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Title ENZYMATIC AND RESPIRATORY STUDIES ON THE ROLE OF COBALT IN LEGUMES

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Manometric experiments with soybean bacteroids and pure cultures of Rhizobium japonicum and Rhizobium meliloti showed that these organisms oxidized fatty acids with chain lengths of two to six carbons. In addition, glutamate, succinate, methylmalonate, malonate, glucose, and pyruvate were also oxidized. It was especially interesting to find that propionate was utilized as a substrate, since cobalt in the form of vitamin B_{12} coenzyme has been shown to be directly involved in the metabolism of propionate in animal tissues and in Propionibacterium shermanii.

Radiorespirometric experiments provided conclusive evidence that Rhizobium cells did indeed oxidize propionate. The rates of recovery of $C^{14}O_2$ from propionate labeled in specific positions indicated that propionate was converted to succinate and that the succinate was subsequently oxidized via the citric acid cycle. Additional support for this conclusion was obtained from experiments in which propionate-1-, -2-, and -3-C^{14} were incubated with R. meliloti cells and radioactive methylmalonate,succinate, fumarate,

and malate were isolated and identified by paper chromatographic procedures.

From manometric and radiorespirometric experiments with *R. meliloti* cells grown with different concentrations of cobalt in culture media, it was shown that cobalt deficiency resulted in a reduced capacity of the cells to oxidize propionate. In contrast, comparable experiments showed that the oxidation of glutamate was not affected by changes in the concentration of cobalt in the growth medium.

Experiments with cell-free extracts of soybean bacteroids, *R. japonicum*, and *R. meliloti* have shown that these organisms contain a propionate or acetate activating enzyme, a propionyl-CoA carboxylase, and a methylmalonyl-CoA mutase. The carboxylase and mutase were found to be widely distributed in bacteroids from several legumes and in five pure culture species of *Rhizobium*.

The propionyl-CoA carboxylase from soybean bacteroids, *R. japonicum*, and *R. meliloti* preferentially carboxylated propionyl-CoA and required an ATP-Mg^{++} mixture for activity. Acetyl-CoA and butyryl-CoA were carboxylated but at greatly reduced rates.

The B_{12} coenzyme of *R. meliloti* was easily removed from the methylmalonyl-CoA mutase apoenzyme by exposure of the extracts to direct light. Activity was restored by the addition of the dimethylbenzimidazolylcobamide coenzyme and to a lesser degree by the addition of the benzimidazolylcobamide coenzyme. The adenylcobamide coenzyme was ineffective in restoring the activity.

Propionyl-CoA carboxylase in cell-free extracts of _R. meliloti_ was not appreciably affected by the concentration of cobalt in the medium used to culture the organisms. On the other hand, methylmalonyl-CoA mutase activity in the extracts was strikingly influenced by the cobalt concentration in the culture medium. Extracts of cobalt deficient cells exhibited extremely low levels of mutase activity. Enzyme activity comparable to that obtained with extracts of cells grown with adequate cobalt was obtained by the addition of the dimethylbenzimidazolylcobamide coenzyme to extracts of cobalt deficient cells or to extracts of cells grown with 0.01 ppb cobalt.

No direct evidence has been presented to indicate the metabolic role or roles of the propionate to succinate pathway in the general metabolism of legume nodules or _Rhizobium_ species grown in pure culture.

ENZYMATIC AND RESPIRATORY STUDIES ON THE ROLE OF COBALT IN LEGUMES

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ENZYMATIC AND RESPIRATORY STUDIES ON THE ROLE OF COBALT IN LEGUMES

INTRODUCTION

Biological nitrogen fixation is considered to be a fundamental process in the continuation of life. The air we breathe is a mixture of gases of which approximately 78 percent by volume is nitrogen. It has been estimated (31, p. 3) that approximately 35,000 tons of nitrogen gas is above every acre of the Earth's surface. Nitrogen in this form is useless to both plants and animals, yet combined forms of the element are essential for these organisms. Although some natural phenomena occur, e.g. lightning, which transforms nitrogen gas into usable compounds, the contribution of these processes is considered to be small (97, p. 540). Combined forms of nitrogen are also produced by the chemical industry, but the total contribution of fixed nitrogen by this industry is relatively small (97, p. 540-541).

Virtanen and Miettinen (97, p. 539-542) state that the primary source of combined nitrogen is that which is produced by the organisms capable of fixing atmospheric nitrogen. They feel too that the contribution to the total amount of fixed nitrogen by the free-living nitrogen fixing organisms is relatively small and that the legumes are the principal organisms providing fixed nitrogen compounds. They contend, however, that the contribution of the blue-green algae may be greater than is presently acknowledged and that this area of

research needs to be throughly investigated. It has been stated (31, p. 1) that more than 10,000 species of legumes are known and that a well inoculated field of any of these species may fix 50 to 100 pounds of nitrogen per acre per year. It becomes clear that, a basic knowledge is needed of the metabolism and nutrition of the legume plants and of the bacteria that grow in a symbiotic association within the nodules of the legumes in order to understand the basic mechanism by which legumes fix atmospheric nitrogen.

It has been established that legumes, when grown under conditions in which they are forced to fix nitrogen, require cobalt (3, 27, 44). Since Ahmed and Evans (3) observed no response to cobalt when soybean plants were supplied with adequate quantities of fixed nitrogen, it was implied that the bacteria within the nodules required the added cobalt and not the legumes _per se_. Lowe _et al_. (67) and Lowe and Evans (65) have subsequently demonstrated a cobalt requirement for five species of _Rhizobium_. This was followed by the identification of B_{12} coenzyme in several _Rhizobium_ species and in the bacteroids from the nodules of several legumes (56, 57). This finding suggests that cobalt is functional in the form of B_{12} coenzyme and not as inorganic cobalt.

The primary objective of this investigation was to identify biochemical sites where cobalt in the form of B_{12} coenzyme functions in the metabolism of both legumes and their associated _Rhizobium_ species and to characterize the enzyme components of the sites identified.

REVIEW OF LITERATURE

Cobalt Nutrition

It is generally agreed by most reviewers (35, p. 109; 47, p. 322-323; 74, p. 504) that, under our present criteria, cobalt is not essential for the growth of higher plants *per se*. There are, however, isolated reports on attempts to demonstrate a cobalt requirement for certain higher plants. Bolle-Jones and Mallikarjuneswara (17, 18) have studied the effects of different cobalt concentrations on the growth of rubber (*Hevea brasiliensis*) and tomato (*Lycopersicon esculentum*) and found no deficiency symptoms that could be attributed to a lack of the element. The rubber plants grown without added cobalt, however, showed a reduction in height, stem girth, and dry weight of the roots when compared with plants grown with cobalt. Tomato plants grown without added cobalt showed no significant reduction in the dry weight of the shoots or roots, but the fresh weight of fruit was reduced some 50 percent. Miller (73) and Thimann (91) have reported that the addition of cobalt to a medium containing sucrose and indoleacetic acid (IAA) increased elongation of pea stem fragments, but this type of experiment does not establish cobalt essentiality.

Either cobalt or vitamin B_{12} has been reported to be essential for the growth of some lower plants. Hutner *et al*. (49, p. 152-154) have shown that the algal flagellate *Euglena gracilis* requires an external source of vitamin B_{12}. They showed that the organism

required only minute traces of the vitamin and Euglena gracilis has since been utilized to assay trace amounts of the vitamin from other organisms. Holm-Hansen et al. (48) demonstrated that four species of blue-green algae required inorganic cobalt for growth. Their results also showed that Nostoc muscorum and Calothrix parientina, both of which fix atmospheric nitrogen, required cobalt for growth even in the presence of fixed nitrogen. The authors proposed that cobalt was not directly involved in the nitrogen fixation process.

More recently, there have been findings in several independent laboratories thatcobalt is essential for symbiotic nitrogen fixation by legumes. Ahmed and Evans (1, 2, 3) established a requirement of cobalt for soybeans grown under conditions in which they were forced to fix atmospheric nitrogen. A concentration of approximately 0.1 parts per billion (ppb) cobalt was required for optimum growth. The addition of adequate quantities of cobalt to the purified nutrient solution used for culturing the plants resulted in a striking increase in the chlorophyll content of the leaves and in the vitamin B_{12} and hemoglobin content of the nodules. The addition of twelve elements not considered to be essential for higher plants failed to replace the cobalt requirement, but the addition of nitrogen compounds to the nutrient solutions completely replaced the cobalt requirement. The authors concluded that the cobalt probably was required by the root nodule bacteria and not by the soybean plants per se. Reisenauer (80) and Delwiche et al. (27) obtained similar results with alfalfa (Medicago sativa). Their data showed that the

addition of 0.1 μ moles of $CoSO_4$ prevented nitrogen deficiency symptoms and resulted in increased total nitrogen and fresh weight of the stems and leaves. Using isotopic nitrogen (N^{15}), they also demonstrated that nodules from alfalfa plants grown with added cobalt possessed a markedly greater nitrogen fixing capacity than those from plants grown without cobalt. Hallsworth *et al*. (44) have reported that the addition of cobalt to cultures of subterranean clover resulted in an increase in: (a) the size of the nodules, (b) a higher content of hemoglobin in the nodules, and (c) the number of leaves on the plants. Powri (79) reported that the addition of cobalt sulphate at the rate of eight ounces per acre to fields, known to be deficient in many micronutrients, increased the yield and total nitrogen content of subterranean clover.

Lowe *et al*. (67) and Lowe and Evans (65) demonstrated that 0.1 ppb cobalt was essential for maximum growth of five species of *Rhizobium*. Subsequently, Nicholas *et al*. (76) confirmed the report that *Rhizobium japonicum* required cobalt and in a later publication (75) presented evidence that *Azotobacter vinelandii* OP also required cobalt for maximum growth. During the investigations on the cobalt requirements of symbiotically grown legumes and of *Rhizobium* species in pure culture, observations were made that led Lowe and Evans (66) to demonstrate that carbon dioxide is also indispensable for the growth of *Rhizobium*. This finding is very significant since, as will be discussed in a later section of this dissertation, the oxidation of propionate requires the fixation of one molecule of carbon

dioxide.

The presence of vitamin B_{12} in legume nodules and pure cultures of Rhizobium has been known for some time (21, 64), but it was not until recently that the cobamide coenzymes, which Barker et al. (10, 11) isolated from Clostridium tetanomorphum and Propionibacterium shermanii, were detected in nitrogen fixing organisms. Kliewer and Evans (54, 55, 56, 57) analyzed nodules from a variety of symbionts and cells from several pure cultures of Rhizobium and demonstrated the presence of B_{12} coenzyme in each of the organisms analyzed. They also showed that the B_{12} coenzyme content of Rhizobium meliloti and Rhizobium japonicum was strikingly increased by increasing the cobalt content of the culture medium. The B_{12} coenzyme content of a variety of pure culture strains of R. meliloti and R. japonicum was determined (57), and it was found that parasitic and ineffective strains (in respect to ability to fix nitrogen symbiotically) contained considerably less coenzyme than the effective strains. In addition, B_{12} coenzyme was detected in extracts from the nodules of two nonleguminous nitrogen fixers, Alnus oregona and Ceanothus velutinus (56). This discovery may have provided the necessary lead for Bond and Hewitt (19) to demonstrate a cobalt requirement for Alnus and Casaurina, when the organisms were cultured under conditions where nitrogen fixation was obligatory. Further purification (56) of the cobamide coenzyme from pure culture R. meliloti and from bacteroids from soybean nodules showed it to be the dimethylbenzimidazolylcobamide

(DBC)* coenzyme. This was established by determining the activity of the coenzyme in the glutamate isomerase reaction of *C. tetanomorphum* and by its spectral and chromatographic properties.

Nicholas *et al.* (76) have attempted to demonstrate a role for cobalt in *R. japonicum*. They showed that nitrate reductase activity was markedly reduced in cobalt deficient cells of *R. japonicum*, but concluded that cobalt was not required for enzyme activity and that the effect was indirect. In a later report, Nicholas *et al.* (75) showed a correlation between the localization of administered hydroxycobalamin-Co^{58} and nitrate reductase activity on ribosomal subparticles from *A. vinelandii*. From this evidence they suggested that cobalt in the form of a B_{12} derivative is needed for the adaptive formation of the enzyme by the organism.

Propionate Metabolism

Because of the recent findings (63, 86, 87) that B_{12} coenzyme is directly involved in propionate metabolism in animals and microorganisms, it seemed logical to investigate this area in attempting to identify a role for cobalt in legumes. No evidence was found in the literature indicating the presence or absence of a pathway for the metabolism of propionate in nodules of legumes or in pure cultures of *Rhizobium*.

* The abbreviations used for the vitamin B_{12} coenzymes in this dissertation are: dimethylbenzimidazolylcobamide, DBC; benzimidazolylcobamide, BC; adenylcobamide, AC.

Formation of Propionate. Our present knowledge of the formation of propionate in living organisms is quite limited. Propionate is known to be a product of the catabolism of certain amino acids and to be an end product of the fermentation of carbohydrate materials by specific bacteria.

The catabolism of valine by rat liver has been shown by Kinnory *et al*. (53) to lead to the formation of one molecule of propionate and two molecules of carbon dioxide. Using DL-valine-4, 4'-C^{14} they isolated and identified radioactive α-ketobutyrate, isobutyrate, β-hydroxyisobutyrate, and propionate. Their data also showed that carbons 3, 4, and 4' of valine became the three carbon atoms of propionate and that the conversion of isobutyrate proceeds by way of β-hydroxyisobutyrate. The incubation of DL-valine-2-C^{14} resulted in little radioactivity being incorporated into propionate, but the specific activity of the CO_2 which was recovered was high. In contrast, when DL-valine-4, 4'-C^{14} was used the specific activity of the CO_2 recovered was low. Atchley (7) using a fatty acid oxidizing system from rabbit kidney showed that isobutyrate was converted to propionate. This evidence, together with the early work of Rose *el al*. (83) who showed that valine and α-ketoisovalerate contributed three of their five carbons to the formation of glucose, led Kinnory *et al*. (53) to propose a pathway for the catabolism of valine. In this proposed sequence of reactions, oxidation of valine is initiated by transamination, resulting in the formation of α-ketoisovalerate. The decarboxylation and oxidation of α-ketoisovalerate

gives rise to isobutyrate. The isobutyrate subsequently undergoes β -oxidation to form β -hydroxybutyrate which, by decarboxylation and oxidation, is converted to propionate. There is evidence that this pathway exists in rumen bacteria since Dehority *et al.* (26), using an *in vitro* rumen fermentation procedure, found that up to 43 percent of the label from DL-valine-1-C^{14} could be recovered as $C^{14}O_2$. In addition, they isolated and identified α -ketoisovalerate-1-C^{14} from the fermentation extracts. Enzymatic evidence for parts of this pathway has been presented by Robinson *et al.* (82) who demonstrated that isobutryl-CoA undergoes a dehydrogenation to methylacryl-CoA and a subsequent hydrogenation to form β -hydroxy-isobutyryl-CoA. Evidence also was presented showing that the β -hydroxyisobutyryl-CoA is converted to methylmalonic semialdehyde by an enzyme extract.

Isoleucine is another amino acid from which propionate can be formed. Greenberg (39, p. 106) has proposed a scheme in which the end products of the catabolic pathway are acetyl-CoA and propionyl-CoA. The proposed pathway has been derived primarily from results obtained by using rat liver preparations and 2-methylbutyrate (24, 25) labeled in specific positions. This work has been supported by the isolation of some of the enzymes in the pathway (81). Coon and Abrahamsen (24) initially showed that the carboxyl carbon of 2-methylbutyrate-1-C^{14} did not contribute to the formation of aceto-acetate which is formed by the condensation of two molecules of acetyl-CoA. The CO_2 recovered, however, was radioactive. They

found that carbons 3 and 4 of the 2-methylbutyrate were converted into acetoacetate but that the methyl group of the compound was not. It was proposed that 2-methylbutyrate undergoes β -oxidation on the longer carbon chain of the molecule and that cleavage occurs between carbons 2 and 3 forming one molecule of acetate and one molecule of propionate.

Two pathways have been proposed for the catabolism of threonine (39, p. 94). In one pathway propionate may be formed by the oxidation of α -ketobutyrate, which is a proposed intermediate. In the alternate pathway threonine is cleaved between carbons two and three to form acetaldehyde and glycine. It has been reported also (69) that propionate may be formed by the catabolism of homoserine. In this pathway α -ketobutyrate is again an intermediate of the pathway.

It has long been known that propionate is produced by bacterial fermentations of lactate and carbohydrates (28, 50). The intermediates in this conversion were suggested when Swick and Wood (90) provided evidence for the following set of reactions in *P. shermanii*:

(1)	Succinyl-CoA	$\leftrightarrow$	Methylmalonyl-CoA
(2)	E^* + Methylmalonyl-CoA	$\leftrightarrow$	Propionyl-CoA + E^*-C_1
(3)	E^*-C_1 + Pyruvate	$\leftrightarrow$	Oxaloacetic + E^*
(4)	Oxaloacetate + 4 H^+	$\leftrightarrow$	Succinate
(5)	Succinate + Propionyl-CoA	$\leftrightarrow$	Succinyl-CoA + Propionate

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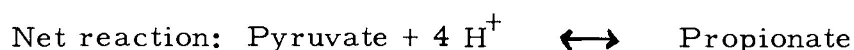


Net reaction: Pyruvate + 4 H^+ $\leftrightarrow$ Propionate

The enzymes which catalyze this series of reactions are: reaction 1, methylmalonyl-CoA mutase*; reactions 2 and 3, methylmalonyl-oxaloacetic transcarboxylase; and reaction 5, propionyl-CoA transferase. Oxaloacetate is reduced to succinate by the citric acid cycle enzymes. Allen *et al.* (4) and Overath *et al.* (77) have modified this scheme with the findings that the enzyme methylmalonyl-CoA racemase, which catalyzes the conversion of one isomer of methylmalonate to another, is present in *P. shermanii*.

Wood and his coworkers (102, 104) have isolated and partially purified the transcarboxylase from *P. shermanii*. The reaction was found to be readily reversible and the equilibrium constant at pH 6.5 and 30°C was 1.9. The enzyme exhibited a broad specificity for the acyl-CoA component. With oxaloacetic acid as the carboxyl donor propionyl-CoA, acetyl-CoA, and acetoacetyl coenzyme A

* This enzyme has previously been referred to in the literature as methylmalonyl isomerase. The enzyme will be referred to in this dissertation by the currently accepted name, methylmalonyl-CoA mutase.

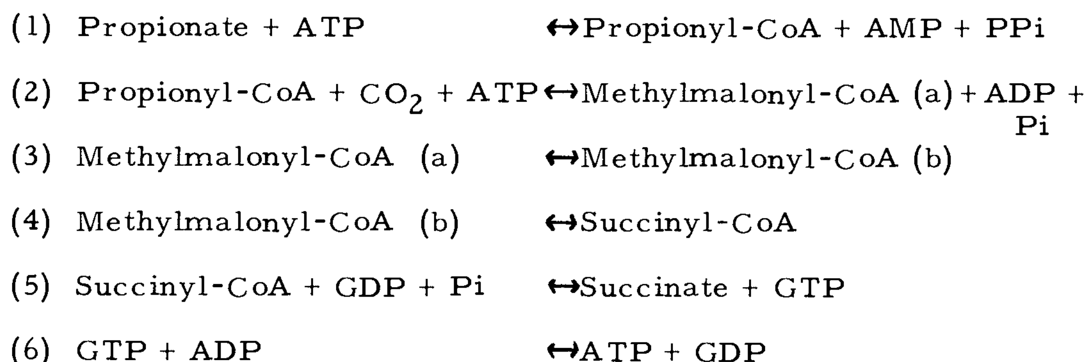
served as the carboxyl acceptor. The authors (102) suggested that the enzyme may possess a broad potential for carboxylation reactions other than the methylmalonyl-oxaloacetic transcarboxylation reaction. When pyruvate was used as the carboxyl acceptor, methylmalonyl-CoA and to a lesser extent malonyl-CoA served as the carboxyl donor. The transcarboxylase like propionyl-CoA carboxylase was shown to contain biotin (102), but no formation of methylmalonyl-CoA could be detected when the enzyme was incubated with ATP, Mg^{++}, HCO_3^-, and propionyl-CoA. The mechanism by which the carboxyl group is transfered has been established recently by Wood et al. (103) who presented evidence showing that the active form of the enzyme-bound biotin was N-carboxybiotin. Their results are consistent, therefore, with those previously reported by Lynen et al. (68) for β -methyl-crotonyl-CoA carboxylase. In a further investigation on the mechanism of the transcarboxylase, Allen et al. (5) presented evidence that showed that the ureido carbon of biotin does not function in the transfer of the carboxyl group. This evidence and the report of Lane and Lynen (61) that N-carboxybiotin is the active form of propionyl-CoA carboxylase casts much doubt on the validity of the work of Waite and Wakil (99) and Wakil and Waite (100) who maintain that the ureido carbon of biotin participates in the carboxylation of acetyl-CoA by an enzyme prepared from chicken liver.

Since methylmalonyl-CoA mutase and methylmalonyl-CoA racemase were first identified in animal tissues and later were

found to occur in extracts of P. shermanii, the properties of these enzymes from P. shermanii will be discussed in a later section of this dissertation.

Existing evidence supports the conclusions that propionate may be formed by the catabolism of certain amino acids, such as valine and isoleucine. In addition, propionate has been shown to be an end product of fermentation of certain microorganisms.

Propionate Oxidation. It has been shown (13, 33, 71) that the main oxidative pathway of propionate in animal tissues proceeds as shown in the following set of reactions:



(1) Propionate + ATP ↔ Propionyl-CoA + AMP + PPi

(2) Propionyl-CoA + CO_2 + ATP ↔ Methylmalonyl-CoA (a) + ADP + Pi

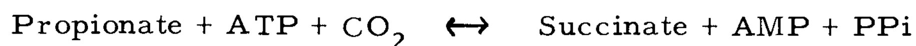
(3) Methylmalonyl-CoA (a) ↔ Methylmalonyl-CoA (b)

(4) Methylmalonyl-CoA (b) ↔ Succinyl-CoA

(5) Succinyl-CoA + GDP + Pi ↔ Succinate + GTP

(6) GTP + ADP ↔ ATP + GDP

Net reaction:



Propionate + ATP + CO_2 ↔ Succinate + AMP + PPi

The enzymes catalyzing this series of reactions are: reaction 1, acetyl-CoA kinase; reaction 2, propionyl-CoA carboxylase; reaction 3, methylmalonyl-CoA racemase; reaction 4, methylmalonyl-CoA mutase; reaction 5, succinyl thiokinase; and reaction 6,

nucleoside-diphosphate kinase. Succinate once formed can be oxidized by the citric acid cycle or the intermediates of the cycle can be converted into various other compounds.

Propionyl-CoA carboxylase has been studied intensively both by Ochoa and his coworkers (32, 51, 52, 93) and by Lane and his coworkers (41, 42, 43, 60) and has been crystallized from pig heart (52) and beef liver (59). The substrate specificity of the enzyme prepared from the two sources has been shown to be similar. Of the three substrates tested, propionyl-CoA was preferentially carboxylated (41, p. 880; 52, p. 1922) followed in turn by butyryl-CoA and acetyl-CoA. Propionyl-CoA, ATP, Mg^{++}, and HCO_3^- are required for maximum enzyme activity (41, p. 880; 93, p. 1397). Like other carboxylases propionyl-CoA carboxylase contains biotin as a prosthetic group (51, 41). The crystalline pig heart enzyme has been shown to contain four moles of biotin per mole of protein (52). The formation of an enzyme-biotin-CO_2 complex by the system was first suggested by Kaziro _et al._ (51), however, direct evidence for the presence of the complex was provided by Friedman and Stern (34) and Halenz and Lane (42, 43). These authors showed that an exchange takes place between 1-C^{14} propionyl-CoA and methylmalonyl-CoA in the absence of Mg^{++}, ADP, and inorganic phosphate. The exchange was inhibited by avidin and the inhibition could be reversed by the addition of D-biotin (34) to the reaction mixture.

Methylmalonyl-CoA mutase was discovered by Ochoa and coworkers (13, 14, 33) in animal tissues. Swick and Wood (90) later

presented evidence for the presence of the enzyme in cell-free extracts of *P. shermanii*. The bacterial enzyme has been purified some 200-fold (88) and at this stage of purification was found to be free of lactic dehydrogenase, transcarboxylase, malic dehydrogenase, CoA deacylase, and propionyl-CoA transferase. The equilibrium constant (succinyl-CoA/methylmalonyl-CoA) at pH 7.0 and $25^{\circ}C$ was 10.5. Mazumder *et al.* (72) subsequently purified the mutase from sheep liver some 5000-fold.

The involvement of vitamin B_{12} in the conversion of methylmalonyl-CoA to succinyl-CoA was first implicated by the work of Smith and Monty (85), who found that extracts prepared from livers taken from vitamin B_{12} deficient rats were low in methylmalonyl-CoA mutase activity. After this, Gurnani *et al.* (40), who also used extracts of livers taken from vitamin B_{12} deficient rats, demonstrated that the addition of DBC coenzyme to the reaction mixtures strikingly increased enzyme activity. Stadtman *et al.* (86) resolved the apoenzyme from the coenzyme in extracts of *P. shermanii* by means of charcoal extraction and showed that DBC coenzyme would restore enzyme activity. This was followed by a report (87) that the DBC coenzyme stimulated the activity of the mutase in an extract prepared from liver taken from a normal ox. Lengyel *et al.* (63) have subsequently carried out a detailed study of the properties of the mutase in extracts of both sheep kidney and *P. shermanii*. This investigation revealed that the DBC and BC coenzymes functioned with the sheep kidney enzyme and that the adenyl derivative was

inactive. In contrast, the apoenzyme in extracts of P. shermanii was activated by all three coenzymes. They also found that in the sheep kidney preparations the coenzyme was tightly bound to the apoenzyme. This was not the case with the mutase from P. shermanii. In the sheep kidney extracts, the coenzyme was removed from the apoenzyme only by acid precipitation from ammonium sulphate solutions.

A partial explanation of the mechanism by which the conversion of methylmalonyl-CoA to succinyl-CoA takes place has been furnished by Eggerer et al. (29. 30). Using an enzyme extract of P. shermanii, they found that approximately 80 percent of the label from 2-C^{14}-methylmalonyl-CoA was found in carbon 3 of the reaction product, succinyl-CoA. Swick (89) using purified enzyme preparations from P. shermanii and sheep kidney found that methyl-labeled methylmalonyl-CoA was converted to 2-C^{14}-succinyl-CoA. Hegre et al. (46) obtained similar results using a partially purified enzyme preparation from beef liver. These investigators have individually proposed that the thioester carboxyl of propionyl-CoA and the free carboxyl group (from CO_2) of methylmalonyl-CoA become the thiolester carboxyl and the free carboxyl of succinyl-CoA respectively. It remains to be seen whether this is an intermolecular or intramolecular exchange.

Recently, methylmalonyl-CoA mutase was found to contain a second enzyme which has been named methylmalonyl-CoA racemase (70, 71). The enzyme has subsequently been identified in

extracts of *P. shermanii* by Allen *et al.* (4) and Overath *et al.* (77). Mazumder *et al.* (71) showed that the racemase did not catalyze an exchange between methyl-labeled methylmalonate-C^{14} and methylmalonyl-CoA. This eliminates the possibility that the racemation occurs by an exchange of the thiolester (coenzyme A) between two molecules of methylmalonate. Experiments, in which methylmalonyl-CoA was incubated in tritium-enriched water at 30°C in the presence of the racemase, showed that a measurable amount of tritium was incorporated into methylmalonyl-CoA. A brief heating of the methylmalonyl-CoA at 100°C with the tritium-labeled water produced identical results. The authors have proposed that the racemation takes place by a mechanism which involves the loss of the proton from the tertiary carbon of methylmalonyl-CoA and a subsequent incorporation of a proton from the medium to form the other isomer of the compound. Overath *et al.* (77) using the racemase of *P. shermanii* have reported similar results and proposed the same mechanism of action.

In contrast to the above scheme of propionate oxidation, Giovanelli and Stumpf (36, 37) and Hatch and Stumpf (45) have shown that various plant tissues will oxidize propionate by yet another pathway. This pathway is outlined as follows:

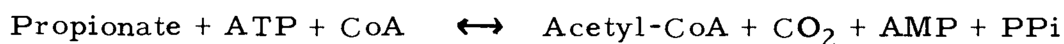
(1) Propionate + ATP + CoA ⟷ Propionyl-CoA + AMP + PPi

(2) Propionyl-CoA ⟷ β-Hydroxypropionate + CoA

(3) β-Hydroxypropionate ⟷ Malonic Semialdehyde

(4) Malonic Semialdehyde + CoA ⟷ Malonyl-CoA

(5) Malonyl-CoA ⟷ Acetyl-CoA + CO_2



Net reaction:

Propionate + ATP + CoA ⟷ Acetyl-CoA + CO_2 + AMP + PPi

The pathway has been demonstrated in peanut mitochondria; the roots and epicotyl of wheat; the cotyledons, stems, and roots of safflower; and the leaves, stem, cotyledons, and roots of peas. One of the interesting points of this pathway is that β-alanine, which is a consitiuent of coenzyme A, can be formed by the reductive amination of β-hydroxypropionate. By administering propionate labeled in specific positions to these tissues, Stumpf and his coworkers (36, 37, 45) have isolated and identified β-hydroxypropionate and β-alanine and found them to be radioactive. When the tissues were administered propionate-1-C^{14}, the specific activity of respired CO_2 was high and the citric acid cycle intermediates were not extensively labeled. When propionate-2-C^{14} and propionate-3-C^{14} were administered the citric cycle acids, as well as β-hydroxypropionate and β-alanine, were found to be extensively labeled. The recovery of the label as $C^{14}O_2$ was lower for each substrate than that recovered when propionate-1-C^{14} was utilized by the tissues. From these results, they concluded that propionate

was oxidized to malonyl-CoA via a series of reactions involving β - hydroxypropionate as one of the intermediates. The carboxyl carbon of propionate is then recovered as CO_2 by the decarboxylation of the malonyl-CoA. The methyl carbon of propionate becomes the carboxyl carbon of acetate and the methylene carbon of propionate becomes the methyl carbon of acetate.

Vagelos and Earl (96) have reported a similar pathway for the oxidation of propionate in Clostridium kluyverii. They found, however, that thiolester remains attached to the carboxyl carbon of propionate and that it is not hydrolyzed as is the case in plant tissues. Thus, the carboxyl carbon of propionate becomes the carboxyl carbon of acetate. The methyl carbon of propionate is oxidized and subsequently released as CO_2 by decarboxylation of the malonyl-CoA which is formed. Carbon 2 of propionate again becomes the methyl carbon of acetate.

There are also reports in the literature that the protozoan Ochromonas malhamensis (6) and an unidentified species of Flavobacterium (8, 9) will oxidize propionate to succinate via methylmalonate and that the pathway is dependent on vitamin B_{12}.

MATERIALS AND METHODS

Biological Materials

The biological materials utilized in this investigation were: soybeans (*Glycine max* Merr. variety Chippewa), peas (*Pisum sativum* L. variety American Wonder), lupines (*Lupinus angustifolius* L. variety Borre Blue), cowpeas (*Vigna sinensis* Savi, variety Iron Clay), *Rhizobium meliloti* (F-29), *Rhizobium japonicum* (61A76), *Rhizobium phaseoli* (K-11), *Rhizobium leguminosarum* (C-56), and *Rhizobium trifolii* (P-28). The five *Rhizobium* species were a generous gift from Dr. Joe C. Burton, Nitragin Company, Milwaukee, Wisconsin.

Cultural Procedures

Seedlings of the various legumes, inoculated with the appropriate *Rhizobium* species, were grown in a greenhouse using the cultural method described by Ahmed and Evans (2, p. 205; 3, p. 25-26). Briefly the method consists of: (a) growing the seedlings in two gallon vessels filled with sterilized "Perlite", (b) supplying the vessels every other day with about 500 ml of a nitrogen-free nutrient solution, and (c) flushing the vessels with water on those days when the nutrient solution was not applied. The nodules were harvested at the times considered appropriate for the different species.

R. japonicum, *R. phaseoli*, *R. leguminosarum*, and *R. trifolii* were cultured in a medium containing the following constituents per liter: K_2HPO_4, 1.00 g; KH_2PO_4, 1.00 g; $MgSO_4 \cdot 7H_2O$, 0.36 g; $CaSO_4 \cdot 2H_2O$, 0.13 g; KNO_3, 0.70 g; $FeCl_3 \cdot 6H_2O$, 4.00 mg; yeast extract, (Difco), 1.00 g; L-arabinose, 1.00 g; and 7.00 ml of glycerol at a final pH of 6.3. This medium is designated medium I. *R. meliloti* was cultured on a medium containing the following constituents per liter: K_2HPO_4, 1.00 g; KH_2PO_4, 1.00 g; $MgSO_4 \cdot 7H_2O$, 0.36 g; $CaSO_4 \cdot 2H_2O$, 0.13 g; mannitol, 3.00 g; yeast extract, (Difco), 1.00 g; $FeCl_2 \cdot 6H_2O$, 4.00 mg; and 0.06 mg of $CoCl_2 \cdot 6H_2O$ at a final pH of 6.3 and is designated as medium II. The two media were modified for some experiments by the addition of 100 mg per liter each of: succinic acid, methylmalonic acid, and sodium propionate. Media I and II to which these acids were added are designated media III and IV respectively. The basic purified medium used for experiments in which *R. meliloti* was grown with varying concentrations of cobalt is presented in Table 1. The methods used for cleaning glassware and for purifying and preparing nutrient stock solutions have been described earlier (2, p. 205-206; 3, p. 25; 67, p. 676).

The cells were cultured on a rotary shaker at 30°C 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 three to four days; and *R. meliloti*, grown in the purified medium, was harvested 36 to 48

Table 1. Basic Purified Culture Medium for *Rhizobium meliloti*

Nutrient and concentration of stock solutions*	Stock solution in medium		Final concentration
Macroelements	Molarity	ml/liter	Molarity
K_2PO_4	1.0	6.00	0.006
KH_2PO_4	1.0	6.00	0.006
$MgSO_4 \cdot 7H_2O$	1.0	1.15	0.0015
KNO_3	2.0	2.00	0.004
$Ca(NO_3)_2$	1.0	0.25	0.00025
$(NH_4)_2SO_4$	2.0	0.50	0.001
Carbon sources			
Mannitol	0.82	20.00	0.0165
Sodium propionate	0.0417	25.00***	0.00104
Succinic acid	0.0339		0.00093
Methylmalonic acid	0.0339		0.00093
Microelements	ppm	ml/liter	ppm
Micronutrient mixture**		0.25	
$MnSO_4$	300		0.075
H_3BO_3	200		0.050
$ZnSO_4$	100		0.025
Na_2MoO_4	40		0.010
$CuSO_4$	20		0.005
Ferric citrate	3400	0.14	0.850
Vitamins			
Riboflavin	200	0.10	0.02
p-Aminobenzoic acid	200	0.10	0.02
Nicotinic acid	200	0.10	0.02
Biotin	200	0.10	0.02
Thiamin hydrochloride	200	0.10	0.02
Pyridoxine hydrochloride	200	0.10	0.02
Calcium panthothenate	200	0.10	0.02
Inositol	1200	0.10	0.12

* Cobalt was added as required from a stock solution of $CoCl_2$.
** All microelements except ferric citrate were combined in a mixed stock solution and were added to the medium as a single solution.
*** Propionate, succinate, and methylmalonate were combined in a mixed stock solution and were added to the medium as a single solution.

hours after inoculation.

Preparation of Cell Suspensions and Extracts

All operations, unless otherwise indicated, were carried out at 0-4°C.

The isolation of the bacteroids from legume nodules was carried out by a procedure essentially the same as that described by Cheniae and Evans (21, p. 141-142). The method consisted of: (a) washing the freshly harvested nodules thoroughly with ice-cold distilled water, (b) crushing the washed nodules in a mortar with two volumes of buffer [0.067 M potassium phosphate buffer pH 6.8 for manometric and radiorespirometric experiments and 0.01 M tris (hydroxymethyl)aminomethane (Tris) buffer pH 8.5 for enzyme experiments], (c) squeezing the homogenate through four layers of cheesecloth, and (d) isolating the bacteroids by centrifugation procedures.

Cells were harvested from pure culture media by centrifugation at 11,000 x g for ten minutes. Cell suspensions for the manometric and respirometric studies were prepared by washing and suspending the cells in 0.067 M potassium phosphate buffer at pH 6.8. Cells grown in the purified medium were harvested in acid-washing centrifuge tubes and purified 0.067 M potassium phosphate buffer at pH 6.8 was used. Aliquots of the cell suspensions were retained and the nitrogen content determined as reported under "Quantitative Determinations".

Cell-free extracts for enzyme experiments were obtained by grinding the cells for ten minutes under dim light with three volumes of 0.01 M Tris buffer [0.001 M with respect to reduced glutathione (GSH)] at pH 8.5 and three weights of levigated alumina. The exposure of the extracts to direct light was avoided to prevent the destruction of enzyme-bound B_{12} coenzyme. After grinding, the alumina and cell debris were removed by centrifugation at 34,000 x g for 20 minutes. The resulting supernatant was used for all enzyme assays.

Purification of Propionyl-CoA Carboxylase from Beef Liver

A detailed description of the purification and crystallization of propionyl-CoA carboxylase from beef liver has been reported by Lane and Halenz (59). This procedure was followed up to the first ammonium sulphate fractionation step. At this point, the carboxylase has a specific activity of 20-25 units per mg of protein and was found to be essentially free of methylmalonyl-CoA mutase. Briefly, the procedure was as follows: (a) fresh beef liver was homogenized in a Waring Blendor with ice-cold 0.25 M sucrose, (b) the resulting solution was centrifuged at a low speed (1900 x g) to remove the debris, (c) the mitochondria were collected by high speed (34,000 x g) centrifugation, (d) a mitochondrial acetone powder was prepared, and (e) an extract of the acetone powder was aged for 20 hours at $30^{o}C$ and then fractionated with saturated ammonium sulphate. The purified enzyme was resuspended in 0.0025 M Tris buffer at pH 7.2

and stored at $-15^{o}C$. Before freezing the enzyme solution was divided into small aliquots sufficient for the assays to be preformed each day.

Manometric Procedures

Oxygen uptake was measured by the conventional manometric techniques (95, p. 1-17). The final volume in each flask was 3.2 ml. This consisted of 2.5 ml of buffered cell suspension in the main compartment, 0.5 ml of buffered substrate in the side arm, and 0.2 ml of 20 percent KOH in the center well. The final concentration of buffer at pH 6.8 was 0.067 M and of each substrate was 0.05 M, except propionate which was 0.017 M. The temperature was maintained at $30^{o}C$. Each experiment was carried out in duplicate and the results reported are average values.

Radiorespirometric Procedures

The procedure utilized for the oxidation of specifically labeled C^{14}-propionate has been described by Wang (101). The contents of each flask in a final volume of 11 ml were made up of ten ml of cell suspension in 0.067 M potassium phosphate buffer pH 6.8 and one ml of the C^{14}-propionate (1.0 μ C in 3.5 μ moles) at pH 6.8. When suspensions of *R. meliloti* which had been grown with varying concentrations of cobalt were used, the flasks contained one-half the quantity of the components listed. The flasks were shaken continuously in a controlled temperature bath at $30^{o}C$. The air-flow

was maintained at 60 ml per minute and the $C^{14}O_2$ was trapped in ten ml of a mixture composed of two parts ethanol and one part 2-aminoethanol. Samples were collected at 20 minute intervals. To determine the amount of $C^{14}O_2$ recovered, the trapping solution was diluted to 15 ml and a five ml aliquot counted with ten ml of phosphor solution in a Packard Tri-Carb (model 314) liquid scintillation counter at 1330 volts. The phosphor solution consisted of 0.3 percent 2,5-diphenyloxazole and 0.003 percent 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in toluene.

Paper Chromatography Procedures

To provide additional information on the oxidative pathway of propionate, the organic acids were recovered from _R. meliloti_ cells which had been incubated with specifically labeled propionate. The cell suspensions, prepared from _R. meliloti_ cultures grown on medium IV, were incubated with the substrates for ten minutes at 30°C. The suspensions were subsequently poured into 90 ml of boiling 95 percent ethanol. The solution was boiled for five minutes, cooled, and the cell debris removed by centrifugation. The ethanol was removed by heating the solution at 75°C and at the same time maintaining a constant air-stream over the solution. The resulting aqueous solution was passed through a Dowex-50 column (50-100 mesh H^+ form, 1.5 x 6 cm) and the column washed with four bed volumes of distilled water. The volume of the effluent was reduced to 0.5 ml and 100 μl spotted on Whatman No. 1 paper.

To isolate the products of the various reaction mixtures, an aliquot of the reaction mixture was extracted overnight with diethyl-ether (33, p. 968). The ether was dried for five hours by the addition of anhydrous sodium sulphate. The solution was evaporated to dryness and the residue taken up in 1.0 ml of distilled water. A 150 μ l aliquot was then applied to Whatman No. 1 paper.

The organic acids were identified by co-chromatography with authentic compounds using isoamyl alcohol saturated with 4.0 N formic acid as the solvent system (33, p. 969). The chromatograms were developed using the descending technique and radioactive areas identified with a strip counter (Vanguard, model 880). The acids were detected by spraying with 0.04 percent bromophenol blue at pH 6.8 (78, p. 13-14).

Enzyme Assays

The procedure used for the determination of propionate and acetate activation is a modification of that described by Berg (15, p. 461-462). The reaction mixture in a final volume of 1.0 ml consisted of the following components in μ moles: Tris buffer pH 8.0, 100; Tris-acetate pH 8.0, or Tris-propionate pH 8.0,10; MgCl, 20; ATP, 10; GSH, 10; NH_2OH, 20; KCl, 50; coenzyme A, 0.25; and enzyme extract containing 2-3 mg of protein. The reaction mixtures were incubated at 37°C for 20 minutes and the activity determined by the formation of either propionhydroxamate or acethydroxamate upon the addition of a $FeCl_3$ solution to the reaction mixture.

Propionyl-CoA carboxylase was assayed by the procedure described by Lane and Halenz (59, p. 566-567). The reaction mixture in a final volume of 1.0 ml consisted of the following components in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$, 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; and enzyme extract containing 1-3 mg of protein. For some experiments other acyl-CoA compounds were substituted for propionyl-CoA. All acyl-CoA compounds were prepared using the method of Simon and Shemin (84). The reaction mixtures were incubated at 37°C for 20 minutes. Enzyme activity was determined by the incorporation of $HC^{14}O_3^-$ into acid stable compounds. The radioactivity was detected with a Nuclear Chicago (D-47) gas-flow counter.

Methylmalonyl-CoA mutase activity was determined using a modification of the procedure of Hegre _et al_. (46, p. 539-540), 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 1.1 ml consisted of the following components in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$, 15; ATP, 4.0; MgCl, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units per mg protein), 1.0-3 mg; and enzyme extract containing 0.5 - 1.0 mg of protein. The enzyme extract was added following a ten minute preincubation at 37°C of all other reaction components. When coenzyme was added to the reaction mixture, it was added immediately after the enzyme extract. The complete system was then incubated an additional 20 minutes.

Enzyme activity was determined by the incorporation of the $HC^{14}O_3^-$ into permanganate stable compounds. The radioactivity was detected with a Nuclear Chicago (D-47) gas-flow counter.

Quantitative Determinations

Protein determinations of the enzyme extracts were made using the Folin-phenol method described by Layne (62, p. 448-450). Bovine serum albumin was used as the standard.

Nitrogen content of the cell suspensions was determined using the modified Kjeldahl method described by Umbreit *et al*. (95, p. 274). Crystalline $(NH_4)_2SO_4$ was used as the standard.

Source of Chemicals

The dipotassium salt of ATP and GSH were obtained from Nutritional Biochemicals Corporation of Cleveland, Ohio; coenzyme A from Sigma Chemical Company of St. Louis, Missouri; propionate-1-C^{14}, propionate-2-C^{14}, propionate-3-C^{14}, and acetate-2-C^{14} from New England Nuclear Corporation, Boston, Massachusetts. The adenylcobamide coenzyme was a gift from Dr. Mark Kliewer, Department of Enology and Viticulture, University of California, Davis, California. The benzimidazolylcobamide coenzyme was a gift from Dr. David Pearlman, Squibb Institute for Medical Research, New Brunswick, New Jersey, and the dimethylbenzimidazolylcobamide coenzyme was a gift of Dr. Karl Folkers, Merck Institute for Therapeutic Research, Rahway, New Jersey.

RESULTS

Manometric Experiments

A series of manometric experiments were conducted to determine whether bacteroids from soybean nodules possessed the capacity to oxidize propionate and certain other fatty acids (Table 2). In addition to these substrates, succinate, glutamate, and malonate were used since these compounds have been reported to be readily oxidized by bacteroids from nodules of several legumes (92, 94).

The data presented in Table 2 show that the bacteroids from soybean nodules not only oxidize propionate but also oxidize the other fatty acids with a carbon chain length of six or less. The rates of oxidation of heptanoate and caprylate by the bacteroids was quite variable. Perhaps this is related to their low solubility in aqueous media and a failure of these compounds to enter the cells. This same type of variability was observed with pure cultures of *Rhizobium*.

As a result of these data, experiments were conducted to determine the capacity of pure cultures of *Rhizobium* to oxidize these same substrates. Effective nitrogen fixing strains of *R. japonicum* and *R. meliloti* were selected for the experiments. Since it was desired to determine whether the addition of certain organic acids to the culture medium would enhance the oxidation of propionate and possibly other substrates, two different media were utilized

Table 2. Effect of Various Substrates on the Rate of Oxygen Uptake by Cell Suspensions of Soybean Bacteroids, R. japonicum, and R. meliloti*

Substrate**	Soybean Bacteroids	R. japonicum***		R. meliloti***	
		Medium I	Medium III	Medium II	Medium IV
Succinate	336	652	629	198	204
Propionate	136	376	377	169	230
Valerate	175	496	459	222	132
Heptanoate	91	201	113	229	97
Methylmalonate	142	212	208	178	158
Glutamate	177	228	202	263	240
Acetate	137	403	308	154	159
Butyrate	217	531	435	229	163
Caproate	155	585	411	235	111
Caprylate	56	92	12	60	23
Glucose	--	199	152	215	201
Pyruvate	--	663	464	246	286
Malonate	148	243	230	196	156
Endogenous	100	100	100	100	100

* All values are expressed as percentage of endogenous respiration. The values are based on total oxygen uptake per mg N per hour.
** Each flask contained: 2.5 ml of buffered cell suspension in the main compartment, 0.5 ml of buffered substrate in the side arm, and 0.2 ml of 20 percent KOH in the center well. The concentration of the substrates added was 0.3 M except propionate which was 0.1 M. The initial pH was 6.8 and the temperature was 30°C.
*** Media I and II are the basic culture media for R. japonicum and R. meliloti respectively. Media III and IV are the same basic media modified by the addition of 100 mg per liter each of: sodium propionate, succinate, and methylmalonate.

for each organism. One medium contained sodium propionate, succinate, and methylmalonate and the other medium lacked these acids. Details of these media have been described under "Cultural Procedures". In these experiments, the capacity of the cells to oxidize glucose and pyruvate also was examined.

All of the substrates, except heptanoate and caprylate, were consistently oxidized by the two organisms regardless of the composition of media utilized for culturing the organisms (Table 2). Propionate oxidation by R. meliloti was enhanced by the addition of the organic acids to the medium, but the modification of the medium did not affect propionate oxidation by R. japonicum. There was, however, a decrease in the capacity of both organisms to oxidize many of the other substrates when the media were modified. Since B_{12} coenzyme has been shown to be directly involved in propionate metabolism in other organisms (40, 86), propionate, succinate, and methylmalonate were routinely added to both the nonpurified and purified media to take advantage of any enhancement in the metabolism of propionate.

To determine the effect of the cobalt concentration in the culture medium on the oxidation of propionate and glutamate R. meliloti was grown in the purified medium with 0.00, 0.01, and 1.00 ppb cobalt. The 0.01 and 1.00 ppb levels of cobalt resulted in suboptimal and optimal growth respectively. The addition of propionate to suspensions of cobalt deficient cells failed to stimulate oxygen uptake (Figure 1). In contrast, the oxygen uptake by suspensions of

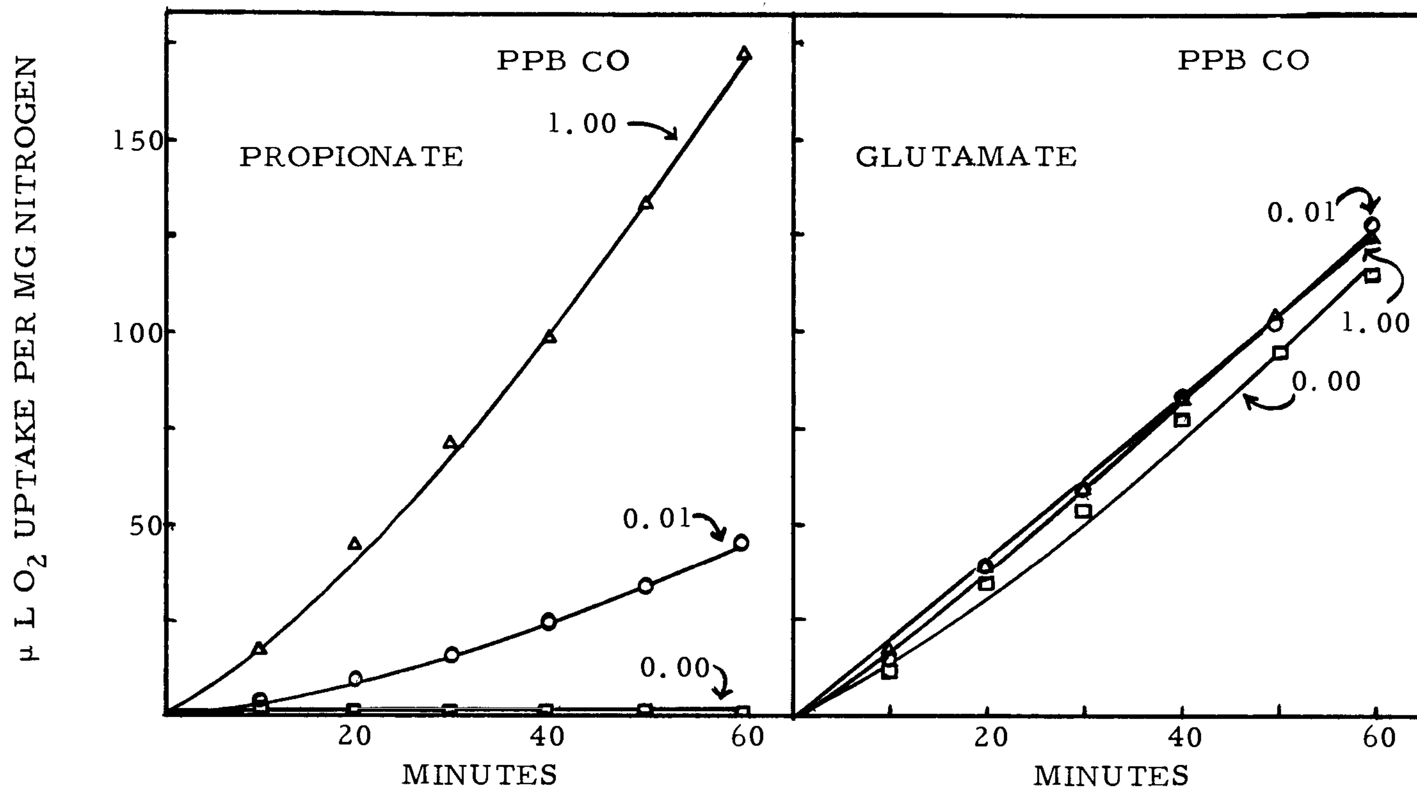


Fig. 1. Effect of cobalt concentration in the basic purified culture medium on the capacity of R. meliloti to oxidize propionate and glutamate. Each flask contained: 2.5 ml of buffered cell suspension in the main compartment, 0.5 ml of buffered substrate in the side arm, and 0.2 ml of 20 percent KOH in the center well. The concentraion of the propionate added was 0.1 M and that of the glutamate 0.3 M. The initial pH was 6.8 and the temperature was 30°C. The nitrogen content per flask was: 1.35, 1.34, and 1.30 mg for the 0.00, 0.01, and 1.00 ppb Co levels respectively.

cells grown with 0.01 ppb cobalt was markedly increased by the addition of propionate and suspensions of cells grown with 1.00 ppb cobalt exhibited an even higher capacity to utilize propionate. On the other hand, the capacity of the cells to oxidize glutamate was not significantly influenced by the concentration of cobalt in the medium (Figure 1). Also, the endogenous oxygen uptake, which was substracted from the total oxygen uptake obtained when both propionate and glutamate were utilized and which represents approximately 30-50 percent of the total oxygen uptake in the experiments, was not influenced by the cobalt content of the culture medium. From these data it is apparent that cobalt deficiency definitely interferes with the capacity of R. *meliloti* to oxidize propionate.

Radiorespirometric Experiments

The radiorespirometric patterns for the oxidation of specifically labeled propionate by bacteroids from soybean nodules (Figure 2) show that propionate was oxidized without an appreciable lag period. This suggests that the enzymes required for the oxidation of propionate are present in the organisms. The results also suggest that propionate is present in soybean nodules and that this substrate is metabolized *in vivo*.

Figures 3 and 4 show that cell suspensions of R. *japonicum* and R. *meliloti* will oxidize propionate. It is interesting to note that the patterns of $C^{14}O_2$ recovery from propionate labeled in specific positions is very similar for R. *japonicum* grown in both

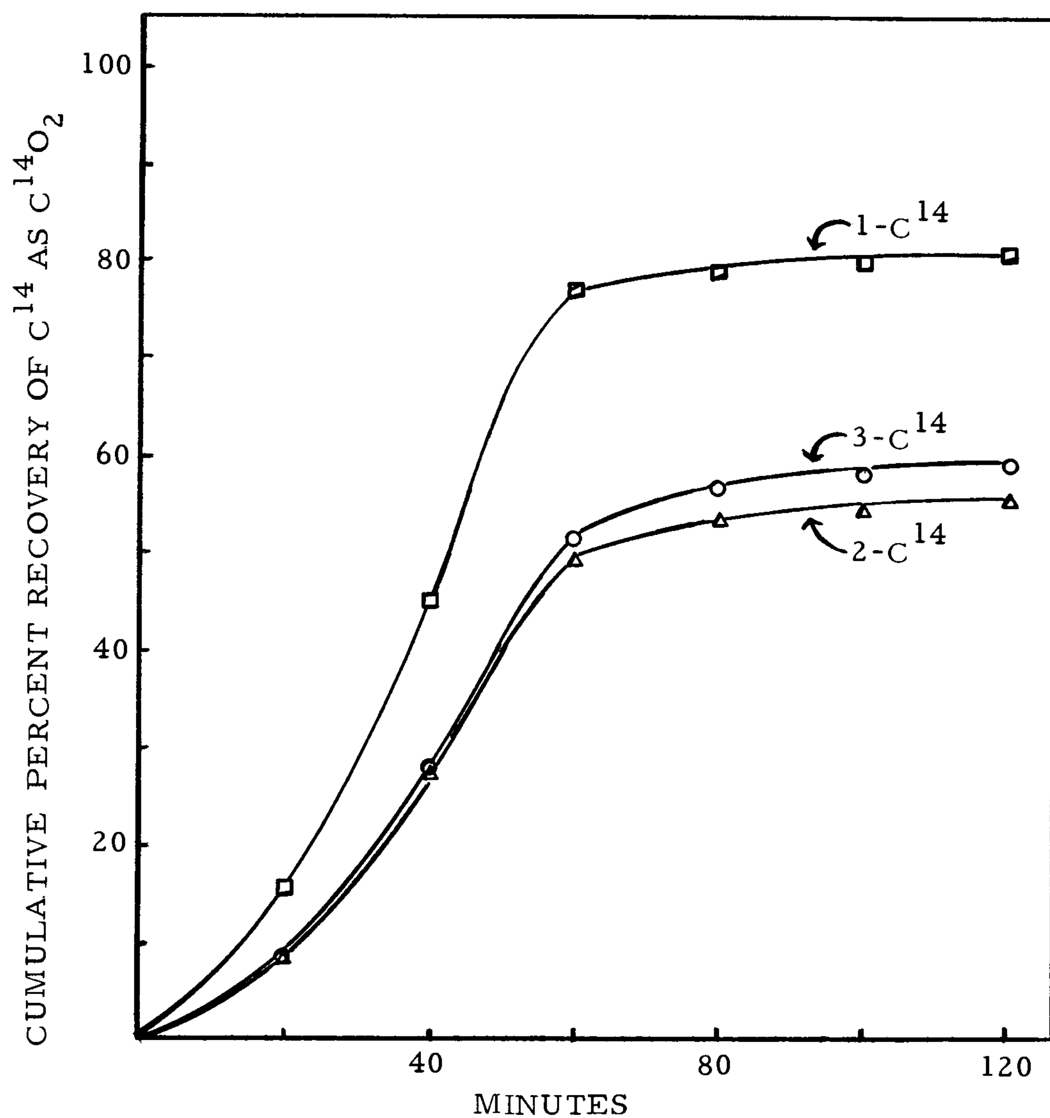


Fig. 2. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-C^{14} by bacteroids from soybean nodules. Each flask contained ten ml of buffered cell suspension (0.355 mg N/ml) and 1.0 ml of buffered propionate (3.5 μ moles). The initial pH was 6.8 and the temperature was 30°C.

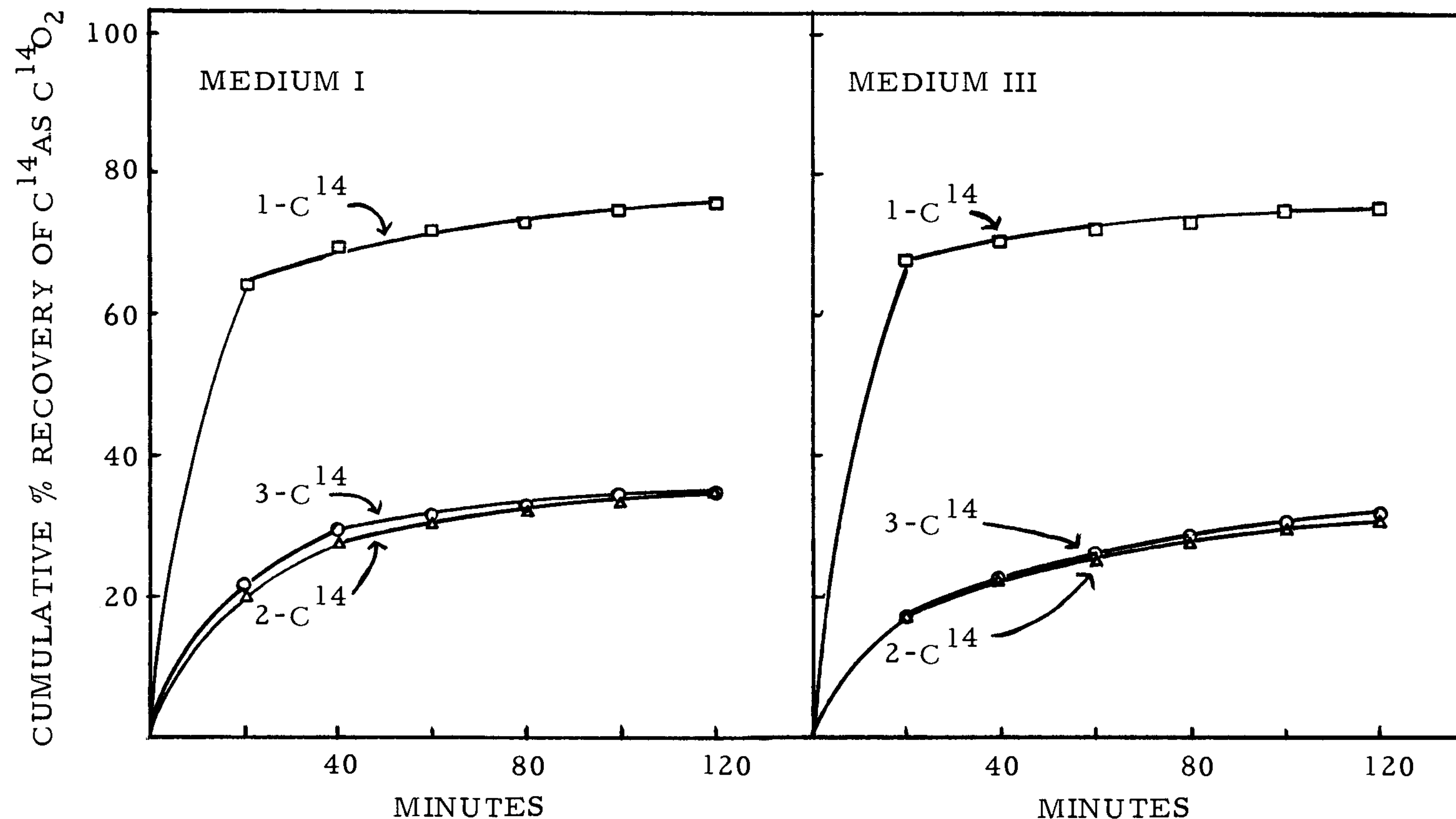


Fig. 3. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-C^{14} by R. japonicum grown on media I and III. Medium I is the basic nonpurified medium for R. japonicum. Medium III is the same medium modified by the addition of 100 mg per liter each of: sodium propionate, succinate, and methylmalonate. Each flask contained: ten ml of buffered cell suspension and 1.0 ml of buffered propionate (3.5 μ moles). The initial pH was 6.8 and the temperature was 30°C. The flasks contained 0.500 and 0.515 mg nitrogen per ml of cell suspension for media I and III respectively.

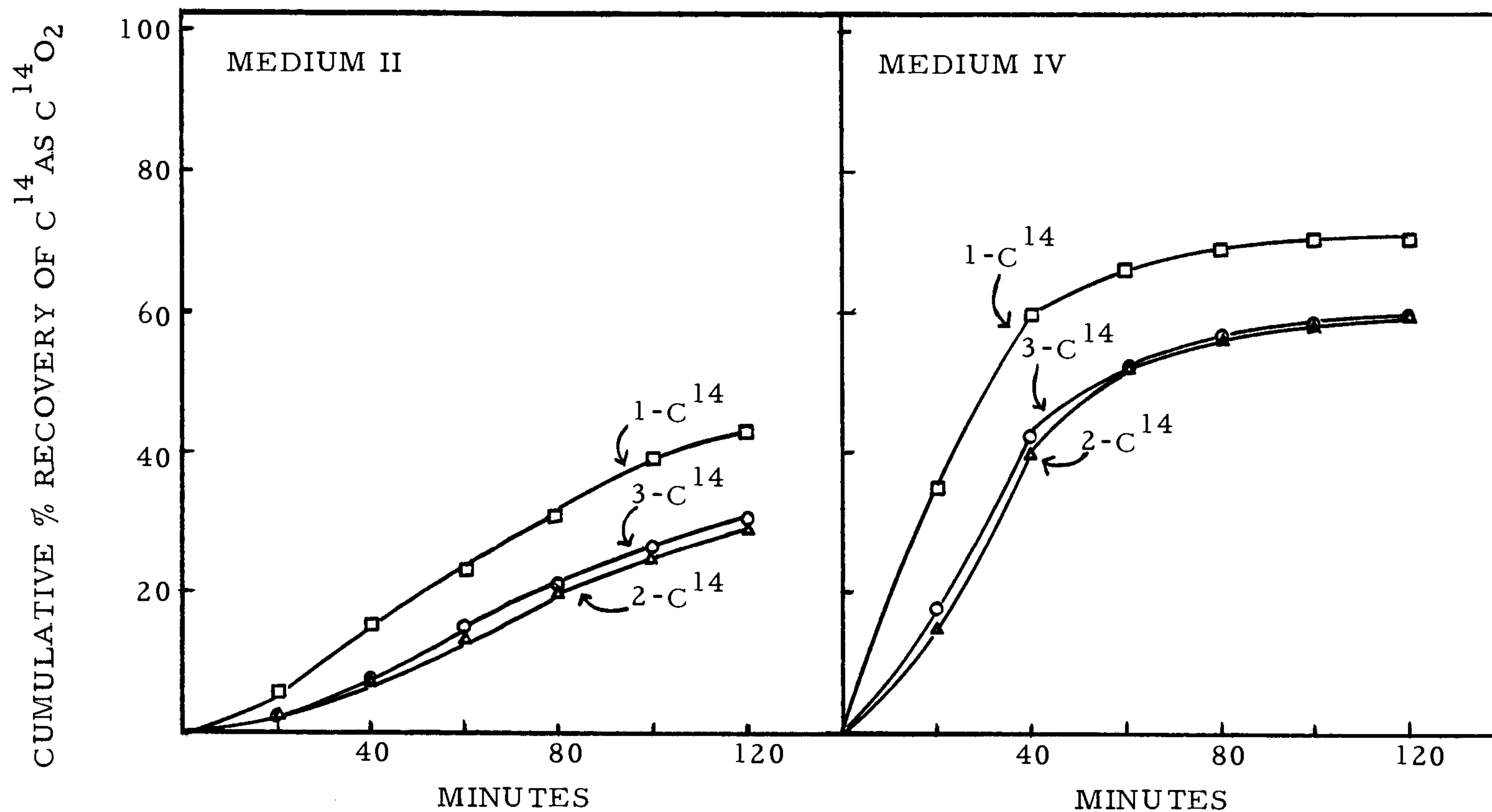


Fig. 4. The radiorespirometric patterns for the oxidation of propionate-1-, -2-, and -3-C^{14} by R. meliloti grown on media II and IV. Medium II is the basic nonpurified medium for R. meliloti. Medium IV is the same medium modified by the addition of 100 mg per liter each of: sodium propionate, succinate, and methylmalonate. Each flask contained: ten ml of buffered cell suspension and 1.0 ml of buffered propionate (3.5 μ moles). The initial pH was 6.8 and the temperature was 30°C. The flasks contained 0.425 and 0.410 mg nitrogen per ml of cell suspension for media II and IV respectively.

media and for *R. meliloti* grown in the modified media. These data (Figure 4) also confirm the results of the manometric experiments, which showed that the modification of the culture medium for *R. meliloti* resulted in an enhanced capacity of the cells to oxidize propionate. Since the patterns of $C^{14}O_2$ recovery for propionate-2-C^{14} and propionate-3-C^{14} (Figures 2, 3, 4) are essentially identical for soybean bacteroids, *R. meliloti*, and *R. japonicum*, it appears that a pathway whereby propionate is converted to succinate is operative in these organisms.

The effect of varying concentrations of cobalt on the capacity of *R. meliloti* to oxidize propionate labeled in different positions is presented in Figures 5, 6, and 7. The trends of the results obtained are very similar to those previously found with the manometric experiments. Cobalt deficient cells exhibited a greatly reduced capacity to oxidize propionate as indicated by the rates of evolution of $C^{14}O_2$ the specifically labeled propionate. The rate of formation of $C^{14}O_2$ from the deficient cells increased after a lag period. This may have been caused by contamination of the cell suspensions with cobalt which resulted in the synthesis of B_{12} coenzyme. The effect of the intermediate concentration of cobalt on the rate of oxidation of propionate was not as striking as that previously observed with the manometric experiments. There was, however, a decreased rate of oxidation of propionate with each of the substrates utilized. The respiratory patterns observed with the cells grown with the varying concentrations of cobalt are in

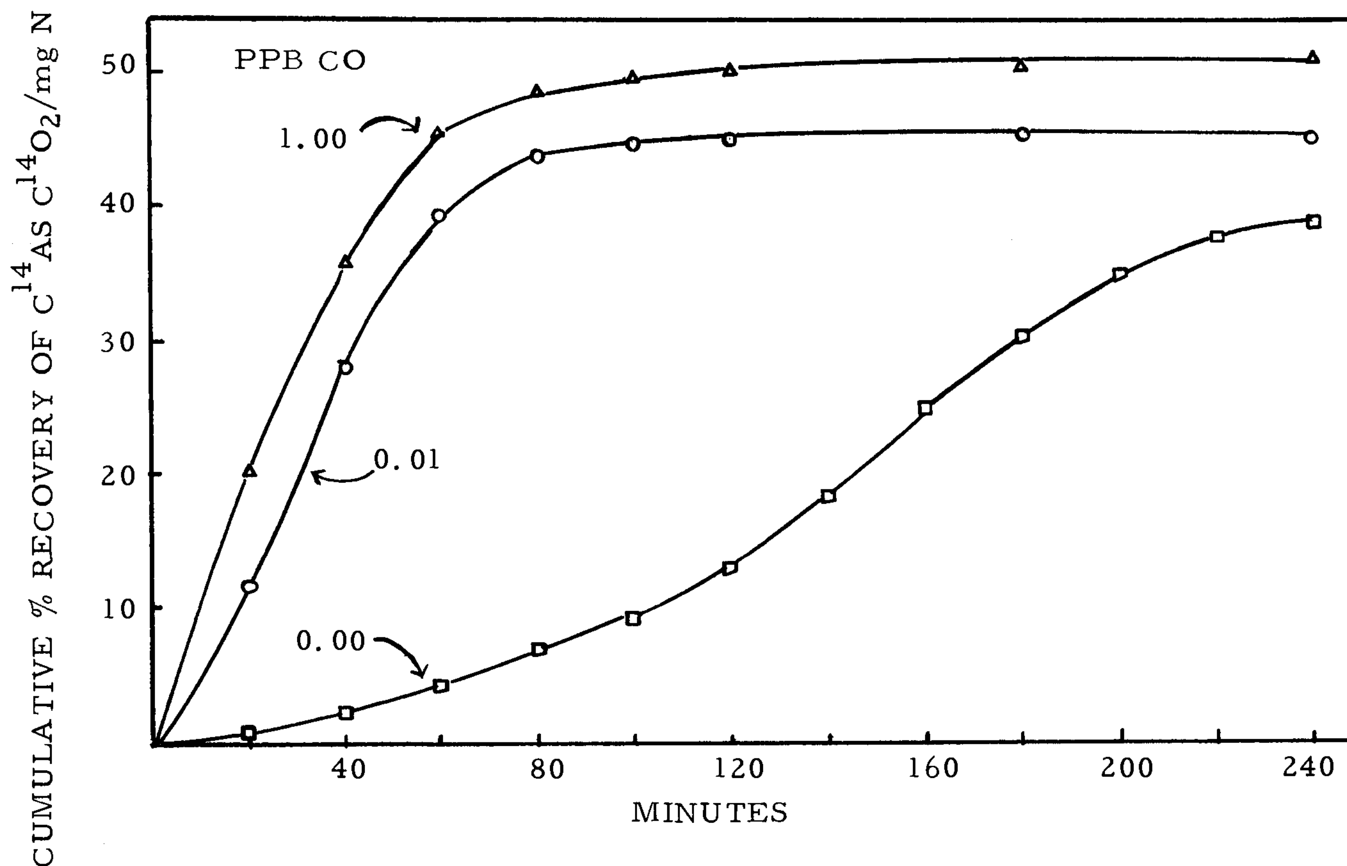


Fig. 5. The radiorespirometric patterns for the oxidation of propionate-1-C^{14} by R. melilot grown with varying concentrations of cobalt in the basic purified culture medium. Each flask contained 5.0 ml of buffered cell suspension and 0.5 ml of buffered propionate (1.75 μ moles). The initial pH was 6.8 and the temperature was 30°C. The nitrogen content per flask was: 1.68, 1.75, and 1.65 mg for the 0.00, 0.01, and 1.00 levels respectively.

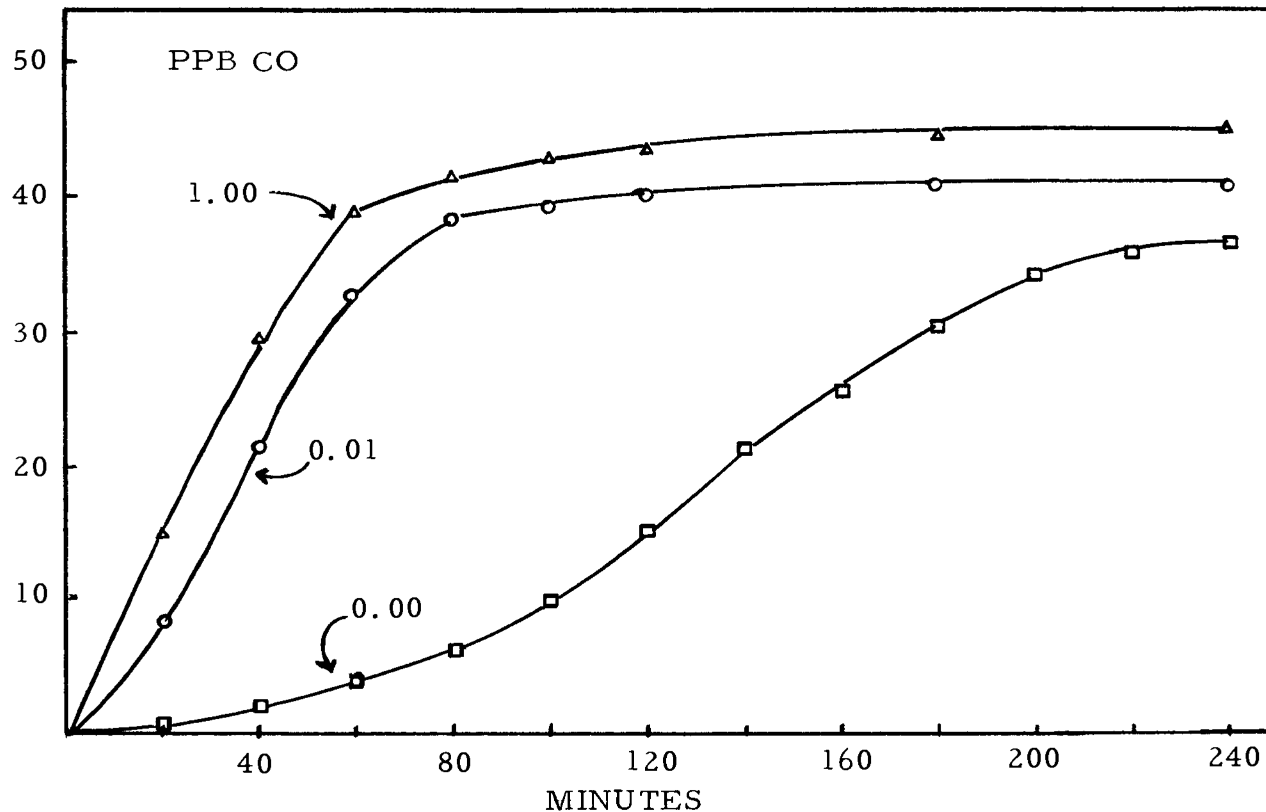


Fig. 6. The radiorespirometric patterns for the oxidation of propionate-2-C^{14} by R. meliloti grown with varying concentrations of cobalt in the basic purified culture medium. Each flask contained 5.0 ml of buffered cell suspension and 0.5 ml of buffered propionate (1.75 μ moles). The initial pH was 6.8 and the temperature was 30°C. The nitrogen content per flask was: 1.68, 1.75, and 1.65 mg for the 0.00, 0.01, and 1.00 levels respectively.

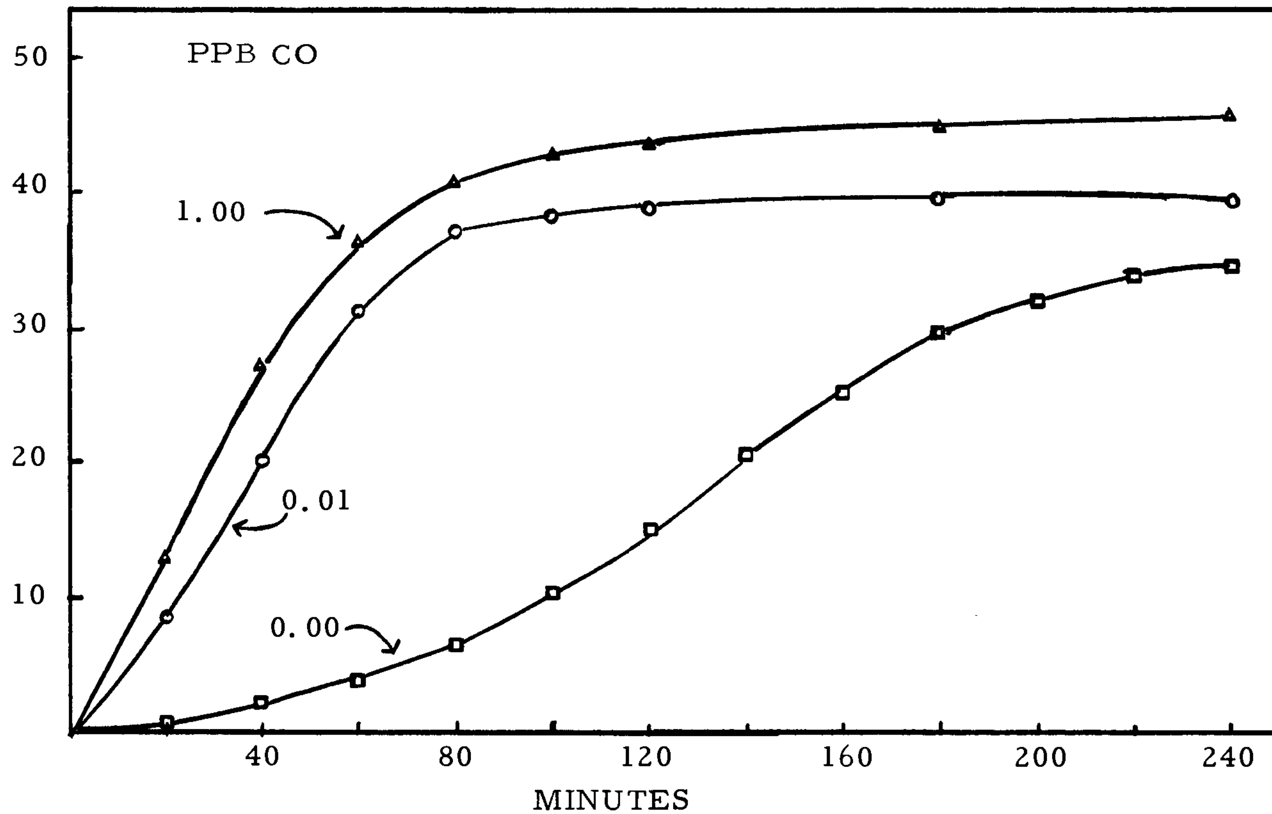


Fig. 7. The radiorespirometric patterns for the oxidation of propionate-3-C^{14} by *R. meliloti* grown with varying concentrations of cobalt in the basic purified culture medium. Each flask contained 5.0 ml of buffered cell suspension and 0.5 ml of buffered propionate (1.75 μ moles). The initial pH was 6.8 and the temperature was 30°C. The nitrogen content per flask was: 1.68, 1.75, and 1.65 mg for the 0.00, 0.01, and 1.00 levels respectively.

agreement with the results of previous radiorespirometric experiments, since they suggest that propionate was oxidized via a pathway in which succinate is an intermediate.

Figure 8 shows the radioactivity in organic acids which were isolated and identified in an extract obtained from R. meliloti cells which had been incubated with propionate-1-C^{14}. The acids identified by co-chromotography with authentic compounds were: malate, succinate, fumarate, and methylmalonate. Radioactivity was not detected in the region (R_F 0.57) where β-hydroxypropionate would be expected with the particular solvent used. The incubation of R. meliloti with propionate-2-C^{14} and propionate-3-C^{14} resulted in identical patterns of radioactivity. These findings are consistent with the radiorespirometric data, since propionate apparently was oxidized via the citric acid cycle. It was interesting to note that no radioactivity was detected in the region (R_F 0.27) where citrate should have migrated. An experiment was conducted therefore, using acetate-2-C^{14} as the substrate and the organic acids were isolated and identified using the same procedures. Again, the region where citrate should have migrated was not radioactive. Malate was found to be the organic acid predominately labeled in each of the experiments conducted.

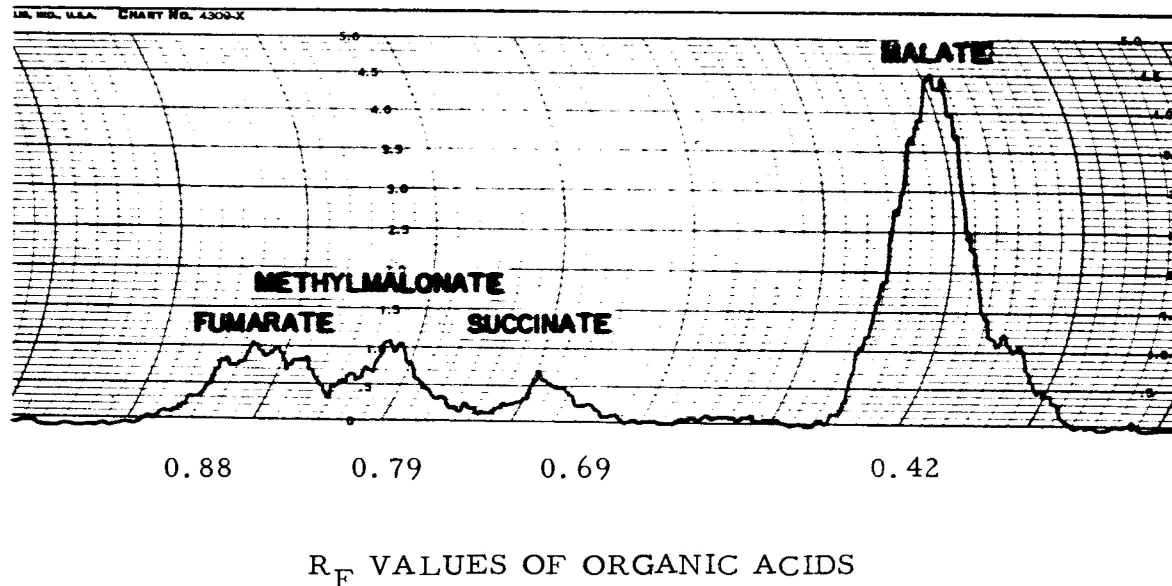


Fig. 8. Radiogram of chromatogram of organic acids isolated from R. meliloti. The cells were grown on medium IV and were incubated with 1.0 μC of propionate-1-C^{14} for ten minutes at 37°C. The chromatogram was developed ascendingly using isoamyl alcohol saturated with 4.0 N formic acid as the solvent. Identical labeling patterns were obtained when either propionate-2-C^{14} or propionate-3-C^{14} was utilized as the substrate under the same conditions.

Enzymes of the Propionate Oxidative Pathway

Propionate Activation. Propionate utilization by living organisms is, in all known cases, 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 (38, p. 308). 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 3 which show that these extracts contained an enzyme system capable of activating both substrates.

Table 3. Activation of Propionate and Acetate by Cell-free Extracts of Soybean Bacteroids, R. meliloti, and R. japonicum

	Enzyme activity **	
Enzyme source*	(μ moles acethydroxamate formed/mg protein/20 minutes)	(μ moles propionhydroxamate formed/mg protein/20 minutes)
Soybean bacteroids	0.475	0.339
R. japonicum	0.230	0.201
R. meliloti	0.084	0.089

* R. japonicum and R. meliloti were grown on media III and IV respectively. Bacteroids were isolated from nodules of 26 day old soybean plants.

** The reaction mixture in a final volume of 1.0 ml consisted of the following constituents in μ moles: Tris buffer pH 8.0, 100; $MgCl_2$, 20; ATP, 10; GSH, 10; NH_2OH, 20; KCl, 50; coenzyme A, 0.25; and enzyme extract containing 2-3 mg of protein. Reaction mixtures were incubated at 37°C for 20 minutes. The complete reaction mixture minus substrate served as negative control. Tris-acetate (10 μmoles) or Tris-propionate (10 μmoles) at pH 8.0 was used as the substrate.

Propionyl-CoA Carboxylase. The second reaction in the oxidation of propionate to succinate is the carboxylation of propionyl-CoA to form methylmalonyl-CoA. Using extracts prepared from soybean bacteroids and pure cultures of R. japonicum and R. meliloti, experiments were carried out to determine if a carboxylase was present which would utilize propionyl-CoA as a substrate. The data presented in Table 4 show that propionyl-CoA was indeed 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 (40) have described a carboxylase from beef liver

Table 4. Requirements of Propionyl-CoA Carboxylase in Cell-free Extracts of Soybean Bacteroids, R. japonicum, and R. meliloti

	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/mg protein/hour)		
System*	Soybean bacteroids**	R. japonicum	R. meliloti
Complete	0.890	1.100	0.160
ATP-$MgCl_2$ omitted	0.000	0.000	0.000
Propionyl-CoA omitted	0.000	0.056	0.015
GSH omitted	0.715	1.290	0.132

* The reaction mixture in a final volume of 1.0 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17 μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; and enzyme extract containing 1-2 mg of protein. Reaction mixtures were incubated at 37°C for 20 minutes. The complete reaction mixture minus enzyme served as negative control.

** R. japonicum and R. meliloti were grown on media III and IV respectively. Bacteroids were isolated from nodules of 31 day old soybean plants.

that is specific for propionyl-CoA, experiments were carried out to determine the substrate specificity of the carboxylase in extracts of soybean bacteroids and of pure cultures of R. japonicum, and R. meliloti. The results indicate (Table 5) that propionyl-CoA is carboxylated at a greater rate than either butyryl-CoA or acetyl-CoA. Since Waite and Wakil (98) have isolated a carboxylase from chicken liver that has an absolute requirement for Mn^{++} and preferentially carboxylates acetyl-CoA, a preliminary experiment was carried out to determine if manganese ions would increase the carboxylation of acetyl-CoA. The results indicated that the carboxylation of acetyl-CoA was not increased when $MnCl_2$ was used in place of $MgCl_2$ in the reaction mixture. Using propionyl-CoA as the substrate, extracts of bacteroids from the nodules of four legumes and of five pure culture Rhizobium species were then assayed to determine whether or not they have the ability to carboxylate propionate. A propionyl-CoA carboxylase was found in extracts of each of the organisms studied (Table 6).

Methylmalonyl-CoA Mutase. As previously mentioned, methylmalonyl-CoA mutase from animal tissues (40, 63, 87) and from P. shermanii (86, 88) has been shown to require B_{12} coenzyme for activity. The results of a survey to determine the occurence of this enzyme in extracts of bacteroids from nodules of four legumes and of five pure culture Rhizobium species are presented in Table 6. From the data plotted in Figure 9, it is apparent that the enzyme activity,

in a cell-free extract of R. meliloti, is proportional to the amount of protein in the extract in the range between 0.0 and 1.0 mg.

Table 5. Substrate Specificity of Propionyl-CoA Carboxylase in Cell-free Extracts of Soybean Bacteroids, R. japonicum, and R. meliloti

	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/mg protein/hour)		
Substrate *	Soybean bacteroids **	R. japonicum	R. meliloti
Acetyl-CoA	0.020	0.010	0.042
Butyryl-CoA	0.051	0.226	0.085
Propionyl-CoA	1.060	1.610	0.213

* The reaction mixture in a final volume of 1.0 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17 μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; acyl-CoA, 1.0; and enzyme extract containing 1-3 mg of protein. Reaction mixtures were incubated at 37°C for 20 minutes. The complete reaction mixture minus acyl-CoA served as negative control.

** R. japonicum and R. meliloti were grown on media III and IV respectively. The bacteroids were isolated from nodules of 30 day old soybean plants.

Table 6. Distribution of Propionyl-CoA Carboxylase and Methylmalonyl-CoA Mutase in Bacteroids from Nodules of Legumes and in Pure Cultures of *Rhizobium*

Enzyme source	Age (days)	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/mg protein/hour)	
		Carboxylase*	Mutase**
Soybean bacteroids	35	0.190	---
Soybean bacteroids	32	---	0.840
Pea bacteroids	35	0.100	---
Pea bacteroids	32	---	0.150
Lupine bacteroids	37	0.079	---
Lupine bacteroids	54	---	0.690
Cowpea bacteroids	37	0.130	---
Cowpea bacteroids	54	---	0.690
R. meliloti	1	0.100	0.700
R. japonicum	4	0.400	0.170
R. phaseoli	1	0.061	0.330
R. leguminosarum	1	0.065	0.360
R. trifolii	1	0.058	0.200

* The reaction mixture in a final volume of 1.0 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; and enzyme extract containing 1-3 mg of protein. Reaction mixtures were incubated at 37°C for 20 minutes. The complete reaction mixture minus propionyl-CoA served as negative control.

** The reaction mixture in a final volume of 1.1 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units/mg protein), 3.0 mg, and enzyme extract containing 0.5-1.0 mg of protein. The enzyme extract was added following a ten minute preincubation of all other reaction components at 37°C. The system was then incubated for an additional 20 minutes.

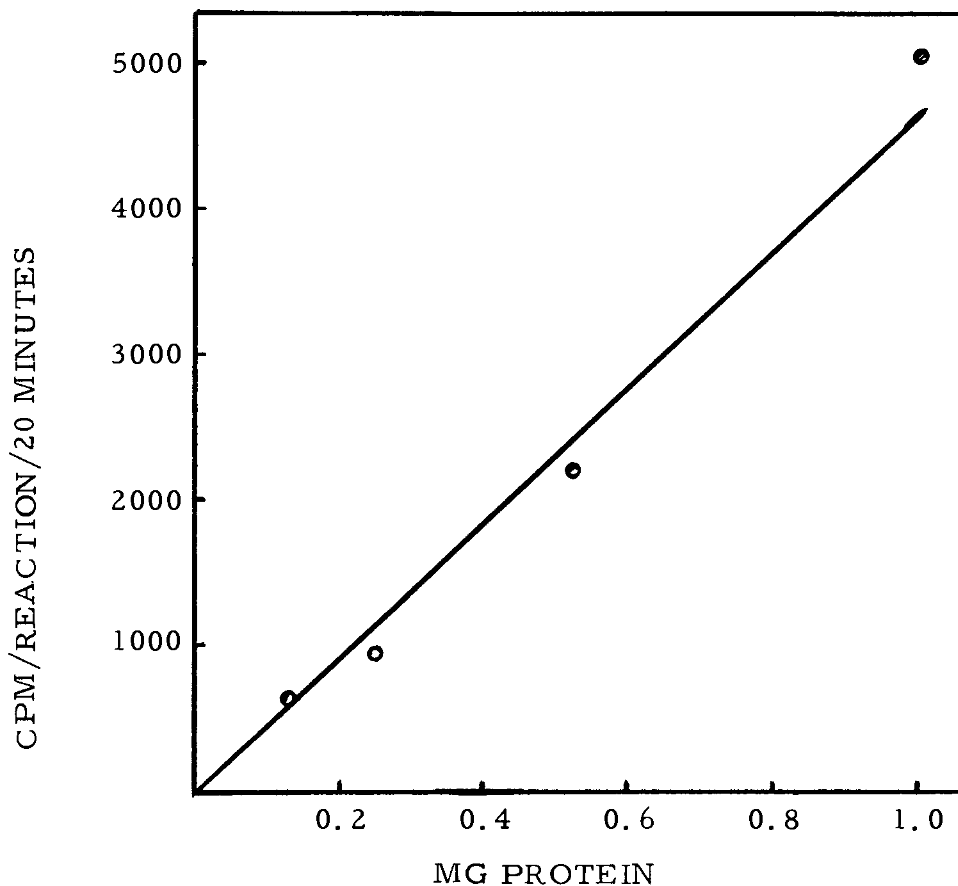


Fig. 9. Fixation of C^{14}-bicarbonate into permanganate stable acids as a function of the protein concentration. Enzyme extract was prepared from R. meliloti grown on medium II. The reaction mixture in a final volume of 1.1 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17 μC/μM), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units/mg protein), 3.0 mg; and enzyme extract as indicated. The enzyme extract was added following a ten minute preincubation of all other reaction components at 37°C. The system was then incubated an additional 20 minutes.

Since Hatch and Stumpf (45) have reported that C^{14}-methylmalonate was not metabolized at an appreciable rate by the plant tissues which they investigated, an experiment was conducted to determine the localization of methylmalonyl-CoA mutase within the root system of nodulated soybean plants. The data presented in Table 7 show that the enzyme was present in the isolated bacteroids, but it was absent in the other fractions assayed. These results support the conclusion of Hatch and Stumpf (45) and are consistent with the conclusion of Ahmed and Evans (3) that cobalt is required by the nodule bacteria and not by the soybean plants _per se_.

Table 7. Localization of Methylmalonyl-CoA Mutase in Soybean Tissues

Enzyme Source*	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/mg protein/hour)
Bacteroids	0.48
Extract from roots	0.00
Hemoglobin fraction	0.00
Extract of nodular debris	0.00

*The reaction mixture in a final volume of 1.1 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17 μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units/mg protein), 3.0 mg, DBC coenzyme, 2.3×10^{-3}; and enzyme extract containing 1-3 mg of protein. The enzyme extracts and DBC coenzyme were added following a ten minute preincubation of all other reaction components at 37°C. The system was then incubated for an additional 20 minutes. The complete reaction mixture minus enzyme extract served as negative control.

Since Kliewer and Evans (56) have isolated and characterized the DBC coenzyme from R. meliloti, experiments were conducted to determine the coenzyme specificity of the methylmalonyl-CoA mutase in extracts of R. meliloti. For these experiments R. meliloti was cultured on medium IV and a cell-free extract prepared as described under "Preparation of Cell Suspensions and Extracts" using reduced light conditions. A portion of the extract, maintained in an ice bath at $0^{o}C$, was subsequently subjected to direct sunlight for a period of 30 minutes. This is the procedure utilized by Barker et al. (10, p. 182) to destroy the AC coenzyme in cell-free extracts of C. tetanomorphum. That portion of the extract which was not illuminated was used for specific control reactions. As illustrated by the data in Table 8, 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 coenzymes to reaction mixtures containing the illuminated enzyme extract resulted in 67, 40, and three percent restoration of the activity respectively. This evidence together with that of Kliewer and Evans (56) indicates strongly that the B_{12} coenzyme (DBC) is the natural cobamide coenzyme associated with the methylmalonyl-CoA mutase in Rhizobium meliloti.

The effect of varying concentrations of cobalt in the growth medium on the activity of propionyl-CoA carboxylase and

Table 8. Coenzyme Specificity of Methylmalonyl-CoA Mutase from *R. meliloti*

System	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/ mg protein/hour)
Complete*	1.167
Complete plus AC**	1.056
Complete plus BC	1.257
Complete plus DBC	1.224
Complete (extract illuminated), plus AC***	0.024
Complete (extract illuminated), plus BC	0.474
Complete (extract illuminated), plus DBC	0.780

* The reaction mixture in a final volume of 1.1 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.38μ C/μ M), 15; propionyl-CoA, 1.0; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA carboxylase (20 units/mg protein) 0.89 mg; and enzyme extract containing 0.90 mg of protein. Enzyme extract and coenzyme, when utilized, was added following a ten minute preincubation of all other reaction components at 37°C. The system was then incubated for an additional 20 minutes. Complete reaction mixture with enzyme extract omitted served as negative control.

** The concentration of the coenzymes added was 2.6×10^{-3} μ moles for the AC and BC coenzymes and 2.3×10^{-3} μ moles for the DBC coenzyme. The complete reaction mixture with enzyme extract omitted and coenzyme added served as negative control for all coenzyme reactions.

*** The enzyme extract was illuminate in direct sunlight for 30 minutes at 0°C.

methylmalonyl-CoA mutase in extracts of R. meliloti was studied and the results are presented in Table 9. The activity of the carboxylase was slightly lower in extracts of deficient cells than in extracts of normal cells but the difference does not appear to be sufficient to influence the overall reaction. On the other hand, the activity of the methylmalonyl-CoA mutase in extracts of the deficient cells is strikingly reduced when compared with the results obtained with extracts of normal cells. The addition of 1.65×10^{-3} μ moles of DBC coenzyme to the reaction mixtures restored full activity to the enzyme extracts of cells grown with no cobalt and with 0.01 ppb of the element. The addition of DBC coenzyme to extracts of cells grown with 1.0 ppb cobalt, however, failed to stimulate enzyme activity. 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 and manometric experiments support this conclusion.

Paper chromatography of the methylmalonyl-CoA mutase reaction mixtures showed that after the permanganate treatment only succinate was radioactive, however, if the propionyl-CoA carboxylase reaction mixtures were extracted and chromatographed, methylmalonate was also found to be radioactive.

Table 9. Effect of Varying Concentrations of Cobalt in the Growth Medium on the Activity of Propionyl-CoA Carboxylase and Methylmalonyl-CoA Mutase of *R. meliloti*

Concentration of cobalt (ppb)	Enzyme activity (μ moles of $HC^{14}O_3^-$ fixed/mg protein/hour)		
	Carboxylase*	Mutase**	
		-DBC	+DBC***
None	1.83	0.0021	1.218
0.01	2.75	0.0504	1.146
1.00	2.41	1.0680	1.068

* The reaction mixture in a final volume of 1.0 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.17 μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; and enzyme extract containing 1-2 mg of protein. Reaction mixtures were incubated at 37°C for 20 minutes. The complete reaction mixture minus propionyl-CoA served as negative control.

** The reaction mixture in a final volume of 1.1 ml consisted of the following constituents in μ moles: Tris buffer pH 8.5, 160; $KHC^{14}O_3$ (0.38 μ C/μ M), 15; ATP, 4.0; $MgCl_2$, 4.0; GSH, 5.0; propionyl-CoA, 1.0; propionyl-CoA carboxylase (20 units/mg protein), 0.89 mg; and enzyme extract containing 0.90 mg of protein. The enzyme extract and the coenzyme, when utilized, was added following a ten minute preincubation of all other reaction components at 37°C. The system was then incubated for an additional 20 minutes. The complete reaction mixture minus enzyme extract served as the negative control.

*** The concentration of the DBC coenzyme added was 1.65×10^{-3} μ moles.

DISCUSSION

The fact that cell suspensions of *R. meliloti*, *R. japonicum*, and bacteroids from soybean nodules oxidize fatty acids with chain lengths of two to six carbons (Table 2) indicates that these organisms have the necessary enzymes to carry out β-oxidation and suggests that a citric acid cycle is operative in their metabolism. At present there is no enzymatic evidence demonstrating the presence or absence of these systems in pure culture *Rhizobium* or in bacteroids from nodules. The observation that glucose, pyruvate, succinate, glutamate, and malonate are oxidized by cell suspensions of pure culture *Rhizobium* and soybean bacteroids confirms in part the earlier reports of Tuzimura and Meguro (94) and Thorne and Burris (92). It was especially interesting to find that propionate and valerate were readily oxidized by cell suspensions of bacteroids from soybean nodules and of cells from pure cultures of *R. meliloti* and *R. japonicum*. Because of these findings and the established fact that B_{12} coenzyme is directly involved in the metabolism of propionate in animals and certain microorganisms (40, 63, 86, 87), the propionate metabolism in certain representative *Rhizobium* species was investigated in detail.

To obtain additional information on the capacity of *Rhizobium* to oxidize propionate and to provide information on the oxidative pathway, radiorespirometric experiments were carried out utilizing propionate labeled in specific positions. The different pathways

(Table 10) whereby propionate is known to be oxidized would provide differences not only in the products which would become radioactive, but also would result in different patterns of $C^{14}O_2$ evolution. The modified β-oxidation pathway, described by Giovanelli and Stumpf (36, 37) for pea, wheat, and safflower, would result in the loss of the carboxyl carbon of propionate by decarboxylation of the malonyl-CoA formed. This decarboxylation reaction would result in carbons 2 and 3 of propionate becoming the methyl and carboxyl carbons respectively of acetate. The rate of recovery of CO_2 from propionate-3-C^{14} would be greater than that from propionate-2-C^{14}, but the recovery of C^{14} from both substrates would be less than that from propionate-1-C^{14}. There is no evidence that cobalt is involved in the oxidation of propionate by this pathway. A pathway with similar intermediates has been reported to be operative in *Clostridium kluyverii* (96). In this system the carboxyl carbon of propionate becomes the carboxyl carbon of acetate, carbon 2 becomes the methyl carbon of acetate, and carbon 3 is recovered as CO_2 when the malonyl-CoA formed is decarboxylated. If this pathway is operative, the rate of recovery of the specifically labeled carbons as $C^{14}O_2$ would be greatest for the methyl carbon of propionate followed in turn by the carboxyl carbon, and then the methylene carbon. In the propionate to succinate pathway, which was first described by Flavin and Ochoa (33), the carboxyl carbon of propionate would become the carboxyl carbons of succinate, and carbons 2 and 3 would become the methylene carbons of succinate. Thus, the

Table 10. Summary of Pathways for Propionate Oxidation

System	Expected rates of recovery of carbon atoms	Carbon atoms entering citric acid cycle	Fate of carbon atoms of propionate		
			carbon 1	carbon 2	carbon 3
Propionate to succinate pathway (animal tissues, 33)*	$1 > 2 = 3$	1, 2, 3	Carboxyl carbon of succinate	Methylene carbons of succinate	
β-Oxidation pathway (plant tissues, 37, 45)	$1 > 3 > 2$	2, 3	CO_2	Methyl carbon of acetate	Carboxyl carbon of acetate
β-Oxidation pathway (*C. kluyverii*, 96)	$3 > 1 > 2$	1, 2	Carboxyl carbon of acetate	Methyl carbon of acetate	CO_2

* Numbers refer to references cited in Bibliography.

rate of recovery of the C^{14} as $C^{14}O_2$ would be greatest from propionate-1-C^{14}. The recovery from propionate-2-C^{14} and propionate-3-C^{14} would be identical because of the randomization of succinate in the citric acid cycle. This pathway would result in an extensive labeling of the citric acid cycle intermediates regardless of the specific label utilized. This would not occur if either of the modified β-oxidation pathways were operative, unless there was an appreciable fixation of the released $C^{14}O_2$ by enzymes such as phosphoenolpyruvate carboxylase.

It was found (Figures 2, 3, 4) that the rate of recovery of C^{14} as $C^{14}O_2$ from propionate labeled in different positions was greatest when the cells were incubated with propionate-1-C^{14}. In addition, the radiorespirometric patterns for $C^{14}O_2$ formation from propionate-2-C^{14} and propionate-3-C^{14} were essentially identical regardless of the conditions or organisms used. These patterns of recovery would be expected only if the propionate to succinate pathway was operative and the succinate formed was subsequently oxidized via the citric acid cycle.

Additional support for this conclusion was obtained when the organic acids were isolated from reaction mixtures containing cell suspensions of _R. meliloti_ and propionate labeled in different positions. Regardless of the position in which propionate was labeled, the radioactive organic acids isolated from the reaction mixtures were malate, succinate, fumerate, and methylmalonate (Figure 9). Malate always was the most radioactive compound detected. It was

interesting to note that the region where citrate should have migrated was not radioactive when the cells were incubated with C^{14}-propionate. An experiment was carried out therefore, to determine if this region would become radioactive when the cells were incubated with acetate-2-C^{14}. Again, the region was not found to be radioactive. Since citrate apparently was not labeled by the oxidation of either propionate or acetate and the patterns of $C^{14}O_2$ recovery from specifically labeled propionate are consistent with that of a complete citric acid cycle, it is proposed that citrate, when formed, is immediately oxidized and does not accumulate in the cells. The fact that methylmalonate was identified as being radioactive by chromatographic procedures is additional evidence that the pathway whereby propionate is converted to succinate via methylmalonate is the primary pathway of propionate oxidation in *Rhizobium* and in bacteroids from nodules of legumes.

Manometric (Figure 1) and radiorespirometric (Figures 5, 6, 7) experiments show that, 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. The rate of oxidation of propionate by the cells was strikingly increased by an increasing cobalt supply in the culture medium suggesting that cobalt is involved in the oxidation of propionate. In contrast, manometric experiments (Figure 1) showed that the oxidation of glutamate was not affected by changes in the concentration of cobalt in culture media. Arnstein and White (6) using *O.*

malhamensis and Ayers (8, 9) using *Flavobacterium spp.* were successful in increasing the rate of propionate oxidation by vitamin B_{12} deficient cells by the addition of vitamin B_{12} and certain B_{12} analogs to the cell suspensions, therefore, experiments were conducted to determine whether B_{12} 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 regardless of the procedure used. On the other hand, the addition of B_{12} coenzyme to extracts of cobalt deficient *R. meliloti* enhanced the methylmalonyl-CoA mutase activity of the extracts (Table 9). Very likely the reason that the addition of B_{12} coenzyme to cobalt deficient bacteria in the manometric and radiorespirometric studies did not increase propionate oxidation was that the coenzyme was not absorbed by the whole cells. The *R. meliloti* cells will take up inorganic cobalt and synthesize B_{12} compounds under normal conditions, but *O. malhamensis* and *Flavobacterium spp.* require preformed vitamin B_{12}, and, therefore, must be capable of absorbing vitamin B_{12} from external sources.

The results presented (Tables 3, 6) show that *R. meliloti*, *R. japonicum*, and soybean bacteroids 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 proviced evidence (Table 5) that the enzyme preferentially carboxylated propionyl-CoA. The enzyme also carboxylated butyryl-CoA and

acetyl-CoA but at greatly reduced rates. In addition to propionyl-CoA the carboxylase required an ATP-Mg^{++} mixture for activity (Table 4). The carboxylase from these organisms, therefore, exhibits properties similar to the propionyl-CoA carboxylase purified from beef liver (41, p. 880) and pig heart (51, p. 1922; 93, p. 1397). The propionyl-CoA carboxylase is widely distributed in bacteroids of leguminous species and in pure cultures of *Rhizobium* (Table 6).

Methylmalonyl-CoA mutase activity was demonstrated in a variety of nodules of legumes and in pure cultures of *Rhizobium* (Table 6). Extracts from the hemoglobin fraction, roots, nodular debris (relatively free of bacteroids), and bacteroids from nodules of soybean plants were assayed for methylmalonyl-CoA mutase activity to determine the localization of the mutase. The finding that the enzyme was present in bacteroids only is consistent with an earlier proposal by Ahmed and Evans (3, p. 33-34) that cobalt probably is required for the metabolism of the nodule bacteria and not by the leguminous plants *per se*.

It is of interest to compare the properties of the methylmalonyl-CoA mutase isolated from *R. meliloti* with those reported for the enzyme isolated from other sources. Lengyel *et al.* (63) carried out 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.

The apoenzyme from sheep kidney showed the greatest affinity for the DBC anolog. The Michaelis constants (K_m) reported were 2.1×10^{-8} and 2.0×10^{-7} for the DBC and BC coenzymes respectively. The AC coenzyme was inactive in the sheep kidney preparation. On the other hand, the activity of the coenzyme-free enzyme from P. shermanii was restored by the addition of each of the coenzymes. The K_m's reported for the DBC, BC, and AC coenzymes were 2.4×10^{-8}, 1.3×10^{-7}, and 1.0×10^{-7} respectively. The data presented in this dissertation show that the coenzyme-free methylmalonyl-CoA mutase from R. meliloti was activated to the greatest degree by the DBC coenzyme, and to a lesser extent by the BC coenzyme (Table 8). The R. meliloti enzyme 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 B_{12} 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 sulphate solution (63). Exposure to charcoal or intrinsic factor failed to remove the coenzyme. Thus, methylmalonyl-CoA mutase from R. meliloti has properties in common with both the animal and bacterial systems.

Both vitamin B_{12} and its benzimidazole analog will cure pernicious anemia in humans (23, p. 47-49), while the adenyl analog of the vitamin will not cure the disease. Recently Barness et al.

(12) have reported that methylmalonate was excreted in the urine of B_{12} deficient rats and that this condition could be prevented by the addition of vitamin B_{12} to the diet. These results suggest that vitamin B_{12} is involved in the biosynthesis of hemoglobin in animals. It is known that legume nodules contain appreciable quantities of hemoglobin when they are fixing atmospheric nitrogen (97, p. 609-621), thus, it is possible that the metabolic route in legume bacteroids whereby propionate is activated to propionyl-CoA then carboxylated to methylmalonyl-CoA and subsequently converted to succinyl-CoA (an essential precursor for the synthesis of heme compounds) may be indispensible in the biosynthetic pathway for the synthesis of hemoglobin. This postulation may partially explain the findings of Kliewer and Evans (57) who showed that the vitamin B_{12} content and the hemoglobin content of soybean nodules were nearly parallel throughout the development of the plants. Also, the postulation may be related to the observation of Ahmed and Evans (3) that nodules of soybean plants grown in a purified culture medium without added cobalt were low in hemoglobin as well as vitamin B_{12}.

The effect of varying concentrations of cobalt in the culture medium upon the activity of propionyl-CoA carboxylase and methylmalonyl-CoA mutase in _R. meliloti_ was determined (Table 9). It was found that the activity of the carboxylase enzyme was slightly reduced in extracts of deficient cells, however, the magnitude of the reduction is not considered significant. The activity of

methylmalonyl-CoA mutase was markedly reduced in extracts of deficient cells and of cells grown with 0.01 ppb cobalt. When B_{12} coenzyme was added to the reaction mixtures, the activity of the extracts was comparable to that observed with extracts prepared from cells grown with 1.00 ppb cobalt. These data indicate that the apoenzyme of methylmalonyl-CoA mutase was present in cells grown without sufficient cobalt and that the holoenzyme was formed immediately upon the addition of the B_{12} coenzyme.

No direct evidence has been provided in this dissertation that establishes the role or roles of the propionate to succinate pathway in the general metabolism of legume nodules or pure culture *Rhizobium*. Evidence is presented that conclusively demonstrates that soybean bacteroids, and pure cultures of *R. japonicum* and *R. meliloti* have an enzyme system whereby propionate can be converted to succinate via methylmalonate. It has been clearly established that B_{12} coenzyme is directly involved in this pathway as a cofactor for the methylmalonyl-CoA mutase enzyme. It seems probable that this is not the only role of B_{12} coenzyme in these organisms and that additional research will reveal other B_{12} requiring systems.

The data suggests that propionate may serve as a source of energy for the organisms by being converted to succinate and the succinate then being oxidized in the citric acid cycle. The possibility is not excluded that propionate may be involved in the synthesis of hemoglobin and other heme compounds by the bacteria in the

nodules. A biochemical study in which the significance of the propionate to succinate pathway in hemoglobin synthesis in nodules is determined would seem highly desirable. The fact that propionate was readily oxidized by bacteroids from soybean nodules suggests that propionate is present in nodules. Perhaps it is formed by the catabolism of certain amino acids but the possibility that other catabolic pathways may exist can not be excluded. In addition, there is no evidence to discount the possibility that propionate may be formed as an end product of metabolism of Rhizobium under conditions that exist in the nodules. In this regard it is known that the internal environment of nodules is partially anaerobic (12), a condition that would be expected to retard succinate oxidation and might promote propionate formation by the pathway described by Swick and Wood (90). Propionate, thus formed, would be available for the synthesis of odd-numbered fatty acids not only by the bacteroids but by the associated plant cells. Methylmalonate would also be available for the synthesis of branch-chained fatty acids. Since there is little direct evidence on the basic metabolic pathways of root nodule bacteria and of pure culture of Rhizobium, the significance of the propionate to succinate pathway will not be fully appreciated until additional details on the metabolism of these organisms have been provided. Certainly additional evidence of this type is needed if symbiotic nitrogen fixation is to be fully understood.

SUMMARY

An investigation was conducted to determine biochemical sites where cobalt, in the form of B_{12} coenzyme, functions in the metabolism of legumes and their associated *Rhizobium* species. This was carried out by means of manometric, radiorespirometric, and enzyme experiments. The results of these experiments may be summarized as follows:

1. Cell suspensions of *R. meliloti*, *R. japonicum*, and soybean bacteroids will oxidize fatty acids with chain lengths of two to six carbons.

2. The oxidation of propionate by cell suspensions of *R. meliloti* was enhanced by the addition of sodium propionate, succinate, and methylmalonate to a nonpurified culture medium This effect was not observed with *R. japonicum*.

3. The rate of propionate oxidation by *R. meliloti* was affected by the concentration of cobalt in the culture medium. In contrast, the oxidation of glutamate was not affected by the concentration of cobalt in the culture medium.

4. The radiorespirometric patterns obtained by incubation of propionate labeled in specific positions with cell suspensions of *R. meliloti*, *R. japonicum*, and soybean bacteroids suggested that propionate was converted to succinate via methylmalonate and was oxidized by the citric acid cycle.

5. Isolation and identification of the organic acids from *R. meliloti* cells incubated with propionate labeled in specific positions showed that methylmalonate, succinate, fumarate, and malate were radioactive with malate being the most radioactive acid.

6. *R. meliloti*, *R. japonicum*, and soybean bacteroids contain an enzyme which will catalyze the activation of propionate and acetate.

7. The carboxylase enzyme from *R. meliloti*, *R. japonicum*, and soybean bacteroids specifically carboxylated propionyl-CoA and required an ATP-Mg^{++} mixture for activity.

8. Propionyl-CoA carboxylase and methylmalonyl-CoA mutase are widely distributed in the bacteroids of legumes and in pure cultures of *Rhizobium*.

9. The B_{12} coenzyme of methylmalonyl-CoA mutase from *R. meliloti* was easily removed from the apoenzyme by exposure to direct light. Activity was restored by the addition of DBC coenzyme and partially restored by addition of BC coenzyme. The AC coenzyme was completely ineffective in restoring the activity.

10. Propionyl-CoA carboxylase activity in extracts of *R. meliloti* cells was not appreciably affected by the concentration of cobalt in the culture medium. On the other hand, the activity of the methylmalonyl-CoA mutase in the extracts was strikingly influenced by the cobalt level in the culture medium. Activity comparable to that obtained with extracts from cells grown with adequate cobalt was obtained by the addition of DBC coenzyme to enzyme extracts

of cobalt deficient cells or to extracts of cells grown with 0.01 ppb Co.

11. It is concluded, therefore, that cobalt deficiency in *R. meliloti* prevents the synthesis of adequate quantities of B_{12} coenzyme which is necessary for the methylmalonyl-CoA mutase and that the inactive mutase results in the failure of the organisms to oxidize propionate.

BIBLIOGRAPHY

1. Ahmed, Shaukat and Harold J. Evans. Effect of cobalt on the growth of soybeans in the absence of supplied nitrogen. Biochemical and Biophysical Research Communications 1:271-275. 1959.

2. Ahmed, Shaukat and Harold J. Evans. Cobalt: A micronutrient element for the growth of soybean plants under symbiotic conditions. Soil Science 90:205-210. 1960.

3. Ahmed, Shaukat and Harold J. Evans. The essentiality of cobalt for soybean plants grown under symbiotic conditions. Proceedings of the National Academy of Sciences 47:24-36. 1961.

4. Allen, S. H. G. et al. The isolation, purification, and properties of methylmalonyl racemase. Journal of Biological Chemistry 238:1637-1642. 1963.

5. Allen, S. H. G., Rune Stjernholm and Harland G. Wood. The noninvolvement of the ureido carbon of biotin in transcarboxylation. Journal of Biological Chemistry 238:PC2889-PC2892. 1963.

6. Arnstein, H. R. V. and A. M. White. The function of vitamin B_{12} in the metabolism of propionate by the protozoan Ochromonas malhamensis. The Biochemical Journal 83:264-270. 1962.

7. Atchley, William A. Studies on the cyclophorase system. IV. Direct demonstration of β oxidation. Journal of Biological Chemistry 176:123-131. 1948.

8. Ayers, William A. Effect of vitamin B_{12} and anologs on the respiration of a marine bacterium. Archives of Biochemistry and Biophysics 96:210-215. 1962.

9. Ayers, William A. The influence of cobamides on the endogenous and exogenous respiration of a marine bacterium. Canadian Journal of Microbiology 8:861-867. 1962.

10. Barker, H.A. et al. Assay, purification, and properties of the adenylcobamide coenzyme. Journal of Biological Chemistry 235:181-190. 1960.

11. Barker, H.A. et al. Isolation and properties of crystalline cobamide coenzymes containing benzimidazole or 5, 6-dimethylbenzimidazole. Journal of Biological Chemistry 235:480-488. 1960.

12. Barness, Lewis A., Diana G. Young and Robert Nocho. Methylmalonate excretion in vitamin B_{12} deficiency. Science 140:76-77. 1963.

13. Beck, William S., Martin Flavin and Severo Ochoa. Metabolism of propionic acid in animal tissues. III. Formation of succinate. Journal of Biological Chemistry 229:997-1010. 1957.

14. Beck, William S. and Severo Ochoa. Metabolism of propionic acid in animal tissues. IV. Further studies on the enzymatic isomerization of methylmalonyl coenzyme A. Journal of Biological Chemistry 232:931-938. 1958.

15. Berg, Paul. Assay and preparation of yeast aceto-CoA-kinase. In: Sidney P. Colowick and Nathen O. Kaplan's Method in enzymology. Vol. 5. New York, Academic Press, 1962. p. 461-466.

16. Bergersen, F. J. The effects of partial pressure of oxygen upon respiration and nitrogen fixation by soybean root nodules. The Journal of General Microbiology 29:113-125. 1962.

17. Bolle-Jones, E. W. and V. R. Mallikarjuneswara. A beneficial effect of cobalt on the growth of the rubber plant (Hevea brasiliensis). Nature 179:738-739. 1957.

18. Bolle-Jones, E. W. and V. R. Mallikarjuneswara. Cobalt: Effects on the growth and composition of Hevea. Journal of the Rubber Research Institute of Malaya 15:128-140. 1957.

19. Bond, G. and E. J. Hewitt. Cobalt and the fixation of nitrogen by root nodules of Alnus and Casuarina. Nature 195:94-95. 1962.

20. Bould, C. and E. J. Hewitt. Mineral nutrition of plants in soils and in culture media. In: F. C. Steward's Plant physiology; A treatise. Vol. 3. New York, Academic Press, 1963. p. 15-133.

21. Burton, Margaret O. and A. G. Lochhead. Production of vitamin B_{12} by Rhizobium species. Canadian Journal of Botany 30: 521-524. 1952.

22. Cheniae, George and Harold J. Evans. Properties of a particulate nitrate reductase from the nodules of the soybean plant. Biochemica et Biophysica Acta 35:140-153. 1959.

23. Coates, M. E. and J. E. Ford. Methods of measurement of vitamin B_{12}. In: The biochemistry of vitamin B_{12}, Ed. by R. T. Williams. London, Cambridge University Press, 1955. p. 36-51. (Biochemical Society Symposia No. 13).

24. Coon, Minor J. and Norman S. B. Abrahamsen. The relation of α-methylbutyrate to isoleucine metabolism. I. Acetoacetate formation. Journal of Biological Chemistry 195:805-812. 1952.

25. Coon, Minor J., Norman S. B. Abrahamsen and Grace S. Greene. The ralation of α-methylbutyrate to isoleucine metabolism. II. Propionate formation. Journal of Biological Chemistry 199:75-84. 1952.

26. Dehority, Burk A. et al. Studies on the metabolism of valine, proline, leucine, and isoleucine by rumen microorganisms in vitro. Archives of Biochemistry and Biophysics 78:15-27. 1958.

27. Delwiche, C. C., C. M. Johnson and H. M. Reisenauer. Influence of cobalt on nitrogen fixation by Medicago. Plant Physiology 36:73-78. 1961.

28. Delwiche, Eugene A. Mechanism of propionic acid formation by Propionibacterium pentosacceum. Journal of Bacteriology 56:811-820. 1948.

29. Eggerer, H. et al. On the mechanism of the cobamide coenzyme dependent isomerization of methylmalonyl-CoA to succinyl-CoA. Journal of the American Chemical Society 82:2643-2644. 1960.

30. Eggerer, H. et al. Zum Mechanismus der durch Cobalamin-Coenzym katalysierten Umlagerung von Methylmalonyl-CoA in Succinyl-CoA. Biochemische Zeitschrift 333:1-9. 1960.

31. Erdman, Lewis W. Legume inoculation: what it is; what it does. Washington, U.S. Government Printing Office, 1953. 20p. (U.S. Dept. of Agriculture. Farmer's Bulletin no. 2003).

32. Flavin, Martin, H. Castro-Mendoza and Severo Ochoa. Metabolism of propionic acid in animal tissues. II. Propionyl coenzyme A carboxylation system. Journal of Biological Chemistry 229:981-996. 1957.

33. Flavin, Martin and Severo Ochoa. Metabolism of propionic acid in animal tissues. I. Enzymatic conversion of propionate to succinate. Journal of Biological Chemistry 229:965-979. 1957.

34. Friedman, Daniel L. and Joseph R. Stern. Enzyme catalyzed exchange of 1-C^{14}-propionyl CoA and methylmalonyl CoA. Biochemical and Biophysical Research Communications 4:266-270. 1961.

35. Gerloff, G.C. Comparative mineral nutrition of plants. Annual Review of Plant Physiology 14:107-124. 1963.

36. Giovanelli, J. and P.K. Stumpf. A new pathway for propionate oxidation. Journal of the American Chemical Society 79:2652-2653. 1957.

37. Giovanelli, J. and P.K. Stumpf. Fat metabolism in higher plants. X. Modified β oxidation of propionate by peanut mitochondria. Journal of Biological Chemistry 231:411-426. 1958.

38. Green, David E. and David M. Gibson. Fatty acid oxidation and synthesis. In: David M. Greenberg's Metabolic pathways. Vol. 1. New York, Academic Press, 1960. p. 301-340.

39. Greenberg, David M. Carbon catabolism of amino acids. In: David M. Greenberg's Metabolic pathways. Vol. 2. New York, Academic Press, 1961. p. 79-172.

40. Gurnani, Shantoo, S.P. Mistry and B. Conner Johnson. Function of vitamin B_{12} in methylmalonate metabolism. I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase. Biochemica et Biophysica Acta 38:187-188. 1960.

41. Halenz, Donald R. and M. Daniel Lane. Properties and purification of mitochondrial propionyl carboxylase. Journal of Biological Chemistry 235:878-884. 1960.

42. Halenz, Donald R. and M. Daniel Lane. Net synthesis of methylmalonyl-CoA by ADP-, ATP-, and Mg-independent transcarboxylation catalyzed by propionyl carboxylase. Biochemical and Biophysical Research Communications 5:27-29. 1961.

43. Halenz, Donald R. and M. Daniel Lane. Further studies on the mechanism of the enzymic carboxylation of propionyl-CoA. Biochemica et Biophysica Acta 48:426-427. 1961.

44. Hallsworth, E.G., S.B. Wilson and E.A.N. Greenwood. Copper and cobalt in nitrogen fixation. Nature 187:79-80. 1960.

45. Hatch, M.D. and P.K. Stumpf. Fat metabolism in higher plants. XVII. Propionate metabolism by plant tissues. Archives of Biochemistry and Biophysics 96:193-198. 1962.

46. Hegre, C.S., S.J. Miller and M. Daniel Lane. Studies on methylmalonyl isomerase. Biochemica et Biophysica Acta 56:538-544. 1962.

47. Hewitt, E.J. The essential nutrient elements: Requirements and interactions in plants. In: F.C. Steward's Plant physiology; A treatise. Vol. 3. New York, Academic Press, 1963. p. 137-360.

48. Holm-Hansen, Osmund, Gerald C. Gerloff and Folke Skoog. Cobalt as an essential element for blue-green algae. Physiologia Plantarum 7:665-675. 1954.

49. Hutner, S.H. et al. Some approaches to the study of the role of metals in the metabolism of microorganisms. Proceedings of the American Philosophical Society 94:152-170. 1950.

50. Johns, A.T. The production of propionic acid by decarboxylation of succinic acid in a bacterial fermentation. The Biochemical Journal, Proceedings of the Biochemical Society 42: ii. 1948.

51. Kaziro, Yoshito, Enzo Leone and Severo Ochoa. Biotin and propionyl carboxylase. Proceedings of the National Academy of Sciences 46:1319-1927. 1960.

52. Kaziro, Yoshito et al. Metabolism of propionic acid in animal tissues. VIII. Crystalline propionyl carboxylase. Journal of Biological Chemistry 236:1917-1923. 1961.

53. Kinnory, David S., Yoshiro Takeda and David M. Greenberg. Isotope studies on the metabolism of valine. Journal of Biological Chemistry 212:385-396. 1955.

54. Kliewer, Mark and Harold J. Evans. B_{12} coenzyme content of the nodules from legumes, alder and of Rhizobium meliloti. Nature 194:108-109. 1962.

55. Kliewer, Mark and Harold J. Evans. Effect of cobalt deficiency on the B_{12} coenzyme content of Rhizobium meliloti. Archives of Biochemistry and Biophysics 97:428-429. 1962.

56. Kliewer, Mark and Harold J. Evans. Identification of cobamide coenzyme in nodules of symbionts and isolation of the B_{12} coenzyme from Rhizobium meliloti. Plant Physiology 38:55-59. 1963.

57. Kliewer, Mark and Harold J. Evans. Cobamide coenzyme contents of soybean nodules and nitrogen fixing bacteria in relation to physiological conditions. Plant Physiology 38:99-104. 1963.

58. Kosow, David P., Shu Chin Huang and M. Daniel Lane. Propionyl holocarboxylase synthesis. I. Preparation and properties of the enzyme system. Journal of Biological Chemistry 237:3633-3639. 1962.

59. Lane, M. Daniel and Donald H. Halenz. Mitochondrial propionyl carboxylase. In: Sidney P. Colowick and Nathan O. Kaplan's Methods in enzymology. Vol. 5. New York, Academic Press, 1962. p. 576-581.

60. Lane, M. Daniel et al. Further studies on mitochondrial propionyl carboxylase. Journal of Biological Chemistry 235: 3082-3086. 1960.

61. Lane, M. Daniel and Feodor Lynen. The biochemical function of biotin. IV. Chemical structure of the carboxylated active site of propionyl carboxylase. Proceedings of the National Academy of Sciences 49:379-385. 1963.

62. Layne, Ennis. Spectrophotometric and turbidimetric methods for measuring proteins. In: Sidney P. Colowick and Nathan O. Kaplan's Methods in enzymology. Vol. 3. New York, Academic Press, 1956. p. 447-454.

63. Lengyel, Peter, Rajarshi Mazumder and Severo Ochoa. Mammalian methylmalonyl isomerase and vitamin B_{12} coenzymes. Proceedings of the National Academy of Sciences 46:1312-1318. 1960.

64. Levin, Alfred P., Helen B. Funk and Moses D. Tendler. Vitamin B_{12}, rhizobia, and leguminous plants. Science 120:784. 1954.

65. Lowe, R.H. and Harold J. Evans. Cobalt requirement for the growth of rhizobia. Journal of Bacteriology 83:210-211. 1962.

66. Lowe, Richie H. and Harold J. Evans. Carbon dioxide requirement for growth of legume nodule bacteria. Soil Science 94: 351-356. 1962.

67. Lowe, Richie H., Harold J. Evans and Shaukat Ahmed. The effect of cobalt on the growth of Rhizobium japonicum. Biochemical and Biophysical Research Communications 3:675-678. 1960.

68. Lynen, F. et al. Die biochemische Funktion des Biotins. Angewandte Chemie 71:481-486. 1959.

69. Matsuo, Yoshihiko, Morton Rothstein and David M. Greenberg. Metabolic pathways of homoserine in the mammal. Journal of Biological Chemistry 221:679-687. 1956.

70. Mazumder, Rajarshi et al. A new enzyme in the conversion of propionyl coenzyme A to succinyl coenzyme A. Journal of Biological Chemistry 236:PC53-PC54. 1961.

71. Mazumder, Rajarshi et al. Metabolism of propionic acid in animal tissues. IX. Methylmalonyl coenzyme A racemase. Journal of Biological Chemistry 237:3065-3068. 1962.

72. Mazumder, Rajarshi, Taiji Sasakawa and Severo Ochoa. Metabolism of propionic acid in animal tissues. X. Methylmalonyl coenzyme A mutase holoenzyme. Journal of Biological Chemistry 238:50-53. 1963.

73. Miller, Carlos O. The influence of cobalt and sugars upon the elongation of etiolated pea stem segments. Plant Physiology 29:79-82. 1954.

74. Nason Alvin and William D. McElroy. Modes of action of the essential mineral elements. In: F.C. Stewards Plant physiology; A treatise. Vol. 3. New York, Academic Press, 1963. p. 451-536.

75. Nicholas, D.J.D., M. Kobayashi and P.W. Wilson. Cobalt requirement for inorganic nitrogen metabolism in microorganisms. Proceedings of the National Academy of Sciences 48:1537-1542. 1962.

76. Nicholas, D.J.D., Yoshiharu Maruyama and D.J. Fisher. The effect of cobalt deficiency on the utilization of nitrate nitrogen in Rhizobium. Biochemica et Biophysica Acta 56:623-626. 1962.

77. Overath, Peter et al. Zum Mechanismus der Umlagerung von Methylmalonyl-CoA in Succinyl-CoA. II. Versuche zur Wirkungsweise von Methylmalonyl-CoA-Isomerase und Methylmalonyl-CoA-Racemase. Biochemische Zeitschrift 335:500-518. 1962.

78. Palmer, J.K. Chemical investigations of the tobacco plant. X. Determination of organic acids by ion exchange chromatography. 1955. 31 p. (Connecticut Agricultural Experiment Station. Bulletin no. 589).

79. Powrie, J.K. A field response by subterranean clover to cobalt fertilizer. The Australian Journal of Science 23:198-199. 1960.

80. Reisenauer, H.M. Cobalt in nitrogen fixation by a legume. Nature 186:375-376. 1960.

81. Robinson, William G., Bimal K. Bachhavat and Minor J. Coon. Tiglyl coenzyme A and α -methylacetoacetyl coenzyme A, intermediates in the enzymatic degradation of isoleucine. Journal of Biological Chemistry 218:391-400. 1956.

82. Robinson, William G. et al. Coenzyme A thiol esters of isobutyric, methylacrylic, and β-hydroxyisobutyric acids as intermediates in the enzymatic degradation of valine. Journal of Biological Chemistry 224:1-11. 1957.

83. Rose, William C., Julius E. Johnson and William J. Haines. The metabolism of valine in phlorhizin glycosuria. Journal of Biological Chemistry 145:679-684. 1942.

84. Simon, Eric J. and David Shemin. The preparation of S-succinyl coenzyme A. Journal of the American Chemical Society 75:2520. 1953.

85. Smith, Richard M. and Kenneth J. Monty. Vitamin B_{12} and propionate metabolism. Biochemical and Biophysical Research Communications 1:105-109. 1959.

86. Stadtman, E.R. et al. The role of biotin and vitamin B_{12} coenzyme in propionate metabolism. Biochemical and Biophysical Research Communications 2:1-7. 1960.

87. Stern, Joseph R. and Daniel L. Friedman. Vitamin B_{12} and methylmalonyl isomerase. Biochemical and Biophysical Research Communications 2:82-87. 1960.

88. Stjernholm, Rune and Harland G. Wood. Methylmalonyl isomerase. II. Purification and properties of the enzyme from propionibacteria. Proceedings of the National Academy of Sciences 47:303-313. 1961.

89. Swick, Robert W. Propionic acid metabolism: Mechanism of the methylmalonyl isomerase reaction and the reduction of acrylyl coenzyme A to propionyl coenzyme A in propionibacteria. Proceedings of the National Academy of Sciences 48:288-293. 1962.

90. Swick, Robert W. and Harland G. Wood. The role of transcarboxylation in propionic acid fermentation. Proceedings of the National Academy of Sciences 46:28-41. 1960.

91. Thimann, Kenneth V. Studies on the growth and inhibition of isolated plant parts. V. The effect of cobalt and other metals. American Journal of Botany 43:241-250. 1956.

92. Thorne, D.W. and R.H. Burris. Respiratory enzyme systems in symbiotic nitrogen fixation. II. The respiration of Rhizobium from legume nodules and laboratory cultures. Journal of Bacteriology 39:187-196. 1940.

93. Tietz, Alisa and Severo Ochoa. Metabolism of propionic acid in animal tissues. V. Purification and properties of propionyl carboxylase. Journal of Biological Chemistry 234:1394-1400. 1959.

94. Tuzimura, Katura and Hirosi Meguro. Respiration substrate of Rhizobium in the nodules. Journal of Biochemistry (Tokyo) 47:391-397. 1960.

95. Umbreit, W.W., R.H. Burris and J.F. Stauffer. Monometric techniques. 3d ed. Minneapolis, Burgess Publishing Company, 1957. 338p.

96. Vagelos, P. Roy and Joan M. Earl. Propionic acid metabolism III. β -hydroxypropionyl-CoA and malonyl semialdehyde coenzyme A, intermediates in propionate metabolism by Clostridium kluyverii. Journal of Biological Chemistry 234: 2272-2280. 1962.

97. Virtanen, Artturi I. and Jorma K. Miettinen. Biological nitrogen fixation. In: F.C. Steward's Plant physiology; A treatise. Vol. 3. New York, Academic Press, 1963. p. 539-668.

98. Waite, Moseley and Salih J. Wakil. Studies on the mechanism of fatty acid synthesis. XII. Acetyl coenzyme A carboxylase. Journal of Biological Chemistry 237:2750-2757. 1962.

99. Waite, Moseley and Salih J. Wakil. Studies on the mechanism of action of acetyl coenzyme A carboxylase. II. On the mechanism of action of enzyme-bound biotin. Journal of Biological Chemistry 238:81-90. 1963.

100. Wakil, Salih J. and Moseley Waite. On the mechanism of action of enzyme-bound biotin. Biochemical Biophysical Research Communications 9:18-24. 1962.

101. Wang, Chih H. Metabolism studies by radiorespirometry. 1962. 14 p. (New England Nuclear Corporation, Boston, Atomlight No. 21).

102. Wood, Harland G. et al. Transcarboxylase. III. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase containing tritiated biotin. Journal of Biological Chemistry 238:547-556. 1963.

103. Wood, Harland G. et al. Transcarboxylase. IV. Function of biotin and the structure and properties of the carboxylated enzyme. Biochemische Zeitschrift 337:247-266. 1963.

104. Wood, Harland G. and Rune Stjernholm. Transcarboxylase. II. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase. Proceedings of the National Academy of Sciences 47:289-303. 1961.