Cobalt is known to be essential for symbiotic nitrogen fixation by legumes (1–5), but a requirement of the element for growth of leguminous plants supplied with adequate fixed nitrogen has not been shown. Ahmed and Evans (3) provided evidence that cobalt was required for the bacteria within the nodules of inoculated legumes but was not required for the leguminous plants per se. Lowe, Evans, and Ahmed (6) and Lowe and Evans (7) subsequently showed that cobalt is necessary for normal growth of five species of Rhizobium. More recently, Kliewer and Evans (8–10) analyzed nodules of a variety of symbionts and cells from several species of Rhizobium and observed the presence of cobamide coenzymes in each of the organisms. In addition, they (9) isolated and identified the dimethylbenzimidazolylcobamide coenzyme in extracts of cells isolated from soybean nodules and in cells of a pure culture of Rhizobium meliloti and presented data (10) showing that cobalt deficiency not only limited the growth of Rhizobium japonicum and R. meliloti but also limited the synthesis of cobamide coenzymes in these organisms.

The B12 coenzyme has been shown to be involved in propionate metabolism in animals (11–13) and in Propionibacterium shermanii (14, 15). In addition, Arnstein and White (16) have shown that B12 deficiency in Ochrobactrum maltophilus resulted in a reduced capacity of the organism to oxidize propionate. Ayers (17) has described similar effects of B12 deficiency in a Flavobacterium species. On the basis of the above reports, experiments were conducted to determine whether Rhizobium would metabolize propionate and the possible effect of cobalt deficiency on propionate utilization in these bacteria. In a preliminary communication (18), evidence was presented showing that the rate of oxidation of propionate by cell suspensions of R. meliloti was strikingly diminished in the presence of cobalt, as compared to the culture of R. japonicum, R. phaseoli, R. leguminosarum, and R. trifolii. In contrast, the oxidation of glutamate was not affected by cobalt deficiency. In this paper, evidence is presented showing that enzymes necessary for the catalysis of the conversion of propionate to succinyl coenzyme A by a pathway involving methylmalonyl coenzyme A are widely distributed in Rhizobium and that cobalt deficiency in R. meliloti results in insufficient B12 coenzyme for the function of methylmalonyl coenzyme A mutase in cell-free extracts.

The Relationship of Cobalt Requirement to Propionate Metabolism in Rhizobium*

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EXPERIMENTAL PROCEDURE

Biological Materials—The biological materials utilized in this investigation were: soybeans (Glycine max (L.) Merr. var. Chippewa), peas (Pisum sativum L. var. American Wonder), lupine (Lupinus angustifolius L. var. Dorre Blue), cowpeas (Vigna sinensis Savi, var. Iron Clay), Rhizobium meliloti F-29, Rhizobium japonicum 61A76, Rhizobium phaseoli K-11, Rhizobium leguminosarum C-56, and Rhizobium trifolii P-28. The seeds of the various legumes were purchased from commercial sources and the Rhizobium species were kindly supplied by Dr. J. C. Burton of the Nitrash Company, Milwaukee, Wisconsin.

Reagents—ATP (dipotassium salt) and GSH were obtained from Nutritional Biochemical Corporation, coenzyme A from Sigma Chemical Company, and propionate-1-14C, propionate-2-14C, and propionate-3-14C from New England Nuclear Corporation. The adenylcobamide coenzyme was a gift from Dr. Mark Kliewer, Department of Viticulture and Enology, University of California, Davis, California. The benzimidazolylcobamide coenzyme was kindly supplied by Dr. Karl Folkers, Merck Institute for Therapeutic Research, Rahway, New Jersey. All other chemicals were reagent grade and were purchased from commercial sources.

Cultural Procedures—Seedlings of the legumes, inoculated with the appropriate Rhizobium species, were grown in a greenhouse according to the cultural method described by Ahmed and Evans (2, 3). Nodules were harvested from roots of plants at ages listed in the legends of figures and tables that present the results of the various experiments.

Medium A, used for the culture of R. japonicum, R. phaseoli, R. leguminosarum, and R. trifolii, contained the following components in a final volume of 1 liter: K2HPO4, 1.00 g; KH2PO4, 1.00 g; MgSO4·7H2O, 0.36 g; CaSO4·2H2O, 0.13 g; KNO3, 0.70 g; FeCl3·6H2O, 4 mg; yeast extract (Difco), 1.00 g; l-arabinose, 1 g; glycerol, 4 ml. The components of the medium were dissolved in distilled water and the pH was adjusted to 6.3 with 1 N HCl or 1 N NaOH.

Medium B, which was used in certain experiments for the culture of R. meliloti, was the same as Medium A except that l-arabinose, glycerol, and KNO3 were omitted and 3.00 g of n-mannitol and 0.06 mg of CoCl2·6H2O were added to each liter. The pH was adjusted to 6.8.

Media C and D were identical with Media A and B, respec-
tively, except that 100 mg each of succinic acid, methylmalonic acid, and sodium propionate were added per liter.

The method employed for purifying components of the culture medium and the cultural procedure used for the growth of *R. meliloti* with varying concentrations of added cobalt have been described in previous communications (2, 3, 7, 18).

The various *Rhizobium* species were transferred from slants to 50-ml culture flasks containing 20 ml of the appropriate medium. When the organisms reached the log phase of growth, a series of 1-liter flasks, each containing 400 ml of the appropriate medium, were inoculated with 4 ml of the appropriate culture. For the study of cobalt deficiency, it was necessary to transfer at least twice to 50-ml flasks containing 20 ml of cobalt-deficient medium before the final inoculation of the cobalt-deficient medium in the 1-liter flasks.

Cells were cultured on a rotary shaker at 30° and were harvested during the log phase of growth. *R. meliloti, R. leguminosarum, R. phaseoli*, and *R. trifolii* were harvested after 18 to 24 hours. *R. japonicum* was harvested after 79 to 96 hours, and *R. meliloti*, grown in the cobalt-deficient medium, was harvested 36 to 48 hours after inoculation.

**Preparation of Cell Suspensions and Extracts**—The operations necessary for the preparation of cell suspensions and cell-free extracts were performed at a temperature of 0-4°. Bacteroids were collected from legume nodules by the procedure described by Cheniae and Evans (19). Cells were harvested from culture media by centrifugation at 11,000 × g for 10 minutes. Four 1-liter flasks containing 400 ml of Medium A, B, C, or D yielded about 1 g of fresh cells. Cells that were collected for radiorespirometric experiments were washed twice in 0.067 M potassium phosphate buffer, pH 6.8, and those used for preparation of cell-free extracts were washed twice with 0.01 M Tris-Cl buffer, pH 8.5. Cells from the cobalt-deficient medium were prepared by the same procedure with the exception that centrifuge tubes were acid-washed and the buffers used for the radiorespirometric experiments were extracted with 1-nitroso-2-naphthol (2, 3) to decrease cobalt contamination. For the radiorespirometric experiments, the bacteroids and cells were suspended in sufficient 0.067 M potassium phosphate buffer, pH 6.8, to obtain 20 mg of wet cells per ml. The nitrogen content of cell suspensions was determined during the log phase of growth. *R. meliloti*, *R. leguminosarum*, *R. phaseoli*, and *R. trifolii* were harvested after 18 to 24 hours. *R. japonicum* was harvested after 79 to 96 hours, and *R. meliloti*, grown in the cobalt-deficient medium, was harvested 36 to 48 hours after inoculation.

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Cell-free extracts for enzyme experiments were obtained by grinding the bacteroids or cells with alumina and 0.01 M Tris-Cl buffer (0.001 M with respect to GSH), pH 8.5 (19). The supernatant solution remaining after the slurry was centrifuged at 34,000 × g for 20 minutes was used for all enzyme assays. Throughout this procedure, exposure to direct light was avoided to prevent destruction of endogenous B12 coenzyme. The protein content of the extracts was determined by the Folin-Ciocalteau method described by Layne (21). Crystalline serum albumin was used as the protein standard.

Purified propionyl-CoA carboxylase was prepared according to the method described by Lane and Halenz (22). The procedure was followed up to the first ammonium sulfate fractionation step. At this point, the carboxylase was found to be essentially free of methylmalonyl-CoA mutase.

**Radiorespirometric Procedures**—The radiorespirometric experiments were performed according to the method described by Wang *et al.* (23) and Wang and Kraekov (24). The contents of each flask in a final volume of 11 ml were made up of 10 ml of cell suspension in 0.067 M potassium phosphate buffer at pH 6.8 and 1.0 ml of 14C-propionate (3.5 μmole containing 1.0 μC). When suspensions of *R. meliloti* were used that had been grown with varying concentrations of cobalt, the flasks contained one-half the quantity of all components listed. The flasks were shaken continuously in a controlled temperature bath at 30°. The air flow was maintained at 60 ml per minute and the 14C02 was trapped in 10 ml of a mixture composed of 2 parts absolute ethanol and 1 part 2-aminoethanol. Samples were collected at 20-minute intervals. To determine the amount of 14CO2 recovered, the trapping solution was collected and diluted to 15 ml with absolute ethanol. A 5-ml aliquot was placed in a 20-ml counting vial containing 10 ml of phosphor solution (0.3% 2, 5-diphenyloxazole and 0.003% 1, 4-bis-(4-methyl-5-phenyloxazoly)benzene in toluene). This was counted in a Packard Tri-Carb model 314 DC liquid scintillation counter. Counting was usually carried out to a standard deviation of no greater than 2%. Counting efficiency was determined by use of 14C-toluene as an internal standard.

**Enzyme Assays**—The procedure used for the determination of propionate and acetate activation is a modification of that described by Berg (25) for the activation of acetate. The complete reaction mixture in a final volume of 1.0 ml consisted of the following components in micromoles: Tris buffer, pH 8.0, 10; Tris-acetate or Tris-propionate, pH 8.0, 10; MgCl2, 20; ATP, 10; GSH, 10; KH14C03, 15; KCl, 50; coenzyme A, 0.25; and enzyme extract containing 1 to 3 mg of protein. In those experiments in which acetate was used as a substrate, the absorbance at 540 nm was converted to micromoles of acetylated malonate with the use of the conversion factor reported by Berg (25). Where Tris-propionate was used as the substrate, absorbance measurements at 540 nm were converted to concentrations of propionyl-hydroxamate by use of a standard curve prepared with propionic anhydride (26).

Propionyl-CoA carboxylase was assayed by the procedure described by Lane and Halenz (22). The complete reaction mixture in a final volume of 1.0 ml consisted of the following components in micromoles: Tris buffer, pH 8.5, 100; KH14C03, 15; ATP, 4.0; MgCl2, 4.0; GSH, 5.0; propionyl-CoA, 1.0; and enzyme extract containing 1 to 3 mg of protein. For some experiments, other acyl-CoA compounds were substituted for propionyl-CoA (26). Methylmalonyl-CoA mutase activity was determined by the procedure of Hegre, Miller, and Lane (28), which involves coupling of a purified propionyl-CoA carboxylase from beef liver and the methylmalonyl-CoA mutase of the enzyme extracts. The complete reaction mixture in a final volume of 1.0 ml consisted of the following components in micromoles: Tris buffer, pH 8.5, 100; KH14C03, 15; ATP, 4.0; MgCl2, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units per mg of protein), 1 to 3 mg; and enzyme extract containing 0.1 to 1.0 mg of protein. The enzyme extract was added following a 10-minute preincubation at 37° of all other reaction components. An amount of coenzyme listed in the legend of the tables was added to the reaction mixture immediately after the enzyme extract. The complete system was then incubated for an additional 20 minutes. The permanganate oxidation and plating procedures were identical with those described by Hegre *et al.*
Fig. 1. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-¹⁴C by bacteroids from soybean nodules. The nitrogen content of cells in each flask was 3.4 mg (See “Experimental Procedure” for further details). ■—■, propionate-¹⁴C; ○—○, propionate-2-¹⁴C; ●—●, propionate-3-¹⁴C.

Fig. 2. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-¹⁴C by *R. japonicum* grown on Media A and C. Medium A is the basic medium for *R. japonicum* Medium C is the same medium modified by the addition of 100 mg per liter each of sodium propionate, succinate, and methylmalonate. The nitrogen content of cells in each flask was 5.0 and 5.2 mg for experiments involving Media A and C, respectively. Symbols are as in Fig. 1. See “Experimental Procedure” for further details.

Fig. 3. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-¹⁴C by *R. meliloti* grown on Media B and D. Medium B is the basic medium for *R. meliloti*. Medium D is the same, except that it is modified by the addition of 100 mg per liter each of sodium propionate, succinate, and methylmalonate. The nitrogen content of cells in each flask was 4.3 and 4.1 mg for experiments in which Media B and D were used, respectively. See “Experimental Procedure” for further details.

Fig. 4. The radiorespirometric patterns for the oxidation of propionate-1-¹⁴C by *R. meliloti* grown with varying concentrations of cobalt in the culture medium purified for cobalt. Each flask contained 5 ml of cell suspension and 0.5 ml of propionate (1.75 μmoles containing 0.5 μe). The nitrogen contents of flasks were: 1.68, 1.75, and 1.65 mg. ○—○, 0.00; ●—●, 0.01; and ■—■, 1.0 p.p.b. cobalt levels, respectively. The abbreviation p.p.b. refers to 1 μg per liter. See “Experimental Procedure” for further details.

This effect was not observed with cell suspensions of *R. japonicum*. Regardless of the organism tested or the medium used for culture, the patterns of ¹⁴CO₂ recovery from propionate labeled in different positions were similar. The rate of recovery of ¹⁴CO₂ from the oxidation of propionate-1-¹⁴C was always greater than rates of recovery of ¹⁴CO₂ from propionate-2- and -3-¹⁴C. The rates of recovery of ¹⁴CO₂ from the latter two compounds were similar.

Since methylmalonyl-CoA mutase from animal tissues (11-13) or from *P. shermanii* (14, 15) has been reported to require cobamide coenzymes, the effect of varying concentrations of cobalt on the capacity of *R. meliloti* to oxidize propionate-1-, -2-, and -3-¹⁴C was studied. The results presented in Fig. 4 show trends in propionate-1-¹⁴C oxidation that are very similar to those previously obtained with manometric experiments (18). Cobalt-deficient cells exhibited a greatly reduced capacity to oxidize propionate as indicated by the rate of evolution of ¹⁴CO₂ from propionate-1-¹⁴C. The increased rate of formation of ¹⁴CO₂ from deficient cells that occurred after a lag period may have been caused by contamination of cell suspensions with cobalt, resulting

**RESULTS**

Radiorespirometric Experiments—The radiorespirometric patterns for the oxidation of specifically labeled propionate by bacteroids isolated from soybean nodules are presented in Fig. 1. These data show that propionate is oxidized rapidly after an initial slower rate of oxidation. The curves in Figs. 2 and 3 indicate that cell suspensions from pure cultures of *R. japonicum* and *R. meliloti* also oxidize propionate. Fig. 3 shows that propionate oxidation by *R. meliloti* was enhanced by the addition of propionate, succinate, and methylmalonate to the culture medium.

(28). The radioactivity of permanganate-stable compounds was measured with a Nuclear Chicago model D-47 gas flow counter. The activity of methylmalonyl-CoA mutase in a cell-free extract of *R. meliloti* was proportional to amounts of extract protein in the range of 0.1 to 1.0 mg.

Products of the methylmalonyl-CoA mutase reaction were isolated and chromatographed by the procedure described by Flavin and Ochoa (29). The radioactive areas of the chromatograms were detected with a Vanguard model 880 strip counter.

This work was supported by Grant 115-44 from the Illinois Agricultural Experiment Station.
in the synthesis of cobamide coenzyme. The effect of the inter-
mediate concentration of cobalt on the rate of oxidation of pro-
pionate was not as striking as that previously observed with
the manometric experiments (18). The results of radiorespirometric
experiments in which propionate-2-14C and -3-14C were utilized as
substrates showed trends similar to those obtained with
propionate-1-14C and are not presented.

**Enzymes of Propionate Oxidative Pathway**—Propionate utiliza-
tion by organisms in all known cases is initiated by a reaction
with ATP and coenzyme A yielding propionyl-CoA, adenylic
acid, and pyrophosphate. The enzyme catalyzing this reaction
will function with either propionate or acetate (30). In the
experiments in which the enzyme activity of cell-free extracts of
soybean bacteroids and pure cultures of *R. japonicum* and *R.
meliloti* were studied, both acetate and propionate were utilized as
substrates. Data are presented in Table I which show that these extracts contained an enzyme system capable of activating both substrates.

The second reaction in the conversion of propionate to succi-
nate is the carboxylation of propionyl-CoA to form methyl-
malonyl-CoA. With the use of extracts prepared from soybean
bacteroids and pure cultures of *R. japonicum* and *R. meliloti*,
experiments were conducted to determine if a carboxylase was
present which would utilize propionyl-CoA as a substrate.

**Table I**

*Activation of propionate and acetate by cell-free extracts of soybean bacteroids, *R. japonicum*, and *R. meliloti***

Bacteroids were isolated from nodules of 26-day-old soybean
plants. *R. japonicum* and *R. meliloti* were grown in Media C
and D, respectively. Enzyme activity was measured as described in
"Experimental Procedure." The complete reaction mixture
minus acetate or propionate served as a negative control. Ess-
entially no activity was obtained when either CoA, enzyme,
acetate, or propionate was omitted from reactions.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Acetate activation</th>
<th>Propionate activation</th>
</tr>
</thead>
</table>
| Soybean bacter-
oids | 0.48 | 0.34 |
| *R. japonicum* | 0.23 | 0.20 |
| *R. meliloti* | 0.08 | 0.09 |

**Table II**

*Requirements of propionyl-CoA carboxylase in cell-free extracts of soybean bacteroids, *R. japonicum*, and *R. meliloti***

Bacteroids were isolated from nodules of 31-day-old soybean
plants. *R. japonicum* and *R. meliloti* were grown in Media C
and D, respectively. Enzyme activity was measured as described in
"Experimental Procedure." The complete reaction mixture
minus enzyme served as a negative control.

**Table III**

*Substrate specificity of propionyl-CoA carboxylase in cell-free extracts of soybean nodule bacteroids, *R. japonicum*, and *R. meliloti***

Bacteroids were isolated from nodules of 31-day-old soybean
plants. *R. japonicum* and *R. meliloti* were grown in Media C
and D, respectively. Enzyme activity was measured as described in
"Experimental Procedure." The complete reaction mixture
minus acyl-CoA served as a negative control.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Soybean bacteroids</th>
<th><em>R. japonicum</em></th>
<th><em>R. meliloti</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>0.09</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>0.05</td>
<td>0.23</td>
<td>0.09</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>1.06</td>
<td>1.61</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Table IV**

*Distribution of propionyl-CoA carboxylase and methylmalonyl-CoA mutase in cell-free extracts of bacteroids from nodules of legumes and of cells from pure cultures of *Rhizobium***

Propionyl-CoA carboxylase and methylmalonyl-CoA mutase activity was measured as described in "Experimental Procedure." For the carboxylase assays, the complete reaction mixture minus propionyl-CoA served as negative control. In the methyl-
malonyl-CoA mutase assay, a complete reaction was grown in
Medium B. *R. japonicum*, *R. phaseoli*, *R. leguminosarum*, and *R. trifolii* were grown in Medium A. Bacteroids were collected
from nodules of the various legumes grown for the number of days
indicated in the table.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Age of organism</th>
<th>Propionyl-CoA carboxylase activity</th>
<th>Methylmalonyl-CoA mutase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean bacteroids</td>
<td>35</td>
<td>0.19</td>
<td>0.84</td>
</tr>
<tr>
<td>Soybean bacteroids</td>
<td>32</td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>Pea bacteroids</td>
<td>37</td>
<td>0.08</td>
<td>0.69</td>
</tr>
<tr>
<td>Lupine bacteroids</td>
<td>54</td>
<td>0.13</td>
<td>0.69</td>
</tr>
<tr>
<td>Cowpea bacteroids</td>
<td>54</td>
<td>0.10</td>
<td>0.70</td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td>1</td>
<td>0.40</td>
<td>0.17</td>
</tr>
<tr>
<td><em>R. japonicum</em></td>
<td>1</td>
<td>0.06</td>
<td>0.33</td>
</tr>
<tr>
<td><em>R. leguminosarum</em></td>
<td>1</td>
<td>0.07</td>
<td>0.36</td>
</tr>
<tr>
<td><em>R. trifolii</em></td>
<td>1</td>
<td>0.06</td>
<td>0.20</td>
</tr>
</tbody>
</table>

From the data presented in Table II, it is apparent that pro-
pionyl-CoA was carboxylated and that the enzyme from these
organisms required an ATP-Mg++ mixture for activity. The
addition or deletion of GSH had no significant effect on the overall
carboxylation reaction. Since Halenz and Lane (31) have
described a carboxylase from beef liver that is specific for pro-
pionyl-CoA, experiments were conducted to determine the sub-
strate specificity of the carboxylase in extracts of soybean
bacteroids and of pure cultures of *R. japonicum* and *R. meliloti*.
The results (Table III) provide evidence that propionyl-CoA is
carboxylated at a greater rate than either butyryl-CoA and
The cell-free extract was prepared from *R. meliloti* grown in Medium D. Enzyme activity was measured by the method described in "Experimental Procedure." A complete reaction mixture (see "Experimental Procedure") without enzyme extract served as a negative control for Reactions 1 and 5. In Reactions 2, 3, and 4 and Reactions 6, 7, and 8, a complete reaction mixture without enzyme extract but with AC, BC, or DBC coenzyme as indicated served as the negative controls. The quantity of AC and BC coenzyme added to reaction mixtures (final volume, 1.1 ml) as indicated was 2.6 × 10⁻³ μmole, and the quantity of DBC coenzyme was 2.3 × 10⁻³ μmole. Certain reaction mixes indicated in the table contained extract that had been illuminated in direct sunlight for 30 minutes at 0°.

### Table V

**Coenzyme specificity of methylmalonyl-CoA mutase in cell-free extract of *R. meliloti***

The cell-free extract was prepared from *R. meliloti* grown in Medium D. Enzyme activity was measured by the method described in "Experimental Procedure." A complete reaction mixture (see "Experimental Procedure") without enzyme extract served as a negative control for Reactions 1 and 5. In Reactions 2, 3, and 4 and Reactions 6, 7, and 8, a complete reaction mixture without enzyme extract but with AC, BC, or DBC coenzyme as indicated served as the negative controls. The quantity of AC and BC coenzyme added to reaction mixtures (final volume, 1.1 ml) as indicated was 2.6 × 10⁻³ μmole, and the quantity of DBC coenzyme was 2.3 × 10⁻³ μmole. Certain reaction mixes indicated in the table contained extract that had been illuminated in direct sunlight for 30 minutes at 0°.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme activity</th>
<th>mumoles H⁺CO₂ fixed/mg protein/hr</th>
<th>stable compounds/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complete</td>
<td>1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Complete + AC</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Complete + BC</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Complete + DBC</td>
<td>1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Complete, extract illuminated</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Complete, extract illuminated, + AC</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Complete, extract illuminated, + BC</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Complete, extract illuminated, + DBC</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Methylmalonyl-CoA mutase in cell-free extracts of *R. meliloti* was prepared as described in "Preparation of Cell Suspensions and Extracts" with reduced light conditions. A portion of the extract, maintained in an ice bath at 0°, was subsequently subjected to direct sunlight for a period of 30 minutes. This is the procedure utilized by Barker et al. (34) to destroy the AC coenzyme in cell-free extracts of *Clostridium tetanomorphum*. That portion of the extract which was not illuminated was used in Reactions 1, 2, 3, and 4 in Table V. As illustrated by the data in Table V, the addition of the DBC and BC coenzymes to the complete reaction mixtures resulted in a slight stimulation of enzyme activity. The addition of the AC coenzyme caused a slight decrease in enzyme activity of the complete reaction mixture. The addition of the DBC, BC, and AC coenzyme to reaction mixtures containing the illuminated enzyme extract resulted in 59, 33, and 0% restoration of the activity, respectively. This evidence, together with that of Kliever and Evans (10), is consistent with the conclusion that the B₁₂ coenzyme (DBC) is the natural cobamide coenzyme associated with the methylmalonyl-CoA mutase in *R. meliloti*.

The effects of varying concentrations of cobalt in the growth medium on the activity of propionyl-CoA carboxylase and methylmalonyl-CoA mutase in cell-free extracts of *R. meliloti* were studied, and the results are presented in Table VI. The activity of propionyl-CoA carboxylase was less in extracts of deficient cells than in extracts of normal cells, but the difference was not striking. In contrast, the activity of methylmalonyl-CoA mutase in extracts of the deficient cells is strikingly reduced when compared with the activity of extracts of normal cells. The addition of 1.65 × 10⁻³ μmole of DBC coenzyme to each reaction mixture restored full activity to the enzyme extracts of cells grown with no cobalt and with 0.01 p.p.b. of the element. The addition of DBC coenzyme to extracts of cells grown with 1.0 p.p.b. of cobalt, however, failed to stimulate enzyme activity.

### Table VI

**Effect of varying concentrations of cobalt in growth medium on activity of propionyl-CoA carboxylase and methylmalonyl-CoA mutase in cell-free extracts of *R. meliloti***

Propionyl-CoA carboxylase and methylmalonyl-CoA mutase activity was measured as described in "Experimental Procedure." For the propionyl-CoA carboxylase assays, the complete reaction mixture without propionyl-CoA served as negative control. For the methylmalonyl-CoA mutase assays in which the coenzyme was omitted, the complete reaction mixture without enzyme extract served as negative control. For the reaction mixture to which DBC coenzyme was added, the complete reaction mixture without enzyme extract plus coenzyme served as the negative control. The quantity of DBC coenzyme added to the reaction mixture (final volume, 1.1 ml) was 1.65 × 10⁻³ μmole. The abbreviations p.p.b. refers to micrograms per liter.

<table>
<thead>
<tr>
<th>Concentration of cobalt added to culture medium</th>
<th>Propionyl-CoA carboxylase activity</th>
<th>Methylmalonyl-CoA mutase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol.</td>
<td>mumoles H⁺CO₂ fixed/mg protein/hr</td>
<td>stable compounds/mg protein/hr</td>
</tr>
<tr>
<td>-DBC</td>
<td>+DBC</td>
<td>-DBC</td>
</tr>
<tr>
<td>None</td>
<td>1.83</td>
<td>0.00</td>
</tr>
<tr>
<td>0.01</td>
<td>2.70</td>
<td>0.09</td>
</tr>
<tr>
<td>1.00</td>
<td>2.41</td>
<td>1.07</td>
</tr>
</tbody>
</table>

The abbreviations used for the cobamide coenzymes are: DBC, dimethylbenzimidazolylcobamide; BC, benzimidazolylcobamide; AC, adenylcobamide.
These results suggest that formation of the apoenzyme is independent of the cobalt supply, but that the catalytic activity of the enzyme may be limited by coenzyme concentration and that synthesis of the coenzyme is directly dependent on cobalt. The data obtained from the radiorespirometric (Fig. 4) and manometric experiments (18) support this conclusion.

Identification of the products of the methylmalonyl-CoA mutase reaction (complete reaction effected with cells grown with 1 p.p.b. of cobalt, Table VI) showed that after the permanganate treatment only succinate was radioactive; however, if the reaction mixture was extracted and chromatographed prior to the permanganate treatment methylmalonate also was found to be radioactive.

An experiment was conducted with normal R. meliloti cells to determine the fate of 14C from sodium propionate-1-14C when this compound was supplied to intact cells. As shown in Fig. 5, the major compounds that were radioactive after a 10-minute incubation period with sodium propionate-1-14C corresponded with malate, succinate, methylmalonate, and fumarate in chromatography experiments. These data are consistent with the other evidence indicating that propionate is converted to succinate via the propionyl-CoA carboxylase and methylmalonyl-CoA mutase route.

**DISCUSSION**

There are three known pathways whereby propionate is oxidized in biological materials. These pathways result in differences not only in the intermediates formed but also in the patterns of 14CO2 evolution from specifically labeled carbon atoms of propionate. In the β-oxidation pathway reported by Giovannelli and Stumpf (33) and Hatch and Stumpf (33) to operate in certain plant tissues, the expected rate of recovery would be greatest for carbon 1 of propionate following in turn by carbon 3 and then carbon 2. The pathway that has been reported to be operative in Clostridium kluyveri (36) and which involves the same intermediates as the higher plant system would be expected to result in the most rapid recovery of the methyl carbon of propionate followed in turn by the carboxyl carbon and then the methylene carbon. In contrast, in the pathway whereby propionate is converted to succinate via methylmalonate (20), the expected rate of recovery is greatest for carbon 1. The recovery of carbon atoms 2 and 3 is expected to be equal since they become the methylene carbon atoms of succinate and should become randomized in the citric acid cycle.

To obtain information on the capacity of rhizobia to oxidize propionate and on the possible pathway whereby it is utilized, a series of radiorespirometric experiments were performed with propionate labeled in specific positions. It was found (Figs. 1, 2, and 3) that the rate of recovery of 14C as 14CO2 from propionate labeled in different positions was greatest when the cells were incubated with propionate-1-14C. In addition, the radiorespirometric patterns for 14CO2 formation from propionate-2-14C and propionate-3-14C were essentially the same regardless of the conditions or the Rhizobium species used. These patterns of recovery would be expected only if propionate were utilized by a sequence of reactions in which it was converted to succinate and then oxidized via the citric acid cycle.

When R. meliloti was grown with varying concentrations of cobalt in the culture medium, the capacity to oxidize propionate was significantly reduced in cobalt-deficient cells (Fig. 4). The rate of oxidation of propionate by the cells was strikingly increased by an increasing cobalt supply in the culture medium. Arnstein and White (18), using O. malhamensis, and Ayers (17), using Flavobacterium sp., have conducted similar experiments in which they showed an increase in the rate of propionate oxidation of vitamin B12-deficient cells by the addition of vitamin B12 and certain B12 analogues to cell suspensions. Experiments were conducted, therefore, to determine whether B12 compounds would restore the capacity of cobalt-deficient R. meliloti cells to oxidize propionate. In each instance, experiments of this type proved...
to be negative. In contrast, the addition of Blz coenzyme to extracts of cobalt-deficient R. meliloti restored activity of methylmalonyl-CoA mutase to a normal level (Table VI). It is apparent, therefore, that cobalt-deficient cells contained a normal level of the apoenzyme of methylmalonyl-CoA mutase and that the content of B12 coenzyme was the limiting factor. These findings are consistent with the report of Kliewer and Evans (10) showing that cobalt deficiency decreased the cobamide coenzyme content of Rhizobium.

The results presented (Tables II and IV) show that cells from pure cultures of R. meliloti, R. japonicum, and bacteroids from soybean nodules have the necessary enzymes to convert propionate to succinate via a pathway in which methylmalonate is an intermediate. Detailed studies of the properties of the propionyl-CoA carboxylase from these sources provided evidence (Table III) that the enzyme preferentially carboxylated propionyl-CoA. The enzyme from these organisms, therefore, exhibits properties similar to those of the propionyl-CoA carboxylase purified from beef liver (31) and pig heart (37). Propionyl-CoA carboxylase is widely distributed in bacteroids from nodules of leguminous species and in pure cultures of Rhizobium (Table IV), and it is suggested that the enzyme may play an important role in the metabolism of these organisms.

Methylmalonyl-CoA mutase activity was observed in a variety of nodules of legumes and in pure cultures of Rhizobium (Table IV). An examination of various fractions from soybean nodules and soybean roots indicated activity only in the nodule bacteroids. It is of interest to compare the properties of the methylmalonyl-CoA mutase in a crude extract from R. meliloti with those reported for the methylmalonyl-CoA from other sources. Lengyel, Mazumder, and Ochoa (13) made a comparative study of the properties of methylmalonyl-CoA mutase isolated from both sheep kidney and P. shermanii. They observed that activity was restored to the coenzyme-free enzyme from sheep kidney by the addition of the DBC and BC coenzymes but was inactive with the AC coenzyme. Unlike the enzyme from sheep kidney, the putative apoenzymes for the DBC and BC coenzymes but inactive with the AC no.

In contrast, the activity of the coenzyme-free enzyme from P. shermanii was restored by the addition of each of the three coenzymes. From our experiments with the coenzyme-free methylmalonyl-CoA mutase from R. meliloti, it is apparent that the greatest activation was obtained by the DBC coenzyme and less activation by the BC coenzyme (Table V). The R. meliloti enzyme, therefore, behaved like the enzyme from sheep kidney in that the AC coenzyme was inactive. The methylmalonyl-CoA mutase isolated from R. meliloti was similar to the enzyme from P. shermanii in that the endogenous Blz coenzyme of both was easily destroyed by light. In contrast, the enzyme from the sheep kidney could be resolved into a coenzyme-free apoenzyme only by acid precipitation of the enzyme from ammonium sulfate solution (13). Further characterization of the methylmalonyl-CoA mutase from Rhizobium must be delayed until a highly purified preparation is obtained from this source.

SUMMARY

An investigation was conducted to determine biochemical sites at which cobalt, in the form of Blz coenzyme, functions in the metabolism of legumes and their associated Rhizobium species. The results of these experiments may be summarized as follows.

1. Data are presented which indicate that propionate is oxidized by cell suspensions of bacteroids from soybean nodules and of Rhizobium japonicum and Rhizobium meliloti. The oxidation of propionate by cell suspensions of R. meliloti was enhanced by the addition of sodium propionate, succinate, and methylmalonate to the culture medium, whereas, the addition of these organic acids to the culture media of R. japonicum failed to produce this effect.

2. The rate of propionate oxidation by cell suspensions of R. meliloti grown with adequate cobalt was markedly greater than that of cells grown in a cobalt-deficient medium.

3. The radiorespirometric patterns obtained by incubation of propionate labeled in specific positions with cell suspensions of R. meliloti, R. japonicum, and soybean bacteroids are consistent with expected patterns if propionate was converted to succinate via methylmalonate and was oxidized by the citric acid cycle.

4. Cell-free extracts of R. meliloti, R. japonicum, and soybean bacteroids exhibit the capacity to catalyze (a) the activation of propionate and acetate, (b) the carboxylation of propionyl coenzyme A, and (c) the conversion of methylmalonate to succinate.

5. The methylmalonyl coenzyme A mutase in extracts of R. meliloti was easily inactivated by exposure of the extracts to direct light. Enzyme activity was restored by the addition of dimethylbenzimidazolylcobamide or benzimidazolylcobamide coenzyme to the extracts but the adenylcobamide coenzyme was ineffective in restoring activity.

6. Propionyl coenzyme A carboxylase activity in extracts of R. meliloti cells was not greatly affected by the concentration of cobalt in the medium in which cells were grown. In contrast, the activity of the methylmalonyl coenzyme A mutase in the extracts was strikingly influenced by the cobalt content of the culture medium. Methylmalonyl coenzyme A mutase activity, comparable to that obtained with extracts from cells grown with adequate cobalt, was obtained by the addition of dimethylbenzimidazolylcobamide coenzyme to enzyme extracts of cobalt-deficient cells or to extracts of cells grown with 0.01 p.p.b. of cobalt.

It is concluded, therefore, that cobalt deficiency in R. meliloti prevents the synthesis of quantities of Blz coenzyme adequate for the normal function of methylmalonyl coenzyme A mutase and that the inactive mutase results in the failure of the organism to oxidize propionate.

REFERENCES

The Relationship of Cobalt Requirement to Propionate Metabolism in *Rhizobium*

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