resin columns. Probably the simplest, quickest, and most frequently used method of preparing the radioactive compounds for chromatography was to add 5 ml of redistilled acetone to aliquots of the solutions to be analyzed; the resulting precipitate was then removed by centrifugation. Regardless of the method of ion removal, the volume of the solution containing the compounds to be analyzed was reduced to about 0.1 ml under a stream of air at room temperature. Samples, and 0.2 μmole of authentic compounds, were deposited on chromatograms and developed in appropriate solvents. Oxalacetic acid was identified by chromatographing the 2,4-dinitrophenylhydrazone. Aliquots of reaction mixtures (containing 5 mg of added carrier oxalacetic acid) were treated with 9 ml of 2 N HCl saturated with 2,4-dinitrophenylhydrazine. The crystals were collected by filtration, washed with 2 N HCl and water, and dried. Solutions for chromatography were obtained by dissolving the crystals in dilute NH₄OH.

The location of the radioactivity on the chromatograms, and thus the characteristic Rf value for the compounds, was determined by three methods. Frequently a quick but rather
indefinite estimate was satisfactory and was obtained by passing the chromatogram beneath the end-window GM tube. The area indicating radioactivity was limited by a strip of cardboard through which a hole the size of the spot had been cut. A relatively accurate estimate of the $R_f$ was obtained by using the Fowler-Rhinehart Scanner. Since the chromatogram and the chart were the same length, the center of the radioactive spots was located by placing the two side by side. The point on the chart tracing which coincided with the center of the radioactive spot was determined by observing numerous known samples. It was found that the center of the spot occurs as the pen reaches the top of the peak. Some variation occurs, however, depending upon the intensity of the radioactivity, and the centers of spots with low activity occur after the top of the peak is reached. Neither of the above methods gives much information concerning the shape of the spot, and final identification was made by radioautography. The chromatograms were placed on Kodak No-Screen medical X-ray film. The length of time necessary for detectable spots to be formed was determined from the following relation:

$$
\text{days} = \frac{7.72 \times 10^2 \times \text{area of the spot (cm}^2\text{)}}{\text{counts per minute}}
$$
This relationship was derived experimentally from the average of the area and activity of several spots. The films were developed in Eastman Kodak D-16 Developer. Following passage through an acetic acid stop bath (Kodak SB-1a), the films were fixed either in sodium thiosulfate (294 g per liter) or in Agfa 201 Fixer. The determination of whether the unknown radioactive compound was identical with the cochromatographed authentic compound was made by superimposing the dried film upon the chromatogram, and comparing the outline of the spot of the known compound on the chromatogram (as revealed by the spraying reagent) with the spot of the radioactive compound on the film. If the outlines were identical in at least two solvent systems (usually 3 or 4), the compounds were considered identical. Development in different solvent systems was accomplished by the two-dimensional technique or by transfer to successive chromatograms as previously described.

The location of the radioactive carbon in oxalacetic acid was determined by the method of Bandurski and Greiner (1953). The 2,4-dinitrophenylhydrazone was boiled in a degradation apparatus similar to that described by Hug (1956), and the $\text{C}^{14}\text{O}_2$ trapped in the NaOH solution was counted as $\text{BaC}^{14}\text{O}_3$.

The protein in the purest *Rhodospirillum rubrum* extracts
was determined by the biuret method (Layne, 1957). The absorbancy was determined either in a Spectronic 20 spectrophotometer (Bausch and Lomb) at 540 m\(\mu\) or in a Klett-Summerson colorimeter using a 540 m\(\mu\) filter. The other extracts were treated with 80% ethanol made acidic with acetic acid to remove the pigments (Vernon and Kamen, 1953) prior to the protein determination by the above procedure.

The protein in the \textit{Nocardia corallina} extracts was estimated by determining the turbidity produced by 10% trichloroacetic acid in the Klett-Summerson colorimeter using a 660 m\(\mu\) filter. The samples were agitated occasionally during a 10 min period before the absorbancy was determined. Crystaline bovine albumin was used as the standard in both protein determination procedures.

The growth of \textit{Nocardia corallina} was determined by measuring the turbidity in the Klett-Summerson colorimeter using the 660 m\(\mu\) filter.
EXPERIMENTAL

Carboxylation of Phosphoenolpyruvate (PEP)

At the beginning of this investigation, the carboxylation of PEP was being studied in this laboratory in the chemoautotroph, Thiobacillus thiooxidans, and the heterotroph, Nocardia corallina. It was considered of interest to determine whether the photosynthetic bacteria also contained the enzymes for this conversion.

Subsequent experiments with cell-free extracts from light-grown cells of Rhodospirillum rubrum showed that $^{14}\text{C}_{\text{O}_2}$ was fixed when the reaction mixture contained PEP or oxalacetate (OAA). Nucleotides stimulated the reaction but some fixation occurred in the absence of added nucleotides. Although the actual values varied with the different extracts, the results in Table 1 are typical for crude and dialyzed extracts and also for extracts treated with activated charcoal or Dowex-3 anion-exchange resin.

Different results were obtained, however, when the reactions were catalyzed by aged extracts or by enzyme preparations obtained with above 40% (v/v) of saturated ammonium sulfate. No significant radioactivity was detected in the
absence of added nucleotides (Table 2). The results from flasks 7-10 indicate that various combinations of pyruvate, malate, and nucleotides did not substitute for either PEP and inosine-5'-diphosphate (IDP) or OAA and inosine-5'-triphosphate (ITP).

Table 1. CO₂ fixation in dialyzed extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Additions</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PEP</td>
<td>304</td>
</tr>
<tr>
<td>2</td>
<td>IDP</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>PEP, IDP</td>
<td>377</td>
</tr>
<tr>
<td>4</td>
<td>OAA</td>
<td>405</td>
</tr>
<tr>
<td>5</td>
<td>ITP</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>OAA, ITP</td>
<td>1437</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each flask contained potassium phosphate buffer (pH 6.5), 50 μmoles; NaH<sup>14</sup>CO₃, 2.5 μmoles (1 x 10<sup>7</sup> cpm); cysteine and MnCl₂, 5 μmoles each; cell-free extract (rocker dialyzed), 0.2 ml; in a total volume of 2.0 ml. 3 μmoles each of PEP, IDP, OAA and ITP were added as indicated and the reaction time was 1 hr.

The fixed C<sup>14</sup> in these reaction mixtures existed in the form of radioactive OAA which was identified by chromatography and radioautography as the 2,4-dinitrophenylhydrazone derivative. The location of the labeled carbon in the β-carboxyl position was determined by trapping the C<sup>14</sup>O₂ liberated during
the degradation of the 2,4-dinitrophenylhydrazone. In this procedure (Bandurski and Greiner, 1953), the β-carboxyl group is decarboxylated.

Table 2. CO₂ fixation in partially purified extracts of Rhodospirillum rubrum

\[
\begin{array}{|c|c|c|}
\hline
\text{Flask No.} & \text{Additions} & \text{Activity fixed (counts/min/0.05 ml)} \\
\hline
1 & OAA & 7 \\
2 & ITP & 4 \\
3 & OAA, ITP & 632 \\
4 & PEP & 5 \\
5 & IDP & 0 \\
6 & PEP, IDP & 219 \\
7 & Pyruvate, ITP & 0 \\
8 & Pyruvate, IDP & 0 \\
9 & Pyruvate, ATP, IDP & 2 \\
10 & Malate, ITP & 2 \\
\hline
\end{array}
\]

\text{Each flask contained potassium phosphate buffer (pH 6.5), 50 μmoles; NaHCl⁴O₃, 2.5 μmoles (1 \times 10⁷ cpm); cysteine, 10 μmoles; MnCl₂, 5 μmoles; and aged ammonium sulfate-treated extract, 0.1 ml (125 μg protein). The substrates and nucleotides, 3 μmoles each, were added as indicated, the reaction time was 45 min, and the total volume was 2.0 ml.}

Some of the characteristics of the nucleotide-dependent reactions were studied in more detail. Table 3 shows the effect of increasing substrate concentrations on both the fixation reaction (substrate: PEP) and the exchange reaction (substrate: OAA). Under the stated conditions, the optimum
activity occurred with 3 \( \mu \)moles of substrate. Conforming to a typical substrate dependence curve, no significant change in activity occurred with higher concentrations of substrate.

Table 3. Effect of substrate concentration on CO\(_2\) fixation by extracts of *Rhodospirillum rubrum*\(^a\)

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>( \mu )moles of substrate (PEP or OAA)</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>188</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>231</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>301</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>292</td>
</tr>
<tr>
<td>6</td>
<td>9.0</td>
<td>670</td>
</tr>
<tr>
<td>7</td>
<td>12.0</td>
<td>250</td>
</tr>
</tbody>
</table>

\(^a\)The flasks containing PEP also contained IDP, 3 \( \mu \)moles; phosphate buffer (pH 6.5) 50 \( \mu \)moles; NaHCl\(_{14}O_3\), 2.5 \( \mu \)moles (1 \( \times \) 10\(^7\) cpm); cysteine, 10 \( \mu \)moles; MnCl\(_2\), 5 \( \mu \)moles; and aged ammonium sulfate-treated extract, 0.1 ml (125 \( \mu \)g protein). The flasks containing OAA also contained ITP, 3 \( \mu \)moles; phosphate buffer (pH 6.5) 50 \( \mu \)moles; NaHCl\(_{14}O_3\), 1.25 \( \mu \)moles (5 \( \times \) 10\(^6\) cpm); cysteine, 10 \( \mu \)moles; MnCl\(_2\), 2.5 \( \mu \)moles; and 0.1 ml (66 \( \mu \)g protein) extract (40-70% ammonium sulfate fraction). In all flasks the total volume was 2.0 ml and the reaction time was 45 min.

Table 4 shows that the highest activity was not reached until about 60 \( \mu \)moles of C\(_{14}O_2\) were added, though more points would be necessary to substantiate this conclusion in the case of PEP.
Table 4. Effect of CO₂ concentration on CO₂ fixation by extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>(\mu)moles NaH(^{14})O₃</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEP, IDP</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>422</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>118</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>842</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>136</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>1082</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>1054</td>
</tr>
</tbody>
</table>

<sup>a</sup>The flasks with PEP (3 \(\mu\)moles) contained 1.5 \(\mu\)moles IDP and those with OAA (3 \(\mu\)moles) contained 1.5 \(\mu\)moles ITP. All flasks contained potassium phosphate buffer (pH 6.5) 50 \(\mu\)moles; cysteine, 10 \(\mu\)moles; MnCl\(_2\), 2.5 \(\mu\)moles; and 0.2 ml extract (aged, ammonium sulfate-treated). All flasks contained 2.0 ml total volume and the reaction time was 1 hr.

Relatively smaller amounts of the nucleotide cofactors were necessary for optimum activity (Table 5), the value being 1.5 \(\mu\)moles or less. Increasing concentrations of nucleotides beyond the optimum concentration had an inhibitory effect.

The specificity of the nucleotide cofactors was also studied; typical results are presented in Table 6. The guanosine counterparts with both PEP and OAA were equally as effective as the inosine nucleotides, uridine nucleotides were less effective, and the others had little or no effect.
Table 5. Effect of nucleotide concentration on CO₂ fixation by extracts of *Rhodospirillum rubrum*¹

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>μmoles of nucleotide</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IDP</td>
</tr>
<tr>
<td>1</td>
<td>0.00</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>564</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>522</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>598</td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>596</td>
</tr>
<tr>
<td>6</td>
<td>3.00</td>
<td>565</td>
</tr>
<tr>
<td>7</td>
<td>4.50</td>
<td>326</td>
</tr>
</tbody>
</table>

¹The flasks contained potassium phosphate buffer (pH 6.5), 50 μmoles; NaHCO₃, 1.25 μmoles (5 x 10⁶ cpm); cysteine, 10 μmoles; MnCl₂, 2.5 μmoles; 0.1 ml (66 μg protein) extract (40-70% ammonium sulfate fraction); PEP, 3 μmoles in the case of IDP; OAA, 3 μmoles in the case of ITP; in a total volume of 2.0 ml. The reaction time was 45 min.

Table 6. Effect of various nucleotides on CO₂ fixation by extracts of *Rhodospirillum rubrum*¹

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
<th>Nucleotide</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEP</td>
<td>OAA</td>
<td></td>
</tr>
<tr>
<td>IDP</td>
<td>142</td>
<td>ITP</td>
<td>514</td>
</tr>
<tr>
<td>GDP</td>
<td>146</td>
<td>GTP</td>
<td>508</td>
</tr>
<tr>
<td>UDP</td>
<td>33</td>
<td>UTP</td>
<td>102</td>
</tr>
<tr>
<td>ADP</td>
<td>17</td>
<td>ATP</td>
<td>66</td>
</tr>
<tr>
<td>CDP</td>
<td>4</td>
<td>CTP</td>
<td>22</td>
</tr>
<tr>
<td>ITP</td>
<td>12</td>
<td>IDP</td>
<td>32</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>None</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 6. (Continued)

The flasks contained PEP, 3 μmoles or OAA, 3 μmoles; various nucleotides, 1 μmole; potassium phosphate buffer (pH 6.5), 50 μmoles; NaHClO₄, 2.5 μmoles (1 x 10⁷ cpm); cysteine, 10 μ moles; MnCl₂, 2.5 μmoles; aged, ammonium sulfate-treated extract, 0.1 ml (125 μg protein); in a total volume of 2.0 ml. The reaction time was 1 hr with OAA and 45 min with PEP.

Abbreviations: IDP, inosine diphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate; ADP, adenosine diphosphate; CDP, cytidine diphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate.

The reactions also required a divalent metal ion cofactor, as indicated by the results of typical experiments shown in Table 7. Mn⁺⁺ fulfilled this requirement while Co⁺⁺ and Mg⁺⁺ were less effective. In experiments not repeated with PEP, Zn⁺⁺ was as effective as Mg⁺⁺ but Sn⁺⁺, Cu⁺⁺, Fe⁺⁺⁺, Ca⁺⁺ and Sr⁺⁺ were inactive. K⁺ and Na⁺ were present in all reaction mixtures and apparently could not substitute for Mn⁺⁺ since no CO₂ fixation occurred in Flask 6. The effect of increasing Mn⁺⁺ concentration is shown in Table 8, with the optimum value at about 2.5 μmoles in both the fixation and exchange reactions.

Sulfhydryl compounds stimulated the reactions as indicated in Table 9; the degree of stimulation apparently depended upon
the age of the extract. For example, the results in Table 9 were obtained with an extract which had been frozen for 8 days after purification, while only about 50% stimulation by cysteine was observed with an extract which had been stored for 2 days after purification.

Table 7. Effect of various divalent metal ions on CO₂ fixation by extracts of *Rhodospirillum rubrum* a

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Divalent ion</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEP, IDP</td>
</tr>
<tr>
<td>1</td>
<td>MnCl₂</td>
<td>146</td>
</tr>
<tr>
<td>2</td>
<td>CoCl₂</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>MgCl₂</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>FeCl₂</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>NiCl₂</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>2</td>
</tr>
</tbody>
</table>

*a* The flasks contained either 3 μmoles of PEP or OAA; the various ions, 5 μmoles; potassium phosphate buffer (pH 6.5) 50 μmoles; NaHCl₁⁴O₃, 1.25 μmoles (5 x 10⁶ cpm); cysteine, 10 μmoles; aged ammonium sulfate-treated extract, 0.1 ml (125 μg protein); IDP or ITP, 3 μmoles; in a total volume of 2.0 ml. The reaction time with OAA was 45 min and with PEP, 1 hr.

The oxidized forms (cystine and GSSG) did not possess the same stimulatory effect and possibly even inhibited to some extent. The sulfhydryl inhibitor p-chloromercuribenzoate (PCMB) completely inhibited the reactions, and the
inhibition was partially reversed by cysteine. The amount of cysteine necessary for optimal activity was 10 \( \mu \)moles in both the fixation and exchange reactions (Table 10).

### Table 8. Effect of MnCl\(_2\) concentration on CO\(_2\) fixation by extracts of Rhodospirillum rubrum\(^a\)

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>( \mu )moles MnCl(_2)</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEP, IDP</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>1.25</td>
<td>128</td>
</tr>
<tr>
<td>4</td>
<td>2.50</td>
<td>234</td>
</tr>
<tr>
<td>5</td>
<td>5.00</td>
<td>257</td>
</tr>
<tr>
<td>6</td>
<td>10.00</td>
<td>221</td>
</tr>
</tbody>
</table>

\(^a\)The flasks with PEP (3 \( \mu \)moles) contained potassium phosphate buffer (pH 6.5), 50 \( \mu \)moles; NaHCl\(^{14}\)O\(_3\), 2.5 \( \mu \)moles (1 \( \times \) 10\(^7\) cpm); cysteine, 10 \( \mu \)moles; MnCl\(_2\), 5 \( \mu \)moles; 0.1 ml (125 \( \mu \)g protein) aged ammonium sulfate-treated extract; and IDP, 3 \( \mu \)moles. The flasks with OAA (3 \( \mu \)moles) contained the same, except for 3 \( \mu \)moles ITP in place of IDP and 5 \( \mu \)moles of cysteine. All flasks contained a total volume of 2.0 ml and the reaction time was 45 min.

The results from a study of the effect of pH on the reactions are presented in Table 11. Although some variation occurred between experiments, the optimum for the exchange reaction appeared to be about pH 7.0, while that for the fixation reaction was somewhat lower at about pH 6.5.
Table 9. Effect of sulfhydryl compounds on CO₂ fixation by extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Additions</th>
<th>Activity fixed (counts/min/0.05 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>PEP, IDP OAA, ITP</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>30 54</td>
</tr>
<tr>
<td>2</td>
<td>Cysteine (10)</td>
<td>283 1380</td>
</tr>
<tr>
<td>3</td>
<td>GSH (10)</td>
<td>154 1420</td>
</tr>
<tr>
<td>4</td>
<td>Cystine (6.5)</td>
<td>38 30</td>
</tr>
<tr>
<td>5</td>
<td>GSSG (10)</td>
<td>4 0</td>
</tr>
<tr>
<td>6</td>
<td>PCMB (0.1)</td>
<td>1 1</td>
</tr>
<tr>
<td>7</td>
<td>PCMB (0.1), cysteine (10)</td>
<td>72 574</td>
</tr>
</tbody>
</table>

<sup>a</sup>The flasks contained potassium phosphate buffer (pH 6.5), 60 μmoles; NaHCl<sup>14</sup>O<sub>3</sub>, 1.25 μmoles (5 x 10<sup>6</sup> cpm); MnCl<sub>2</sub>, 2.5 μmoles; aged ammonium sulfate-treated extract (frozen 8 days before use following purification), 0.2 ml; PEP or OAA; 3 μmoles; IDP or ITP, 0.75 μmoles; in a total volume of 2.0 ml. The reaction time was 1 hr.

Table 10. Effect of cysteine concentration on CO₂ fixation by extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>μmoles cysteine</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>PEP, IDP OAA, ITP</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>19 288</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>161 822</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>190 1002</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>275 1122</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>246</td>
</tr>
<tr>
<td>6</td>
<td>20.0</td>
<td>276 1032</td>
</tr>
<tr>
<td>7</td>
<td>25.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The flask contents and reaction conditions were the same as those presented in Table 2.
Table 11. Effect of pH on CO₂ fixation by extracts of *Rhodospirillum rubrum*

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>pH (phosphate buffer)</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
<th>PEP, IDP</th>
<th>OAA, ITP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7</td>
<td>920</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.0</td>
<td>186</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>6.2</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.35</td>
<td></td>
<td></td>
<td>1036</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>285</td>
<td></td>
<td>1102</td>
</tr>
<tr>
<td>6</td>
<td>6.6</td>
<td>281</td>
<td></td>
<td>1160</td>
</tr>
<tr>
<td>7</td>
<td>6.8</td>
<td>265</td>
<td></td>
<td>1198</td>
</tr>
<tr>
<td>8</td>
<td>7.0</td>
<td>241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7.2</td>
<td>224</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>7.35</td>
<td>884</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>7.4</td>
<td>202</td>
<td></td>
<td>628</td>
</tr>
<tr>
<td>12</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>8.0</td>
<td>76</td>
<td></td>
<td>546</td>
</tr>
</tbody>
</table>

*The flasks with PEP contained 60 μmoles of potassium phosphate buffer at the various pH values and the flasks with OAA contained 100 μmoles of the same buffer. The other components and the reaction conditions were the same as in Table 2.*

The activity of the enzyme was found to be proportional to both the amount of extract and the reaction time - as indicated in Tables 12 and 13, respectively - in both the fixation and exchange reactions.

Preliminary results using an EC-extract from *Rhodospirillum rubrum* grown aerobically in the dark were similar to those in Table 1 under similar conditions.
Table 12. Effect of extract volume on CO₂ fixation by extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>ml Extract</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEP</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>151</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>339</td>
</tr>
</tbody>
</table>

<sup>a</sup>The flasks contained the stated volumes of aged ammonium sulfate-treated extract. The other components and conditions were the same as in Table 2.

Table 13. Effect of reaction time on CO₂ fixation by extracts of *Rhodospirillum rubrum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Minutes</th>
<th>Activity fixed (counts/min/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PEP, IDP</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>142</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>151</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>272</td>
</tr>
</tbody>
</table>

<sup>a</sup>The flask contents and reaction conditions were the same as those in Table 2.

Preliminary results using a crude extract of *Nocardia corallina* indicated a 4-fold increase in CO₂ fixation by sodium pyrophosphate when OAA was the substrate. One flask contained; OAA, 2.5 μmoles; Tris buffer (pH 7.75), 50 μmoles; MgCl₂,
2.5 μmoles; NaHCO\textsubscript{3} \(^{14}\)O\textsubscript{3} (1 x 10\(^7\) cpm) 2.5 μmoles; and 0.3 ml of crude extract. Following incubation the reaction mixture contained 268 cpm/0.1 ml, and the 2,4-dinitrophenylhydrazone prepared from 0.5 ml of the reaction mixture containing added authentic OAA contained 1050 cpm (corrected for background only). Another flask contained, in addition, 2.5 μmoles of sodium pyrophosphate. This reaction mixture contained 976 cpm per 0.1 ml and 4160 cpm in the 2,4-dinitrophenylhydrazone. That the radioactivity was probably largely present in OAA was further substantiated by chromatography and the detection of radioactivity with the Fowler-Rhinehart Scanner over the yellow spots of the 2,4-dinitrophenylhydrazone of OAA.

Carboxylation of Carboxylic Acids

Since acetate, propionate, and butyrate had been found to be carboxylated in extracts of \textit{Nocardia corallina} (Baugh \textit{et al.}, 1961, 1962) it was considered of interest to extend this study. Most of the experiments utilized extracts from cells grown as described in Appendix A. A number of acids was substituted for propionate in the carboxylation reaction; the results with various purifications of the extract are presented in Table 14. Although some variation occurred
Table 14. Carboxylation of acids in various extracts of *Nocardia corallina*<sup>a</sup>

<table>
<thead>
<tr>
<th>Acid</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>Exp 4 Tris</th>
<th>Exp 4 PO₄</th>
<th>Exp 5 Tris</th>
<th>Exp 5 PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>41</td>
<td>23</td>
<td>0</td>
<td>311</td>
<td>462</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Propionic</td>
<td>584</td>
<td>142</td>
<td>46</td>
<td>3580</td>
<td>4030</td>
<td>277</td>
<td>236</td>
</tr>
<tr>
<td>Acetic</td>
<td>229</td>
<td>106</td>
<td>54</td>
<td>3445</td>
<td>3500</td>
<td>153</td>
<td>138</td>
</tr>
<tr>
<td>3-Methylvaleric</td>
<td>403</td>
<td>145</td>
<td>77</td>
<td>3030</td>
<td>3235</td>
<td>210</td>
<td>173</td>
</tr>
<tr>
<td>Butyric</td>
<td>253</td>
<td>62</td>
<td>12</td>
<td>1135&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3645</td>
<td>87</td>
<td>55</td>
</tr>
<tr>
<td>Valeric</td>
<td>197</td>
<td>89</td>
<td>37</td>
<td>2050</td>
<td>1597</td>
<td>177</td>
<td>124</td>
</tr>
<tr>
<td>2-Pentenoic</td>
<td>184</td>
<td>86</td>
<td>24</td>
<td>1535</td>
<td>1865</td>
<td>91</td>
<td>93</td>
</tr>
<tr>
<td>2-Hexenoic</td>
<td>136</td>
<td>32</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiglic</td>
<td>112</td>
<td>56</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crotonic</td>
<td>78</td>
<td>24</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Methylvaleric</td>
<td>68</td>
<td>23</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinylacetic</td>
<td>66</td>
<td>31</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The flasks in experiment 1 contained potassium phosphate buffer (pH 7.5) 40 μmoles; KHCl<sub>4</sub>O₃, 2.5 μmoles (9.3 x 10⁴ cpm); GSH, 2.5 μmoles; MgCl₂, 2.5 μmoles; 0.3 ml crude extract; ATP, 1.5 μmoles; CoA, 0.15 μmoles; and 2.5 μmoles of the various acids. Total volume was 1.0 ml and the reaction time was 45 min.

In experiment 2, the contents and conditions were the same, except that 0.4 ml of dialyzed extract was substituted for the crude extract.

In experiment 3, the contents and conditions were again the same, except that 0.5 ml of extract (20-40% ammonium sulfate fraction) was substituted for the crude extract.

The flasks in experiment 4 contained potassium phosphate buffer (pH 7.5) or Tris buffer (pH 7.5), 40 μmoles; NaHCl<sub>4</sub>O₃ (1.9 x 10⁶ cpm); GSH, 5 μmoles; MgCl₂, 2.5 μmoles; ATP, 1.5 μmoles; CoA, 0.15 μmoles; substrate, 2.5 μmoles; EC-extract, 0.5 ml; and a total of 1.05 ml. The reaction time was 30 min.
Table 14. (Continued)

In experiment 5, the contents and conditions were the same as experiment 4 except 0.5 ml of fraction 8 (Appendix B) of the DEAE-fractionated extract was used and the dilute KHCl\textsubscript{14}O\textsubscript{3} was used, 2.5\mu moles (9.3 \times 10^4 \text{ cpm}).

\textsuperscript{b}3.0 \mu moles ATP.

between extracts, the highest activity was obtained with propionate or 3-methylvalerate and the activity decreased, in general, as the carbon chain increased in length except in the case of acetate. Lower activities were observed with the unsaturated acids. In experiments 4 and 5 (Table 14) little or no difference in total activity was observed between phosphate and Tris buffer. In crude extracts negative results were obtained with \textit{\alpha}-hydroxyisobutyrate, \textit{\beta}-hydroxybutyrate, formate, nonanoate, caprate, isovalerate, 5-methylcaproate, \textit{\alpha}-ketoisovalerate, 4-phenylbutyrate, methacyrlate, 2-methylbutyrate, isobutyrate, dimethylacrylate, caproate, acrylate, \textit{\beta}-hydroxy-\textit{\beta}-methylglutarate, caprylate, and heptanoate.

The compounds which contained the fixed C\textsubscript{14} were identified by chromatography and radioautography or scanning. The radioactive acids probably were in the form of CoA esters since the activity remained at the origin in EaFW (free acids
move in this solvent) and moved in solvents known to separate CoA esters (EA and PIW). After alkaline hydrolysis, the radioactive compounds behaved as free acids and were identified as shown in Table 15. The absence of propylmalonate in the valeric acid reaction mixture was further substantiated by the fact that no radioactivity was observed by radioautography in the spot produced by the hydroxamic acid of authentic propylmalonic acid. Other spots with low Rf values also appeared in various solvents and were assumed to be amino acids. Although not identified conclusively by radioautography, these compounds from acetate or propionate and a crude extract were possibly glycine, and aspartic and glutamic acids. Radioactive spots on chromatograms were detected by the scanner in the appropriate area for these compounds. The primary carboxylation products apparently were identical irrespective of the degree of purification of the extract.

The relative amounts of the radioactive end products were determined by scanning chromatograms developed in OFW with the Fowler-Rhinehart scanner (See Appendix C for information concerning operation and calibration of the instrument). Although phosphate apparently had little effect upon the total CO2 fixation, Table 16 indicates that in the EC-extract,
fixation into malonate was stimulated in the presence of phosphate buffer. This effect is also demonstrated in Figures 1 and 2, which are tracings of the scanner charts. The effect was not observed in the extracts treated with DEAE-cellulose. (Table 17).

Table 15. Major radioactive products formed by extracts of *Nocardia corallina* from various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>Methylmalonate, malonate</td>
</tr>
<tr>
<td>Propionate</td>
<td>Methylmalonate</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Ethylmalonate, methylmalonate</td>
</tr>
<tr>
<td>Valerate</td>
<td>Methylmalonate, malonate</td>
</tr>
<tr>
<td>3-Methylvalerate</td>
<td>Methylmalonate, malonate</td>
</tr>
<tr>
<td>2-Pentenoate</td>
<td>Methylmalonate, malonate</td>
</tr>
<tr>
<td>γ-Methylvalerate</td>
<td>Methylmalonate, malonate</td>
</tr>
<tr>
<td>2-Hexenoate</td>
<td>Ethylmalonate, methylmalonate, malonate</td>
</tr>
<tr>
<td>Crotonate</td>
<td>Malonate, methylmalonate</td>
</tr>
<tr>
<td>Vinylacetate</td>
<td>Malonate, methylmalonate</td>
</tr>
<tr>
<td>Tiglate</td>
<td>Methylmalonate, malonate</td>
</tr>
</tbody>
</table>

The end products were identified from the hydrolyzed reaction mixtures described in Table 14 by radioautography and scanning and decrease in radioactivity from left to right.
Table 16. End products formed by an EC-extract of Nocardia corallina from various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphate</th>
<th>Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Originb</td>
<td>M MM EM</td>
</tr>
<tr>
<td>Acetate</td>
<td>19c</td>
<td>41 43 0</td>
</tr>
<tr>
<td>Propionate</td>
<td>14</td>
<td>9.5 75 0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>7</td>
<td>10 29 68</td>
</tr>
<tr>
<td>Valerate</td>
<td>13</td>
<td>18 70 0</td>
</tr>
<tr>
<td>3-Methylvalerate</td>
<td>13</td>
<td>15 73 0</td>
</tr>
<tr>
<td>2-Pentenoate</td>
<td>37</td>
<td>11 51 0</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>51 27 0</td>
</tr>
</tbody>
</table>

^Reaction mixtures were those from Expt. 4 in Table 14. Chromatograms were developed in OFW.

^Abbreviations and terms: Origin, radioactivity at or near the origin of the chromatograms; M, MM, and EM; radioactivity over spots of added malonic acid, methylmalonic acid, and ethylmalonic acid, respectively.

^Per cent of total activity, calculated from area measurements (sq. cm.).

Table 17. End products formed by a DEAE-extract of Nocardia corallina from various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphate</th>
<th>Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Originb</td>
<td>M MM EM</td>
</tr>
<tr>
<td>Acetate</td>
<td>29c</td>
<td>8 61 0</td>
</tr>
<tr>
<td>Propionate</td>
<td>10</td>
<td>4d 81 0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>28</td>
<td>7 21 42</td>
</tr>
<tr>
<td>Valerate</td>
<td>10</td>
<td>6d 82 0</td>
</tr>
<tr>
<td>3-Methylvalerate</td>
<td>22</td>
<td>6d 70 0</td>
</tr>
<tr>
<td>2-Pentenoate</td>
<td>13</td>
<td>8 74 0</td>
</tr>
</tbody>
</table>

^Reaction mixtures were those from Expt. 5 in Table 14. Chromatograms were developed in OFW.
Table 17. (Continued)

\( ^b \) Abbreviations, same as Table 16.

\( ^c \) Per cent of total activity, calculated from area measurements (sq. cm.)

\( ^d \) Peaks slightly lower than malonic acid.

More details of the carboxylation of acetate and the formation of methylmalonate from acetate were sought by observing the effect of various compounds. Preliminary results in which a crude extract was employed are presented in Table 18. The total stable non-volatile activity after hydrolysis was decreased by the absence of phosphate, and by the presence of OAA, iodoacetamide and hydroxylamine; activity was increased by the presence of malonate and sodium chloride. The total activity just after the reaction was stopped was decreased somewhat by the absence of phosphate or the presence of iodoacetamide, hydroxylamine, and caproate, and was increased by citrate, malonate, and sodium chloride. The activity at the origin, presumably amino acids, apparently was greater in the absence of phosphate and in the presence of hydroxylamine. A comparatively smaller amount of malonate activity was obtained in the absence of phosphate and in the presence of iodoacetamide; greater activity was obtained in
Figure 1. Tracing of the scanner chart obtained from a chromatogram of the reaction mixture from Experiment 4 (Table 14) containing acetate and Tris developed in OFW.

The chart units refer to the printed numbers on the scanner chart (No. 5401, Minneapolis-Honeywell Reg. Co.). The numbered circles are tracings of the acid spots on the chromatogram superimposed on the chart tracing. The arrow indicates the direction of solvent flow. Peak A (26.4 sq cm) corresponds with spot 1 which is the origin of the chromatogram. Peak B (15.3 sq cm) corresponds with Spot 2 which is the spot due to added authentic malonic acid. The same relationship holds for Peak C (62.4 sq cm) and Spot 3 which is due to co-chromatographed methylmalonic acid. The total area is 103.9 sq cm.

The operating conditions were: range 3200, 1650 v, 16 mm from slip to anode, 4 cm Q gas, resolving slip, 3/4 in.
Figure 2. Tracing of the scanner chart obtained from a chromatogram of the reaction mixture from Experiment 4 (Table 14) containing acetate and phosphate obtained under the same conditions as Figure 1.

The notations are also those described for Figure 1. The areas are: Peak A, 18.4 sq cm; Peak B, 41.6 sq cm; Peak C, 42.2 sq cm; and total area, 100.4 sq cm.
Table 18. Effect of various compounds on acetate carboxylation by a crude extract of *Nocardia corallina*\(^a\)

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Stable act. (^b)</th>
<th>Additions</th>
<th>Activity fixed (counts/min/0.025 ml)</th>
<th>Origin (^c)</th>
<th>M</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96.5</td>
<td>Ac, Phos</td>
<td>595</td>
<td>58.0</td>
<td>34.8</td>
<td>8.8</td>
</tr>
<tr>
<td>2</td>
<td>55.3</td>
<td>Ac, Tris</td>
<td>481</td>
<td>84.8</td>
<td>6.9</td>
<td>26.5</td>
</tr>
<tr>
<td>3</td>
<td>17.3</td>
<td>Phos</td>
<td>137</td>
<td>0</td>
<td>55.5</td>
<td>59.0</td>
</tr>
<tr>
<td>4</td>
<td>43.6</td>
<td>OAA, Ac, Phos</td>
<td>589</td>
<td>54.8</td>
<td>38.8</td>
<td>12.4</td>
</tr>
<tr>
<td>5</td>
<td>61.5</td>
<td>Iodoacetamide, Ac, Phos</td>
<td>519</td>
<td>66.8</td>
<td>21.6</td>
<td>12.0</td>
</tr>
<tr>
<td>6</td>
<td>65.7</td>
<td>NH(_2)OH, Ac, Phos</td>
<td>509</td>
<td>48.7</td>
<td>56.8</td>
<td>12.0</td>
</tr>
<tr>
<td>7</td>
<td>111.9</td>
<td>Caproate, Ac, Phos</td>
<td>463</td>
<td>59.0</td>
<td>29.6</td>
<td>10.4</td>
</tr>
<tr>
<td>8</td>
<td>106.5</td>
<td>Citrate, Ac, Phos</td>
<td>730</td>
<td>60.0</td>
<td>30.9</td>
<td>8.6</td>
</tr>
<tr>
<td>9</td>
<td>177.0</td>
<td>Malonate, Ac, Phos</td>
<td>794</td>
<td>51.6</td>
<td>38.5</td>
<td>10.2</td>
</tr>
<tr>
<td>10</td>
<td>141.2</td>
<td>NaCl, Ac, Phos</td>
<td>889</td>
<td>59.6</td>
<td>36.6</td>
<td>9.1</td>
</tr>
</tbody>
</table>

\(^a\)Each flask contained ATP, 1.5 \(\mu\)moles; CoA, 0.15 \(\mu\)moles; MgCl\(_2\), 2.5 \(\mu\)moles; GSH, 5 \(\mu\)moles; NaHCl\(^{14}\)O\(_3\), 2.5 \(\mu\)moles (1 \(\times\) 10\(^7\) cpm). As indicated the following were added: potassium phosphate buffer (pH 7.75), 40 \(\mu\)moles; Tris buffer (pH 7.75), 40 \(\mu\)moles; acetate, iodoacetamide, caproate, citrate, OAA, NH\(_2\)OH, NaCl, 2.5 \(\mu\)moles each and malonate, 5.0 \(\mu\)moles. Reaction time was 30 min. and the total volume, 1.0 ml.

\(^b\)Total radioactivity due to stable, nonvolatile compounds after alkaline hydrolysis, determined by the scanner.

\(^c\)Abbreviations: same as Table 16 and acetate, Ac; phosphate buffer, Phos; Tris buffer, Tris.
the presence of hydroxylamine. Radioactivity in methylmalonate was greater in the absence of phosphate. Both the total and the total stable activity were quite low (as expected) in the absence of acetate (Flask 3), and the activity apparently was limited to malonate and methylmalonate. The relative amount of the various stable products was thus unchanged by OAA, caproate, citrate, malonate, and sodium chloride.

The effect of pH on the inhibition of propionate carboxylation by avidin was studied using the EC-extracts of *Nocardia corallina*. Figure 3 indicates that greater inhibition by 0.0125 unit of avidin occurred on either side of the pH optimum. Similar results occurred in Tris buffer, except that the least inhibition appeared at pH 8. The effect of OAA on propionate carboxylation was also tested in one experiment with a crude extract of *Nocardia corallina*. The total activity was somewhat higher and a small amount of malonate in addition to methylmalonate and the origin activity was detected by scanning, while none was present when OAA was not added to the reaction. A slightly smaller total activity occurred when pyruvate was added to propionate, and a spot just above the origin in OFW appeared which was not seen in the absence of pyruvate. This spot was in the vicinity of
Figure 3. Effect of pH on the inhibition by avidin of propionate carboxylation by extracts of Nocardia corallina. The flasks contained potassium phosphate buffer (various pH values), 50 μmoles; KHCl4O3, 0.25 μmoles (9.3 x 10^4 cpm); GSH, 2.5 μmoles; MgCl2, 2.5 μmoles; propionate, 2.5 μmoles; ATP, 1.5 μmoles; CoA, 0.15 μmoles; avidin, as indicated, 0.05 mg (0.0125 unit ); 0.25 ml of EC-extract; and a total volume of 1.0 ml. Reaction time was 30 min. When avidin was present it was in contact with the extract for 25 min before the reaction was started.

-—— O A: counts per min in the absence of avidin
-—— — B: counts per min in the presence of 0.0125 unit of avidin
-—— — — C: per cent inhibition calculated as follows:

\[
100 - \frac{B \times 100}{A} = \% \text{ inhibition}
\]
malic acid in this solvent (OFW).

Nutrition and Oxygen Assimilation Studies

In a crude experiment designed to determine which acids are used for the growth of Nocardia corallina the results in Table 19 were obtained. The saturated straight-chain and branched-chain acids containing fewer than 7 carbons were utilized for growth. None of the unsaturated acids were used initially, but adaptive systems apparently were formed for the utilization of methacrylic, glutaconic and tiglic acids. Growth also was apparent in the presence of α-ketoisovaleric and β-hydroxybutyric acids.

The results obtained in manometric studies with whole cells of Nocardia corallina are presented in Table 20. Total oxygen uptake decreased with decreasing chain length of the saturated fatty acids. With the exception of acetate, valerate, and caprate, the reverse was true in the rate of oxidation indicated by the QO2 values. In the straight-chain saturated fatty acids, only about thirty to forty per cent of the oxygen necessary for complete combustion was assimilated. Results with the following acids were not significantly different from the endogenous values: methylmalonic, dimethyl-
Table 19. Utilization of various compounds for growth of *Nocardia corallina*\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation time (days)</th>
<th>Substrate</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>L-Valine</td>
<td>±(^b)</td>
<td>Dimethylmalonate</td>
<td>-</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>±</td>
<td>Heptanoate</td>
<td>-</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>±</td>
<td>Tiglate</td>
<td>±</td>
</tr>
<tr>
<td>(\beta,\beta)-Dimethylacrylate</td>
<td>-</td>
<td>(\alpha)-Ketoisovalerate</td>
<td>++</td>
</tr>
<tr>
<td>(\beta)-OH-(\beta)-CH(_3)-glutarate</td>
<td>-</td>
<td>3-CH(_3)-valerate</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>++</td>
<td>Caprylate</td>
<td>-</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>Caproate</td>
<td>+++</td>
</tr>
<tr>
<td>Butyrate</td>
<td>+</td>
<td>Caprate</td>
<td>+</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>++</td>
<td>Tartronate</td>
<td>-</td>
</tr>
<tr>
<td>Valerate</td>
<td>±</td>
<td>(\beta)-OH-butyrinate</td>
<td>+++</td>
</tr>
<tr>
<td>Crotonate</td>
<td>-</td>
<td>(\alpha)-OH-isobutyrate</td>
<td>-</td>
</tr>
<tr>
<td>Mesaconate</td>
<td>-</td>
<td>Acrylate</td>
<td>-</td>
</tr>
<tr>
<td>Methacrylate</td>
<td>-</td>
<td>Glucose</td>
<td>++</td>
</tr>
<tr>
<td>(\gamma)-Methylvalerate</td>
<td>++</td>
<td>Isolevalerate</td>
<td>+++</td>
</tr>
<tr>
<td>Glutaconate</td>
<td>-</td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Growth determined visually by turbidity and characteristic pink pellicle in tubes containing 5 ml of the following medium: \((\text{NH}_4)_2\text{SO}_4, 4.72 \text{ g}; \text{NaCl, 5.00 g; KH}_2\text{PO}_4, 0.6 \text{ g; K}_2\text{HPO}_4\cdot3\text{H}_2\text{O, 16.18 g; traces of MgCl}_2, FeCl}_2, \text{and CaCl}_2; substrates, 10 mg; and 1000 ml of distilled water.\)

\(^b\)++, heavy growth; -, no growth; ±, possibly a small amount of growth.
Table 20. O₂ uptake by *Nocardia corallina*<sup>a</sup>

<table>
<thead>
<tr>
<th>Acid</th>
<th>μl O₂&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Q₀₂&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capric</td>
<td>720</td>
<td>30.2</td>
</tr>
<tr>
<td>Nonanoic</td>
<td>530</td>
<td>23.0</td>
</tr>
<tr>
<td>Caprylic</td>
<td>434</td>
<td>25.8</td>
</tr>
<tr>
<td>Heptanoic</td>
<td>334</td>
<td>27.2</td>
</tr>
<tr>
<td>γ-Methylvaleric</td>
<td>325</td>
<td>23.6</td>
</tr>
<tr>
<td>Caproic</td>
<td>316</td>
<td>30.4</td>
</tr>
<tr>
<td>2-Hexenoic</td>
<td>296</td>
<td>14.5</td>
</tr>
<tr>
<td>Valeric</td>
<td>247</td>
<td>29.0</td>
</tr>
<tr>
<td>Butyric</td>
<td>238</td>
<td>33.2</td>
</tr>
<tr>
<td>Isobutyric</td>
<td>235</td>
<td>17.5</td>
</tr>
<tr>
<td>Isovaleric</td>
<td>222</td>
<td>14.5</td>
</tr>
<tr>
<td>α-Ketoglutaric</td>
<td>184</td>
<td>14.5</td>
</tr>
<tr>
<td>Crotonic</td>
<td>176</td>
<td>24.2</td>
</tr>
<tr>
<td>2-Pentenoic</td>
<td>161</td>
<td>18.5</td>
</tr>
<tr>
<td>Propionic</td>
<td>159</td>
<td>43.5</td>
</tr>
<tr>
<td>Tiglic</td>
<td>156</td>
<td>10.3</td>
</tr>
<tr>
<td>Dimethylacrylic</td>
<td>154</td>
<td>12.9</td>
</tr>
<tr>
<td>Vinylacetic</td>
<td>148</td>
<td>15.1</td>
</tr>
<tr>
<td>Succinic</td>
<td>131</td>
<td>44.7</td>
</tr>
<tr>
<td>Lactic</td>
<td>117</td>
<td>9.7</td>
</tr>
<tr>
<td>β-Hydroxybutyric</td>
<td>104</td>
<td>11.3</td>
</tr>
<tr>
<td>Acetic</td>
<td>104</td>
<td>35.6</td>
</tr>
<tr>
<td>Methacrylic</td>
<td>95</td>
<td>15.8</td>
</tr>
<tr>
<td>3-Methylvaleric</td>
<td>64</td>
<td>27.6</td>
</tr>
<tr>
<td>Acrylic</td>
<td>59</td>
<td>12.1</td>
</tr>
<tr>
<td>Formic</td>
<td>52</td>
<td>9.2</td>
</tr>
<tr>
<td>Pyruvic</td>
<td>48</td>
<td>10.0</td>
</tr>
<tr>
<td>Tartronic</td>
<td>28</td>
<td>8.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each flask contained potassium phosphate buffer (pH 7.5), 100 μmoles; 0.3 ml whole cell suspension (8.28 mg dry wt); 5 μmoles of substrate; and 0.2 ml 10% KOH in the center well. Reactions were continued until the endogenous rate was reached ranging from 110 min for acetate to 420 min for caprate.

<sup>b</sup> Determined by difference between total μl O₂ and μl O₂ of the endogenous flask.

<sup>c</sup> μl mg⁻¹ hr⁻¹ in linear part of the curve.
malonic, glutaconic, \( \beta \)-hydroxyisobutyric, \( \beta \)-hydroxy-\( \beta \)-methylglutaric, mesaconic, hydroxypropionic, and propylmalonic.

Figure 4 is a plot of the results using heptanoic acid as substrate; the shape of the curve is typical of the other acids except dimethylacrylic, glyoxylic, \( \beta \)-hydroxybutyric, \( \alpha \)-ketoglutaric, tartronic, isovaleric and tiglic acids. In these cases, the rate remained constant during the entire experiment, and the slope did not decrease to that of the endogenous.
Figure 4. O$_2$ uptake by *Nocardia corallina* with heptanoate as substrate. Conditions as stated in Table 20

A - no substrate

B - substrate: heptanoate
DISCUSSION

From these results, it is apparent that the photosynthetic bacterium *Rhodospirillum rubrum* contains the enzymes necessary for the carboxylation of phosphoenolpyruvate (PEP), either when grown anaerobically in the light or aerobically in the dark. The main objective, to determine the mechanism by which this conversion occurs, was initially approached by observing the effect of nucleotides and the reversibility of the reaction. The activity with PEP in the absence of added IDP in the relatively crude extracts suggested that PEP carboxylase (Reaction 8) was present, and the nucleotide stimulation and the reversible exchange reaction suggested that PEP carboxykinase (Reaction 5) was present. Since the activity in the absence of added nucleotides could have been due to nucleotides in the extract, the extracts were treated with charcoal or Dowex-3 anion-exchange resin to remove nucleotides. As was noted in Table 1, the exchange reaction operated even after these treatments in the absence of added ITP.

Although the activity in the absence of ITP in the exchange reaction was usually much smaller than that in the absence of IDP in the fixation reaction, it seems question-
able to attribute the activity with PEP, in the absence of IDP, to the irreversible PEP carboxylase when the reverse exchange reaction occurred in the absence of added ITP.

Baugh (1961) identified the two enzymes in *Nocardia corallina* on the basis that Mg$^{4+}$ replaced Mn$^{4+}$ in the exchange reaction but not in the nucleotide independent fixation reaction. This was not the case, however, in the *Rhodospirillum rubrum* extracts (Table 7).

In an attempt to separate the enzymes, the extracts were subjected to ammonium sulfate fractionation. Various procedures were performed with several extracts but when the activity with OAA in the absence of added ITP was absent, no activity was observed with PEP in the absence of IDP.

An enzyme preparation, prepared according to Bandurski (1955), also fixed $^{14}$O$_2$ in the absence of added ITP with OAA. It remains unexplained why spinach extracts failed to exhibit activity when prepared by this method in this laboratory. In only one case (fractionation according to Utter and Kurahashi, 1954a) did activity appear with PEP in the absence of added IDP and not with OAA in the absence of ITP. Since the opposite behavior appeared in numerous other preparations, it seems questionable to conclude that
PEP carboxylase was present in the extracts of *Rhodospirillum rubrum* studied in this investigation.

One explanation for the activity with OAA in the absence of ITP is that the fresh extracts contained a reversible nucleotide-independent mechanism for the carboxylation of PEP. This suggestion is substantiated somewhat by the fact that aged extracts catalyzed neither the fixation reaction nor the exchange reaction in the absence of added nucleotides, indicating that the enzyme became inactive after storage in the freezer.

The apparent stimulation of CO₂ fixation in OAA by pyrophosphate in the crude extracts of *Nocardia corallina* suggests the presence of carboxytransphosphorylase (Reaction 9). This enzyme was discovered (Siu et al., 1961) after the investigation of *Rhodospirillum rubrum*, and therefore the effect of pyrophosphate was not studied. However, a mechanism of this nature could explain the above results if for some reason the pyrophosphate (or other cofactor) were not dialyzable. The fact that activity with PEP in one experiment was about 30% higher in phosphate buffer than in Tris at the same pH, also supports this suggestion. On the other hand, it is possible that the nucleotides were not completely removed by
the methods employed and became inactive during storage.

The data subsequent to Table 1 did, however, indicate that the reversible, nucleotide-dependent PEP carboxykinase was present in both the light-grown and dark-grown cell extracts. The exchange reaction was always greater than the fixation reaction. The reaction was specific for PEP or OAA since pyruvate or malate was inactive. This observation indicated that PEP was not converted to pyruvate and carboxylated via malic enzyme, and that OAA was not converted to malate and pyruvate with a subsequent carboxylation via the same enzyme. The reaction in the partially purified light-grown extracts appeared to be similar to that described by Utter et al. (1954) in mammalian tissues. Inosine or guanosine nucleotides were necessary while the others tested were much less effective. Even this small activity may have been due to contamination since such small amounts are required. Although preliminary evidence indicated that nucleoside diphosphokinase was absent in these extracts, no conclusion can be made as to whether both nucleotides are active or one was converted to the other.

Although Eisenberg (1953) had demonstrated the enzymes of the citric acid cycle in cells of *Rhodospirillum rubrum*
grown under aerobic conditions, the presence of this pathway was somewhat uncertain in cells grown under illumination and in the absence of oxygen. Recently, Ormerod and Gest (1962) suggested an anaerobic light-dependent citric acid cycle which disposes of electrons by the formation of hydrogen.

The decrease in hydrogen evolution due to a lower amount of CO₂ could be due to a smaller amount of OAA produced by PEP carboxykinase.

Several attempts to demonstrate CO₂ fixation in reaction mixtures containing ribose-5-phosphate and extracts of *Rhodospirillum rubrum* were negative.

Probably the most significant observation in the study of the carboxylation of acids by extracts of *Nocardia corallina* was the inability to carboxylate longer carbon chains than that of butyryl-CoA. Although radioactive products were obtained with other acids, they were identical to those produced from acetate, propionate, and butyrate. The formation of acetyl-CoA, propionyl-CoA and butyryl-CoA from higher acids could be explained by normal fatty acid oxidation, notwithstanding the fact that the reactions were performed in the absence of oxygen. Possibly some other compound replaced oxygen as the electron acceptor. Apparently the oxidation
system is present in this organism since the whole cells were able to oxidize the fatty acids. The fact that the acids were not oxidized completely might indicate that certain intermediates were diverted (carboxylated?) into synthetic mechanisms or merely accumulated or eliminated. For example, 2-hexenoic acid could have been converted to the CoA ester, hydrated, dehydrogenated, and cleaved to butyryl-CoA and acetyl-CoA by β-ketoacyl thiolase. Thus the production of ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA would be explained, since ethylmalonyl-CoA and methylmalonyl-CoA were formed from butyrate alone, and methylmalonyl-CoA and malonyl-CoA were formed from acetate alone. It is interesting to note that the higher even-carbon acids were not utilized for carboxylation even though they were oxidized by the whole cells. Possibly the cell-free extracts could not form the CoA esters of the larger compounds. In the case of the odd-carbon fatty acids, the saturated form of the 5-carbon fatty acid (valerate) was utilized as well as the unsaturated form (2-pentenoate). As before, these compounds could have been esterified, converted to 3-ketovaleryl-CoA, and cleaved into propionyl-CoA and acetyl-CoA. The presence of these two compounds would then explain the production of methylmalonate
and malonate from valerate and 2-pentenoate since the same products were formed by these acids separately. Unexpectedly, methylmalonate and malonate were also produced from 3-methylvalerate. However, a spot in the area of propionate was observed when 3-methylvalerate was chromatographed in PA. Although the amount was small, it probably was sufficient to produce most of the methylmalonate. This finding, however hardly accounts for the higher amount of malonate produced than with propionate alone. The cleavage of 3-methylvalerate to compounds which can be carboxylated to methylmalonate and malonate must be left to speculation. It should be noted that many of the results with the various compounds probably could also be explained as a slight contamination by acetate or propionate; however, one would then have expected a rate in the manometric experiments similar to that with acetate or propionate. In this regard, the $Q_{02}$ with 3-methylvalerate was more nearly that of acetate and propionate than the other acids and after the short, rapid rise, the endogenous rate was observed. Thus the small amount of the contaminant probably was the cause of the oxygen uptake, rather than the compound itself. Also when 5 $\mu$moles of the other acids which produced radioactive products were chromatographed in
the PA solvent, no spots were evident in the propionate or acetate area. Since 0.8 μmole of propionate was detectable on the chromatogram and only 2.5 μmoles of the various substrates were used in the reactions, less than 0.4 μmole (if any) of a contaminant could have been present in the reaction mixtures.

The production of methylmalonyl-CoA, malonyl-CoA, and possibly ethylmalonyl-CoA from 4-methylvalerate could indicate a cleavage to acetyl-CoA and isobutyryl-CoA, since the carboxylation of isobutyryl-CoA would produce dimethylmalonyl-CoA, the free acid of which is found in the same chromatographic area as ethylmalonic acid in the solvents employed. This observation was the only suggestion that another compound was carboxylated. The radioactive spot on the film, however, was very slight, and it should be recalled that isobutyrate was inactive. Again the longer odd-numbered fatty acids (heptanoic and nonanoic) were inactive, although they were oxidized by the whole cells.

The unsaturated 4-carbon acid (crotonic acid) and possibly its isomer (vinylacetic acid) may have been converted to crotonyl-CoA and then converted to acetoacetyl-CoA which would have been cleaved to acetyl-CoA. Thus malonyl-CoA and
methylmalonyl-CoA formation would be explained as due to the formation of acetyl-CoA. The slight possibility of ethylmalonyl-CoA could be explained by the reduction to butyryl-CoA and subsequent carboxylation. The 2-methyl derivative of crotonate (tiglic acid) may have been converted to tiglyl-CoA, α-methyl-β-hydroxybutyryl-CoA, α-methylacetoacetyl-CoA, and finally propionyl-CoA and acetyl-CoA, thus explaining the production of methylmalonate and malonate in the hydrolyzed reaction mixture.

In addition to the expected carboxylation products of ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA from butyryl-CoA (Reaction 19), propionyl-CoA (Reaction 13), and acetyl-CoA (Reaction 8), respectively, malonate and methylmalonate were present in the hydrolyzed reaction mixtures with butyrate and acetate as substrates.

The presence of radioactive methylmalonate in hydrolyzed reaction mixtures containing acetate and butyrate in Tris buffer was taken as indirect evidence of transcarboxylation (Baugh et al., 1961). This would seem to necessitate propionyl-CoA as the CO₂ acceptor and OAA as the CO₂ donor (Reaction 21) unless the expected carboxylation products (malonyl-CoA or ethylmalonyl-CoA) could function in this
capacity. Unless these compounds (propionyl-CoA and OAA) were present initially in the extract, several reactions seem necessary for their formation. In the purified extracts, they would not be expected to be present unless somehow attached to the enzymes. However, since the extracts were not purified extensively, several enzymes probably functioned, and the various intermediates could conceivably have been formed. If this were the case, acetyl-CoA or butyryl-CoA would be carboxylated, followed by transcarboxylation (Reaction 23) with the formation and accumulation of methylmalonyl-CoA. The increased amount of malonate in phosphate buffer could be accounted for by the stimulation of acetyl-CoA or malonyl-CoA formation or by the inhibition of the transcarboxylation reaction. On the other hand, acetate and butyrate (via acetate) may be converted to methylmalonyl-CoA followed by the transcarboxylation with acetyl-CoA with the formation of malonyl-CoA. In this case the higher amounts of malonate in the presence of phosphate could be due to the stimulation of the transcarboxylation reaction by phosphate. In this connection it is of interest to note that with the DEAE-extract, very little malonate was detected in the hydrolyzed reaction mixtures with any of the acids tested.
Possibly the necessary cofactors, or the enzyme itself, involved in the transcarboxylation reaction were removed or were inactive.

In discussing the experimental results it should be noted again that the total radioactivity detected by the Geiger- Muller tube in the acidified reaction mixtures could have been due to a combination of radioactive CoA esters of nonvolatile compounds (eg. malonyl-CoA), esters of volatile CoA esters (eg. propionyl-CoA), and nonvolatile free acids including unstable OAA (eg. citrate, malate, or amino acids). The radioactivity detected by the scanner after hydrolysis was due to nonvolatile stable compounds (eg. citrate, malonate, and amino acids, and probably not OAA). The columns in the tables designated "origin" indicate the activity which did not move from the origin of the chromatogram. In these tables the solvent was OFW and the compounds which are immobile (or only slightly mobile) include amino acids and polycarboxylic acids. In addition to the malonic acid derivatives detected, the reaction mixtures of the different acids in the various extracts also contained other radioactive compounds. When extracted with ether, these spots were less obvious on films. When the origins (OFW) of acetate
and propionate reaction mixtures with crude extracts were eluted and developed in EaFW, activity appeared in amino acid spots revealed by ninhydrin in the area of aspartate, glutamate and glycine.

Since most of the acids produced results similar to acetate, the effect of various compounds on the carboxylation of acetate in a crude extract was observed in an attempt to elucidate the mechanism for the formation of methylmalonate.

Since these experiments were not repeated, they should not be considered conclusive, but several possibilities present themselves which may be of value in future investigations of this mechanism. The stimulation by citrate of the total amount of C\(^{14}\)O\(_2\) fixed probably was not due to a stimulation of malonyl-CoA (Martin and Vagelos, 1962), since the radioactivity after hydrolysis was similar to that obtained in the absence of citrate.

Additional malonyl-CoA formed by transcarboxylation of the tertiary carboxyl group of citrate (Abraham et al., 1961) or by the conversion of citrate to oxalosuccinate and transcarboxylation via Reaction 13 would not be detected in this type of experiment since the carboxyl groups transferred are not labeled.
Various metabolic pathways, including the citric and glyoxylic acid cycles, are implicated by the stimulation of citrate and the remaining results in Table 18. It is interesting to note also that radioactive glycolate and succinate were detected in undialyzed extracts in the presence of propionate (Baugh, 1961). However, in the present study a non-radioactive spot in the area of succinate was observed occasionally on various chromatograms. Thus the formation of succinate by the condensation of two acetate molecules should be considered.

In addition to a more thorough study of the above mechanisms, more information concerning the conversion of acetate to methylmalonyl-CoA would be obtained by observing the effect of oxalosuccinate, isocitrate, fluoride, fluoroacetate, and arsenite. The uncertain radioactive compounds need to be identified and possible mechanisms for the formation of propionate, such as the formation and subsequent decarboxylation of α-ketobutyryl-CoA and the reduction of pyruvate, should be investigated. However, more conclusive results would necessitate rigorous enzyme separation and purification as well as a study of the individual reactions employing various radioactive substrates.
SUMMARY AND CONCLUSIONS

1. Evidence has been presented which indicates that cell-free extracts from light-grown cells of *Rhodospirillum rubrum* can carboxylate phosphoenolpyruvate with the formation of oxalacetate by a reversible, nucleotide-requiring reaction similar to that catalyzed by oxalacetic carboxylase (phosphoenolpyruvic carboxykinase). Inosine or guanosine nucleotides are equally effective as cofactors, and manganese is most efficient in satisfying the divalent ion requirement. As the extracts age, sulfhydryl compounds produce an increasing stimulation. The sulfhydryl inhibitor, p-chloromercuribenzoate, inhibits the reaction but cysteine reverses the inhibition. The optimum pH ranges from 6.4 to 7.0. Preliminary results indicate that dark-grown cells also carboxylate phosphoenolpyruvate by the same reaction.

2. Cell-free extracts from light-grown cells of *Rhodospirillum rubrum* may also contain a reversible, nucleotide-independent mechanism for the carboxylation of phosphoenolpyruvate, which becomes inactive on storage at -20 C. This reaction may be similar to that catalyzed by carboxytransphosphorylase.

3. Crude extracts of *Nocardia corallina* probably also
contain the pyrophosphate-stimulated conversion of oxalacetate to phosphoenolpyruvate.

4. A number of carboxylic acids were substituted for propionate in the propionyl carboxylase reaction in variously purified extracts of *Nocardia corallina*, and it was found that none of those tested (other than acetate and butyrate) could be directly carboxylated to any significant degree. However, radioactive malonate and methylmalonate were detected in reaction mixtures containing valerate, 3-methylvalerate, 2-pentenoate, γ-methylvalerate, 2-hexenoate, crotonate, vinylacetate, and tiglate, as well as in acetate, propionate, and butyrate. Butyrate and 2-hexenoate also contained ethylmalonate. The mechanism for the formation of these products was studied and discussed.

5. The least inhibition by avidin in the carboxylation of propionate in cell-free extracts of *Nocardia corallina* occurs at the optimum pH of the enzyme. Inhibition increases on either side of the pH optimum.

6. Whole cells of *Nocardia corallina* oxidize a variety of saturated straight and branched-chain carboxylic acids up through 10 carbons in length. Generally, the rate decreases and the total oxygen uptake increases with increasing chain length.
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Lardy, Henry A. and Adler, Julius

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Utter, M. F.

Utter, Merton F. and Keech, D. Bruce

Utter, M. F. and Kurahashi, K.

Utter, M. F. and Kurahashi, K.

Utter, M. F., Kurahashi, K., and Rose, Irvin A.

Utter, M. R. and Kurahashi, Kiyoshi
Utter, M. F., Lipmann, Fritz, and Werkman, C. H.

Utter, M. F. and Wood, Harland G.

Utter, Merton F. and Wood, Harland G.

Utter, Merton F. and Wood, Harland G.

Vagelos, P. Roy and Alberts, A. W.

Valley, George and Rettger, Leo F.

Veiga Salles, J. B., Harary, Isaac, Banfi, Roberto F., and Ochoa, Severo

Vennesland, Birgit and Conn, Eric E.

Vennesland, Birgit and Evans, E. A., Jr., and Altman, Kurt I.

Vennesland, Birgit and Felsher, Rose Z.
Vennesland, Birgit, Gollub, Miriam C., and Speck, John F.  

Vernon, Leo P. and Kamen, Martin D.  

Vishniac, Wolf and Fuller, R. C.  

Wakil, Salih J.  

Wakil, Salih J., Titchener, Edward B., and Gibson, David M.  

Walker, D. A.  

Warburg, Otto  

Web, R. B., Clark, J. Bennett, and Chance, H. L.  

Weissbach, A., Smyrniotis, P. Z., and Horecker, B. L.  

Werkman, C. H.  
Werkman, C. H.


Werkman, C. H. and Wood, H. G.

Werkman, C. H. and Wood, H. G.

Wessman, G. E. and Werkman, C. H.

Wherry, Wm. B. and Ervin, D. M.

Wieringa, K. T.

Williams, Virginia R. and Fieger, E. A.

Wilson, J., Krampitz, L. O., and Werkman, C. H.

Wood, Harland G.
Wood, Harland, G., Lifson, Nathan, and Lorber, Victor

Wood, Harland, G. and Stjernholm, Rune


Wood, Harland Goff and Werkman, Chester Hamlin

Wood, H. G. and Werkman, C. H.

Wood, Harland Goff and Werkman, Chester Hamlin

Wood, Harland Goff and Werkman, Chester Hamlin

Wood, Harland Goff and Werkman, Chester Hamlin

Wood, Harland Goff and Werkman, Chester Hamlin
Woods, Donald Devereux  

Woody, Barbara R. and Lindstrom, E. S.  

Woronick, Charles L. and Johnson, Marvin J.  
ACKNOWLEDGEMENTS

I wish to express my feeling of great loss at the death of Dr. C. H. Werkman during the final stage of this study. I will always be grateful for the opportunity to study with him. His encouragement, confidence, and direction were instrumental in the completion of this study. His advice, wisdom, and experience were and shall continue to be invaluable in my personal life. Although in poor health, he forced himself to continue working with me to the very last and for this I am extremely grateful. I appreciate the opportunity to pay tribute to a renowned scientist, exceptional major professor, and dear friend.

I wish to thank Dr. W. R. Lockhart for his assistance in constructing the thesis and taking Dr. Werkman's place in the final completion of this degree. I am also grateful to Dr. Lockhart and others concerned for making it possible for me to finish as Dr. Werkman's student.

To Dr. E. B. Fowler and Mr. Wayne Rhinehart, I express my gratitude for their help, advice, and suggestions concerning the operation of the Fowler-Rhinehart scanner.

I appreciate the suggestions and assistance during this investigation by my fellow graduate students: Isamu Suzuki,

Special acknowledgement is also expressed to my wife, Judy, for technical assistance, help in preparing the manuscript, and her unfailing encouragement and understanding during the course of this study.

I wish also to thank Mrs. Carolyn Ringgenberg and Mrs. Anna Lamp for typing parts of the manuscript.
Attempts to follow the growth of *Nocardia corallina* were fraught with difficulty, but a general idea can be obtained as to the stage of growth of the cells when harvested by observing the typical data in Figure 5. The cells were incubated for 36 hr, when they apparently were in the late log phase, and then harvested. Microscopic examination revealed the presence of small, stubby rods and some rather spherical cells. These cells probably correspond to the bacillary and coccoidal forms described by Webb *et al.* (1954).

The growth curves were somewhat questionable for several reasons. The organism undergoes morphological changes during growth, ranging from long coenocytic hyphae to coccoidal forms. Since total mass was measured by light absorption, the curve probably would not be affected but cell numbers would change as the long cells fragmented.

Correction for the turbidity due to the antifoam appeared necessary, although visual observation indicated that the turbidity seemed to disappear soon after inoculation. In the experiment shown in Figure 5, the antifoam added initially
Figure 5. Growth curve of *Nocardia corallina* in the fermentor.
Turbidity was determined by measuring the optical density at 660 nm.
would affect all points equally. Correction for the second antifoam addition would only reduce the absorbance to that indicated by the dotted line.

The greatest problem arose from the fact that the organism grows in clumps which were suspended as well as possible with a syringe prior to the turbidity measurements. The early drop in absorbancy may have been due to the fact that initially the antifoam and inoculum were in a rather homogeneous suspension, but after incubation it appeared that the cells and antifoam went out of suspension and collected upon the parts of the fermentor, leaving the medium relatively clear.
APPENDIX B

Fractionation of *Nocardia corallina* Extracts on DEAE-Cellulose

The results of the fractionation of the crude extract are presented in Table 21. Fraction 8 was the one used for the experiments presented herein. It should be noted that the activity fixed was due to propionate-$^{14}$C and not $^{14}$CO$_2$. Therefore some of the activities could have been due to propionyl-CoA since the total of the fractions was greater than the crude extract itself (270 cpm/0.5 ml extract). The higher activity could have also been the result of the removal of some inhibitor during the fraction procedure.
Table 21. DEAE-cellulose fractionation of *Nocardia corallina* extracts

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Additions to the column</th>
<th>Protein (mg/ml)</th>
<th>Activity fixed (counts/min/0.05 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>crude extract</td>
<td>0.04</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>0.05 M Tris</td>
<td>0.22</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>0.05 M Tris</td>
<td>0.07</td>
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</tr>
<tr>
<td>4</td>
<td>0.025 M KCl</td>
<td>0.00</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>0.05 M KCl</td>
<td>0.01</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>0.1 M KCl</td>
<td>0.02</td>
<td>---</td>
</tr>
<tr>
<td>7</td>
<td>0.2 M KCl</td>
<td>0.06</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>1.0 M KCl</td>
<td>0.40</td>
<td>221</td>
</tr>
<tr>
<td>9</td>
<td>1.0 M KCl</td>
<td>0.60</td>
<td>260</td>
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</table>

The fractions consisted of the effluent from the column after 10 ml of each of the various additions was added to the column. The additions were those described in the "Methods" section. Protein was determined by TCA precipitation as previously described.

The flasks in the radioactivity assay contained potassium phosphate buffer (pH 7.5), 40 μmoles; potassium propionate-1-C$^{14}$, 8 μmoles (5.6 x 10$^6$ cpn); GSH, 5 μmoles; MgCl$_2$, 2.5 μmoles; ATP, 1.5 μmoles; CoA, 0.15 μmoles; and 0.5 ml of the various fractions. Total volume was 1.05 ml and the reaction time was 45 min.
Calibration of the Fowler-Rhinehart Scanner

The various conditions for the operation of the scanner were determined experimentally. The operating voltage (1650 v) was picked from the center of the plateau on a cpm/voltage plot. The plateau was about 100 volts long. No plateau was observed with 2 cm of Q gas (Matheson Company), but increasing pressures above 4 cm did not affect the length of the plateau. The threshold voltage, however, increased with increasing Q gas pressure. Arbitrarily, 4 cm of Q gas was chosen. Theoretically, the longer the distance between the resolving slit and the anode, the more resolving power is obtained. This effect was not observed, however, so 16 mm was arbitrarily chosen. Widening the slit lowers the resolving power, but more sensitivity is gained since more β-rays can enter the detector. Various slit widths were used in the study but the data presented were obtained using the widest slit (3/4 in).

The area of the peaks was proportional to the radioactivity in the spots as shown in Figure 6. Various amounts of a radioactive solution were spotted on paper strips and
Figure 6. The relationship between radioactivity and the area under the peaks on charts from the Fowler-Rhinehart Scanner. Range: 3200
on glass planchets in duplicate. The spots on the strip were scanned several times and the area of the peaks was measured with a planimeter after the curves were arbitrarily extrapolated to background. The averages were used in Figure 6.

The data in Tables 16, 17, and 18 were obtained by scanning the chromatograms twice and determining the average area in sq cm. The difference between the two values was seldom as much as 10%, with the smaller peaks having the largest errors. The per cent of the total was determined by dividing the area of each peak by the total area on the chromatogram.
APPENDIX D

$R_f$ Values of Various Compounds in the Solvents Used
APPENDIX D

Rf Values of Various Compounds in the Solvents Used

<table>
<thead>
<tr>
<th>Compound</th>
<th>OFW</th>
<th>EaFW</th>
<th>EAW-I</th>
<th>PA</th>
<th>BA-I</th>
<th>iPF</th>
<th>PFW</th>
<th>EAW-II</th>
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<td>Acetic acid</td>
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<tr>
<td>Aconitic acid</td>
<td>51</td>
<td>86</td>
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<td>19</td>
<td>27</td>
<td>0</td>
<td>64</td>
<td>13</td>
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<td>Acrylic acid</td>
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<td>53</td>
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<td>γ-Aminobutyric acid</td>
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*Rf (x 100) of the major spots. In some cases as many as two minor spots appeared probably because of impurities.*
### APPENDIX D (Continued)

<table>
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<th>Compound</th>
<th>OFW</th>
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<th>EAW-I</th>
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