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Title PROPIONATE METABOLISM AND ITS RELATIONSHIP TO LEGHEMOGLOBIN BIOSYNTHESIS IN SOYBEAN NODULES

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Experiments are reported which demonstrate that a cobalt deficiency in R. meliloti results in a decreased cytochrome content of bacterial cells. It is concluded that the effect of cobalt deficiency on cytochrome content of Rhizobium cells and on the leghemoglobin content of nodules possibly may be explained by an effect of cobalt deficiency on the utilization of propionate. Radiotracer experiments have provided evidence that propionate may be utilized for heme synthesis as well as for the maintenance of the citric acid cycle in nodules. When soybean nodules are incubated with propionate-2-C^{14}, the intermediates of the citric acid cycle not only become labeled with C^{14} but also the heme moiety of leghemoglobin becomes labeled. The incorporation of propionate-2-C^{14} into heme is linear with time and it appears that propionate is utilized without a lag period. The rate of incorporation of propionate-2-C^{14} into heme is more rapid than the rate of incorporation of succinate-2-C^{14} and citrate-1, 5-C^{14},

however, these rates of incorporation may be influenced by different endogenous pool sizes of organic acids.

It can be concluded from additional radioactive tracer experiments that the supply of succinyl-CoA from propionate is competitive with the supply of succinyl-CoA from the citric acid cycle. It was observed that when the concentration of propionate was high in the incubation mixture, the rate of succinate-2-C^{14} incorporation into heme was inhibited. Furthermore, when a large amount of substrate (succinate or acetate) which can be utilized by the citric acid cycle enzymes is added to the incubation mixture using whole nodules, the rate of incorporation of propionate-2-C^{14} into heme is reduced. The addition of acetate to the incubation mixture reduced the rate of incorporation of propionate-2-C^{14} into heme by 33 percent, yet it stimulated the citric acid cycle activity and increased the rate of incorporation of succinate-2-C^{14} into heme by nearly 50 percent.

Since C^{14}-labeled metabolites were incorporated into the heme moiety of leghemoglobin, a method was developed for the isolation of pure heme from nodules. In brief, the heme was extracted with acid acetone and reextracted with chloroform. After separation and evaporation of the chloroform, the pyridine hemochromogen was isolated by column chromatography from a silicone impregnated cellulose column.

The fact that propionate is readily utilized by bacteroids

suggested that this compound may be a normal metabolite in nodules. No detectable pool size of propionate was found however, in either soybean nodules or in isolated bacteroids. These results indicated that propionate may be utilized as rapidly as it is formed. An investigation was initiated therefore to determine whether or not lactate could be a precursor of propionate in this symbiotic relationship. Tracer experiments have indicated that lactate-1-C^{14} and lactate-2-C^{14} are incorporated into the heme moiety of leghemoglobin at approximately equal rates. The rate of incorporation of lactate-C^{14} into heme is significantly decreased by the addition of non-radioactive propionate to the reaction mixture, and isolated propionate from this mixture shows that propionate becomes radioactive. Further experiments using a cell-free extract from nodule bacteroids demonstrated the direct conversion of lactate to propionate. The cofactor requirements for this enzymatic conversion are ATP, Mg^{++}, NADH and coenzyme A. The rate of C^{14} accumulation in propionate from lactate-1-C^{14} is inhibited by the addition of non-radioactive acrylate, suggesting but not proving that acrylate may be an intermediate in the reaction.

PROPIONATE METABOLISM AND ITS RELATIONSHIP TO LEGHEMOGLOBIN BIOSYNTHESIS IN SOYBEAN NODULES

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INTRODUCTION

Biological fixation of atmospheric nitrogen is an important factor in maintaining the nitrogen balance in nature. Because legumes are major biological nitrogen-fixing organisms, the observation (1, 2, 3, 35, 48, 73, 74) that cobalt is essential for their symbiotic growth is potentially of great importance. Under conditions of cobalt deficiency, symbiotic fixation is impaired and the legume may suffer from nitrogen deficiency. A basic knowledge of the metabolism of the legume-Rhizobium symbiotic relationship is essential for an understanding of the basic mechanism by which symbiotically grown legumes are able to fix atmospheric nitrogen.

Cobalt has been shown to be essential for the nodule bacteroids (61, 63, 70) but has not been demonstrated to be essential for either leguminous or non-leguminous plants per se. The specific role or roles of cobalt in the metabolism of either symbiotic or free-living nitrogen-fixing organisms has not been adequately determined. The status of our present knowledge of the biochemical role of cobalt in leguminous plants may be summarized by stating that cobalt deficiency results in an impaired synthesis of B_{12} coenzyme in Rhizobium cells. The limited supply of this coenzyme results in a block in the activity of the methylmalonyl-CoA mutase which is an essential step in the

metabolism of propionic acid. As a result of this impairment, nodule bacteroids lose their capacity to oxidize propionate. At the present time practically nothing is known regarding the detailed pathways involved in the utilization of propionate or in the formation of this organic acid in the nodules of legumes. It is known that (34) propionate is activated in the presence of ATP and CoA to form propionyl-CoA then carboxylated to form methylmalonyl-CoA and finally converted to succinyl-CoA via the methylmalonyl-CoA mutase reaction.

Since cobalt deficiency is known to impair the pathway of propionate utilization in nodules of leguminous plants and in pure cultures of Rhizobium, the primary objective of this investigation is to determine the importance and subsequent pathways of propionate utilization in the R. japonicum-soybean symbiotic relationship. An additional objective is to obtain information concerning pathways where propionate may be synthesized in components of the symbiotic association.

REVIEW OF LITERATURE

Metabolism of the Soybean-Rhizobium Symbiotic Relationship

Bacteroid Formation

Rhizobium japonicum cells infect the root hairs of soybean plants and penetrate by means of an infection thread. After liberation from the infection thread, the bacteria collect in the cytoplasm of the host cells and multiply causing nodule initiation in the root cortex (22). This gives rise to a homogeneously aged infected tissue in soybean nodules. This situation is not the case in nodules initiated by Rhizobium of the fast-growing group (23). After liberation from the infection thread (78) and initiation of nodule formation (92), a morphological change occurs in the bacteria. The actively growing bacteria become swollen and lose their ability for cell division. At this stage the cells are termed "bacteroids". It is reported that bacteroids are still metabolically active retaining their ability to grow (60) to deposit fat bodies (60) to synthesize amino acids (25, 54, 55) and to utilize organic acids (94). From evidence in the literature there seems to be no significant difference in metabolic pathways between soybean bacteroids and cultured cells of R. japonicum (17, 25, 90, 91). Bergersen (16) concluded that the bacteria must

be fully developed into the bacteroid form before nitrogen fixation could take place.

pO_2 of Soybean Nodules

It is well documented (19, 20, 41, 81, 82) that the interior of soybean nodules is nearly anaerobic. Bergersen (19) found the oxygen tension in soybean nodules to be below 0.005 mm mercury or less than 0.0007 percent. This evidence supported the earlier conclusion (20) that there is an oxygen barrier in the nodules which does not permit the passage of oxygen to the bacteroids until a certain oxygen tension has been exceeded. Even though rhizobia are classified as strict aerobes, both R. japonicum and soybean bacteroids are able to attain maximum respiration at low oxygen tensions (6, 7, 25). Burris and Wilson (25) have reported that soybean bacteroids attain their maximum rate of respiration at 0.03 to 0.04 atmospheres of oxygen. Cells grown in pure culture possess a somewhat higher pO_2 value.

Carbohydrate Metabolism

The partially anaerobic condition in the nodule interior would promote fermentation reactions. Virtanen (100) demonstrated that both crushed nodules and pure cultures of R. trifolii could ferment glucose in an oxygen-free atmosphere. The products of this

fermentation were lactic, butyric and acetic acids and also ethyl alcohol, CO_2 and H_2. These observations were substantiated by Burris and Wilson (25) who showed CO_2 evolution from glucose under an atmosphere of hydrogen. Suspensions of either soybean bacteroids or cultured cells of R. japonicum carried out the fermentation. Lactate and acetate support rapid respiration in various Rhizobium species (25, 91). It would appear, therefore, that there are no apparent peculiar aspects in the metabolism of Rhizobium cells.

Rhizobia are usually divided into groups of fast and slow growers. Soybean, cowpea, and lupine rhizobia belong to the slow-growing group. A generation time on glucose for R. japonicum is 6.09 hours (4). R. meliloti of the fast-growing group required only 3.21 hours for a generation time on the same medium (4). It is now known that R. japonicum and other slow growers (17, 25, 91, 94) are not able to utilize carbohydrates as rapidly as organic acids. Neal (69) concluded that carbohydrates were incompletely oxidized by Rhizobium. According to him the rate of oxygen consumption increased consistently until approximately one-third of the theoretical yield of complete oxidation of various carbohydrates. At this time there was an abrupt decrease in both the rate of oxygen consumption and the respiratory quotient of the organisms. Tuzimura and Meguro (94) suggested that the enzymes necessary for the utilization of glucose are adaptive in R. japonicum. They studied the metabolism of this

organism after subjecting it to three types of environments: namely, those grown on glucose, those grown on succinic acid, and the symbiotic cells isolated from soybean nodules. Glucose was not oxidized by the symbiotic cells, but was oxidized slowly by those organisms grown on succinate. In contrast, cells cultured on glucose oxidized glucose rapidly. The utilization of several intermediates of glycolysis were tested and only fructose-1, 6-diphosphate was oxidized by both the symbiotic and the cultured cells. The fact that other phosphorylated substrates were not utilized however, may be due to a permeability problem.

Thorne and Burris (91) concluded that cowpea bacteroids may lack enolase since the addition of fluoride to suspensions of bacteroids was observed to stimulate respiration. When cultured cells were grown on glucose, the addition of fluoride was inhibitory. According to Wilson (102) a number of inhibitors known to inhibit the operation of the glycolytic sequence had no effect on the anaerobic production of CO_2 from glucose.

Pyruvate can be produced via the Entner-Duodoroff pathway in certain Rhizobium species (the fast-growing group) grown in pure culture (56). Both 6-phosphogluconate and the possible intermediate 2-keto-3-deoxy-6-phosphogluconate were rapidly utilized by these organisms. The participation of the Entner-Duodoroff pathway in rhizobia of the slow-growing group has not been investigated.

Citric Acid Cycle

The existence of the citric acid cycle in soybean bacteroids is supported by considerable evidence (25, 90, 91, 94). Succinate appears to be the best substrate for oxidation by soybean bacteroids, however, oxalacetate and several other four-carbon dicarboxylic acids also are rapidly utilized (17). Citrate is used less effectively as a substrate than other intermediates of the citric acid cycle. De Hertogh et al. (34) have shown that succinate, fumarate, malate, and methylmalonate are present and may be isolated chromatographically in cells from pure cultures of R. meliloti. According to Bergersen (18) soybean bacteroids are incapable of complete oxidation of intermediates of the citric acid cycle. Although no enzymatic evidence has been presented, it may be concluded that the citric acid cycle very likely is functional in rhizobia.

Glyoxylate Cycle

Johnson et al. (53) have reported a relatively high content of fatty acids in soybean nodules and in the bacteroids from nodules. Their investigation of the glyoxylate cycle in nodules showed the presence of malate synthetase in both soybean bacteroids and in R. japonicum cells grown in pure culture. No appreciable activity of isocitrate lyase was detected unless cells were cultured on a

medium containing oleate. They concluded that the glyoxylate cycle probably does not operate in the symbiotic growth of rhizobia.

Leghemoglobin Metabolism

Presence in Effective Nodules. Virtanen and Miettinen (99) have discussed in considerable detail the role of leghemoglobin in nodules of leguminous plants. It is well established that the nitrogen-fixing capacities of legume root nodules is positively correlated with their leghemoglobin contents. Strains of Rhizobium that are effective in symbiotic nitrogen fixation induce the synthesis of high concentrations of leghemoglobin in root nodules, whereas, ineffective strains induce the formation of nodules with little or no leghemoglobin (44, 99). The concentration of leghemoglobin in nodules fluctuates throughout the growing season. The time of maximum pigmentation is at or just before flowering and is coincident with the maximum dry weight and nitrogen content of the plant (97, 98). Leghemoglobin in soybean nodules can reach a concentration of 5×10^{-4} M or 3.25 mg/gm (fresh weight) of nodule tissue (81, 82).

Location of Leghemoglobin. Smith (81) has shown that the appearance of leghemoglobin in nodules coincides with the differentiation of rhizobia cells into bacteroids. This conclusion is supported by the report of Heumann (50). Furthermore, Smith (81) has demonstrated that leghemoglobin is found only in the large plant cells of

the nodules that contain bacteroids. Electron micrographs by Bergersen and Briggs (21) demonstrate that the bacteroids in the infected cells are arranged in groups enclosed by a membrane envelope. This membrane is described as a double membrane probably arising from the host plant. Leghemoglobin is found outside the bacteroids but enclosed by the surrounding membrane. The pocket formed by this membrane, enclosing a group of bacteroids along with leghemoglobin, has been postulated to be the site of nitrogen fixation (21), however, this hypothesis has not been confirmed.

Biosynthesis of Hemoglobin and Leghemoglobin. The pathway of porphyrin and subsequently heme biosynthesis has now been demonstrated by the use of radioisotopes and enzymatic techniques (79, 80). All of the carbon atoms of heme can be derived from acetate and glycine. In animals, only the methylene carbon of glycine is incorporated into the porphyrin molecule. The carboxyl carbon is liberated as CO_2. Glycine contributes nitrogen to all four pyrrole rings and donates eight of the 34 carbon atoms of protoporphyrin IX (39, p. 866). The pathway of acetate utilization in porphyrin synthesis is through the citric acid cycle which leads to the formation of succinyl-CoA. Succinyl-CoA is then condensed with glycine to form the direct precursor of porphyrins, δ-aminolevulinic acid. Richmond and Salomon (76) demonstrated by the use of specifically labeled glycine-C^{14} and acetate-C^{14} that the general sequence of

reactions leading to heme formation in soybean nodules was similar to that reported in animal systems. They observed, however, that the carboxyl carbon atom of glycine was slowly incorporated into heme. The conversion of radioactive sodium carbonate into heme did not account for this incorporation. In additional experiments, the conversion of glycine-2-C^{14} into heme was stimulated by the addition of either fluoride or acetate. It was suggested (76) that the stimulation by acetate might be due to its direct conversion to succinyl-CoA. In soybean nodules the activation of propionate and conversion to succinyl-CoA via methylmalonyl-CoA makes possible a second mechanism for the formation of succinyl-CoA (32, 34).

Neither the plant cells alone nor pure cultured rhizobia cells are capable of synthesizing leghemoglobin. Falk and coworkers (37) have suggested that the capacity for protoporphyrin IX synthesis has degenerated in nodule bacteroids. According to Falk et al. (37) the mature bacteroids from nitrogen-fixing soybean nodules were capable of catalyzing porphyrin synthesis in vitro only as far as uroporphyrin. This conclusion is substantiated by an analysis showing that uroporphyrin is the predominate porphyrin compound in effective soybean nodules. Bacteroids isolated from effective nodules yielded no protoporphyrin and relatively small amounts of copro- and uroporphyrins from δ-aminolevulinic acid.

Effect of Cobalt Deficiency on Leghemoglobin Content. In 1949

Gall (40) reported that a low hemoglobin content in sheep was correlated with cobalt deficiency, and that this condition could be alleviated by the injection of vitamin B_{12}. It is now known that both vitamin B_{12} and its benzimidazole analog will alleviate the symptoms of pernicious anemia in humans (29, p. 47-49). Recently Barness et al. (14) 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; however, information concerning precise sites where the vitamin functions is incomplete. Vitamin B_{12} also may be involved in the biosynthesis of leghemoglobin since Ahmed and Evans (3) demonstrated that a significant reduction in the hemoglobin content of nodules occurs when soybeans are grown under conditions of cobalt deficiency. Furthermore Kliewer and Evans (59) have shown that the rates of increase in concentrations of both B_{12} coenzyme and leghemoglobin are nearly parallel during the development of soybean nodules. Wilson and Reisenauer (101) have shown that the nodules of symbiotically-grown alfalfa contain only a trace of leghemoglobin when 0.001 parts per billion of cobalt are supplied in the nutrient medium. When 0.010 parts per billion of cobalt was supplied, the leghemoglobin content of the nodules increased to 0.73 mg per cc of nodule tissue.

Propionate Metabolism

General Pathways of Propionate Formation

Several pathways for the formation of propionate in microorganisms have been described, but no direct evidence was found in the literature indicating pathways of propionate formation in the nodules of legumes or in pure cultures of Rhizobium. It has been known for some time that propionate is produced by bacterial fermentation of carbohydrates or lactate (13, 36, 52, 65). Ruminant animals derive the major part of their energy requirements from propionate and acetate which accumulate from the fermentation of carbohydrates (65). Two possible routes for the formation of propionate in rumen microorganisms are known. The first is referred to as the acrylate pathway, or in some cases the non-randomizing or direct reductive pathway, whereby lactate is fermented to propionate. The second pathway is referred to as the succinate or dicarboxylic acid pathway.

Baldwin and coworkers (11, 13) have demonstrated that lactate is converted to propionate via the acrylate pathway in the rumen microorganism Peptostreptococcus elsidenii. The intermediates in this sequence are outlined as follows:

1. acetyl-CoA + lactate $\longleftrightarrow$ lactyl-CoA + acetate

2. lactyl-CoA $\longleftrightarrow$ acrylyl-CoA + H_2O

3. acrylyl-CoA + $2H^+$ $\longleftrightarrow$ propionyl-CoA

4. propionyl-CoA + lactate $\longleftrightarrow$ lactyl-CoA + propionate

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Net reaction: lactate + $2H^+$ $\longleftrightarrow$ propionate + H_2O

The enzymes involved in this series of reactions are: (1) CoA transphorase, (2) lactyl-CoA dehydrase, (3) acyl-CoA dehydrogenase, and (4) CoA transphorase. Each of these enzymes has been identified, partially purified and characterized.

This pathway for the conversion of lactate to propionate via acrylate is also present in C. propionicum (27, 64). Cardon (27) showed that C. propionicum could ferment alanine, serine, pyruvate, lactate, and acrylate into propionic and acetic acids. The relative amounts of the two volatile acids depended on the state of oxidation of the substrate. It was suggested that acrylate was an intermediate in this pathway.

From recent work of Baldwin and coworkers (12, 13) it appears that the succinate pathway is the most important pathway of propionate formation in the rumen. It is generally considered that carbohydrates would be catabolized to pyruvate which is then converted to oxalacetate by the fixation of carbon dioxide. Oxalacetate is reduced to succinate, which in turn is esterified with CoA and decarboxylated to propionyl-CoA and CO_2.

An early paper by Johns (52) described the isolation of a Micrococcus species from sheep rumen. This organism was a strict anaerobe, but did not ferment any of the sugars tested. It did ferment pyruvic, oxaloacetic, l-malic and fumaric acids with the production of propionic, acetic and carbonic acids along with H_2. The product of succinic acid fermentation was propionic acid and CO_2. This provided evidence for a decarboxylation of succinic acid. The intermediates in this conversion were suggested when Swick and Wood (87, 88) provided evidence for the following set of reactions in P. shermanii:

1. Succinyl-CoA $\longleftrightarrow$ Methylmalonyl-CoA
2. E + Methylmalonyl-CoA $\longleftrightarrow$ Propionyl-CoA + E-C_1
3. E-C_1 + Pyruvate $\longleftrightarrow$ Oxaloacetic + E
4. Oxaloacetate + $4H^+$ $\longleftrightarrow$ Succinate
5. Succinate + Propionyl-CoA $\longleftrightarrow$ Succinyl-CoA + Propionate

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

The enzymes that 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 reduction to succinate is catalyzed by the citric acid cycle enzymes. Allen et al. (5) and Overath et al. (72) have modified this scheme by the discovery of methylmalonyl-CoA racemase. This enzyme, shown to be present in P. shermanii,

catalyzes the conversion of one isomer of methylmalonate to another. Methylmalonyl-CoA mutase in P. shermanii (84) as well as in other organisms (34, 83) requires a B_{12} coenzyme for activity.

Several other pathways for the formation of propionate in microorganisms are known. Propionate can arise by the catabolism of the following amino acids: valine (58), isoleucine (77; 45, p. 106), threonine (45, p. 94) and homoserine (67). The catabolic pathways by which propionate may arise from each amino acid has been reviewed by De Hertogh (31) and will not be discussed here. In addition, propionate may arise by the alpha-oxidation of long chain fatty acids (66). Once an odd-chain fatty acid is formed, it could be converted to propionate via the beta-oxidation pathway. Propionate may also be formed from α-ketoglutarate. Recent findings by Valentine and Wolfe (96) show that α-ketoglutarate dehydrogenase can be a source of electrons in Micrococcus lactilyticus which can reduce ferredoxin and evolve hydrogen. The reaction proceeds as follows:

$$\alpha\text{-ketoglutarate} \xleftarrow{\text{Ferredoxin}} H_2 + CO_2 + \text{propionyl phosphate}$$

The propionyl phosphate formed possibly could give rise to either propionyl-CoA or propionate.

Pathway of Propionate Utilization in Bacteroids

Except for the work of De Hertogh et al. (32, 34) there is no indication in the literature of propionate utilization either in legume

nodules or by Rhizobium species. Manometric experiments by these workers showed that propionate and other fatty acids were rapidly oxidized by cell suspensions of both R. japonicum from pure cultures and by bacteroids from soybean nodules. The pathway of propionate oxidation in soybean bacteroids (34) is essentially the same as that established for propionate utilization in animals. The pathway proceeds via the following reactions (15, 38, 68):

1. Propionate + ATP $\longleftrightarrow$ Propionyl-CoA + AMP + PPi
2. Propionyl-CoA + CO_2 + ATP $\longleftrightarrow$ Methylmalonyl-CoA (a) + ADP + Pi
3. Methylmalonyl-CoA (a) $\longleftrightarrow$ Methylmalonyl-CoA (b)
4. Methylmalonyl-CoA (b) $\longleftrightarrow$ Succinyl-CoA
5. Succinyl-CoA + GDP + Pi $\longleftrightarrow$ Succinate + GTP
6. GTP + ADP $\longleftrightarrow$ ATP + ADP

Net reaction: Propionate + ATP + CO_2 $\longleftrightarrow$ Succinate + AMP + PPi

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

This reaction sequence was conclusively established in soybean bacteroids and in pure cultured Rhizobium species. Radiorespirometric experiments utilizing propionate-C^{14} labeled in specific positions (34) demonstrated that bacteroids oxidize propionate via the

pathway just previously listed and not appreciably by other pathways (26, 42, 43, 95). Furthermore, De Hertogh et al. (34) showed that cell suspensions of R. meliloti that were incubated with propionate-1-C^{14} converted radioactivity into succinate, fumarate and malate of the citric acid cycle and into methylmalonate. These data indicated that the succinate formed from propionate was further oxidized by the citric acid cycle. Enzymatic experiments demonstrated that propionyl-CoA carboxylase and methylmalonyl-CoA mutase were active and widely distributed in Rhizobium species.

Cobalt Requirement for Propionate Utilization in Rhizobium

The role of B_{12} coenzyme in the conversion of methylmalonyl-CoA to succinyl-CoA was first implicated by the work of Smith and Monty (83) who noted that extracts of livers from B_{12} deficient rats exhibited a low methylmalonyl-CoA mutase activity. Gurnani et al. (46) demonstrated that the addition of dimethylbenzimidazolylcobamide (DBC) coenzyme to extracts prepared from B_{12} deficient rats markedly increased the methylmalonyl-CoA mutase activity of extracts. Stadtman et al. (84) successfully resolved the apoenzyme of methylmalonyl-CoA mutase from the coenzyme in extracts of P. shermanii by use of charcoal treatment and showed that the addition of DBC coenzyme restored activity. This research was followed by

a report by Stern et al. (86) that DBC coenzyme stimulated the activity of methylmalonyl-CoA mutase in extracts of the liver from a normal ox. Furthermore, B_{12} deficient cells of Ochromonas malhamensis (8) and Flavobacterium species (9, 10) oxidize propionate at a significantly reduced rate in comparison to control cultures. The addition of vitamin B_{12} restored the capacity of these organisms to utilize propionate.

The rate of propionate oxidation by R. meliloti cells is known to be strikingly affected by the cobalt concentration in the culture medium (34). Propionyl-CoA carboxylase activity in cell-free extracts of R. meliloti was not appreciably affected by cobalt deficiency but, in contrast, the activity of the methylmalonyl-CoA mutase in the extracts was strikingly influenced by the cobalt level in the culture medium. Methylmalonyl-CoA mutase activity comparable to that obtained with extracts from cells provided with adequate cobalt was obtained by the addition of DBC coenzyme to enzyme extracts of deficient cells. In additional experiments, the endogenous B_{12} coenzyme associated with methylmalonyl-CoA mutase was destroyed by direct light and activity was restored by the addition of DBC coenzyme. Partial restoration of activity was obtained by the addition of benzimidazolylcobamide coenzyme. The adenylcobamide coenzyme was completely ineffective in the methylmalonyl-CoA mutase system from Rhizobium.

MATERIALS AND METHODS

Source of Chemicals

Recrystallized hemin, glutathione and the dipotassium salt of ATP were obtained from Nutritional Biochemicals Corporation. Sodium propionate-2-C^{14} (5.15 mC/m mole), acetate-2-C^{14} (2.0 mC/m mole), citrate-1,5-C^{14} (5.32 mC/m mole), α-ketoglutarate-5-C^{14} (6.94 mC/m mole), succinate-2-C^{14} (9.1 mC/m mole) and δ-aminolevulinate-4-C^{14} (11.7 mC/m mole) were purchased from New England Nuclear Corporation and fumarate-2,3-C^{14} (6.2 mC/m mole), lactate-1-C^{14} (5.47 mC/m mole) and lactate-2-C^{14} (11.7 mC/m mole) were obtained from the Volk Radiochemical Company. Coenzyme A was purchased from the Sigma Chemical Company and other chemicals and solvents used were reagent grade from commercial sources.

The sodium salts of acrylic, lactic, propionic and other organic acids were prepared by neutralization with NaOH. Phosphate buffers were prepared from 0.2 M stock solutions of K_2HPO_4 and KH_2PO_4.

Biological Materials

The biological materials utilized in these experiments were:

soybeans (Glycine max Merr. variety Chippewa), cowpeas (Vigna sinensis L. variety Iron clay mixed) and Rhizobium meliloti (F-29). The Rhizobium strain was a gift from Dr. J. C. Burton of the Nitragin Company.

Cultural Procedures

Soybeans were grown in a greenhouse using the cultural methods described by Ahmed and Evans (2, 3). Inoculated soybean seeds were planted in plastic pots containing sterilized 'Perlite'. Fluorescent lights were utilized to maintain a 16 hour light period. The plants were supplied with about 500 mls of a nitrogen-free nutrient solution four times each week and flushed thoroughly with water on the days when the nutrient solution was not supplied. The nodules were harvested when plants were 38 to 48 days old. Precise ages of nodules are given in the legends describing the various experiments.

The methods employed for purifying the culture medium and growing R. meliloti with and without added cobalt have been described in previous publications (2, 3, 61, 33). Cells were cultured in one-liter flasks containing 400 ml of purified medium grown at 30° C on a rotary shaker. Both the cobalt-deficient cells and the cells receiving 1 ppm cobalt were cultured from a 1 ml inoculum of a cobalt-deficient culture maintained in 50-ml culture flasks.

Determination of Cytochrome Concentration

For these experiments, cells of R. meliloti were grown in liquid culture with and without added cobalt by procedures referred to in the previous section. After 56 hours of growth, cells from several flasks of cobalt deficient cultures were harvested and combined in order to obtain sufficient material. The concentration of the b- and c-type cytochromes was determined by the microspectroscopic method of Hartree (49). Equal weights of normal and cobalt-deficient cells were utilized. A slurry of 0.475 gm (wet weight) of cells in 0.05 M tris succinate buffer was reduced with sodium hydrosulfite and placed in the center well of a culture slide which was 1.6 cm in diameter and 0.3 cm deep. A solution containing 0.01 μmoles per ml of authentic cytochrome C was used as the standard in the movable tray. The concentration was calculated in μmoles per ml by the following equation:

$$\text{Conc. of Cyto. C in tray} \times \frac{\text{Depth of known standard}}{\text{Depth of unknown sample}} \times \frac{\text{vol. of sample}}{\text{vol. of bacteria}} = \frac{\mu\text{moles Cyto. C}}{\text{ml bacteria}}$$

Isolation and Purification of Heme

The method for the isolation of heme from legume nodules was adapted from the methods described by Richmond, Altman, and

Salomon (75), Chu and Chu (28), and Kiese and Kurz (57). Nodules were washed thoroughly and then macerated in a mortar with an equal volume of water. The residue was removed by centrifugation. The supernatant was then adjusted to pH 3 with glacial acetic acid after which sufficient acetone was added to make an 80 percent acetone solution. The precipitate was removed by centrifugation. Twenty mls of chloroform were then added to the acetone solution and the two phases allowed to stand for one to two hours at room temperature. The heme was then thoroughly extracted with chloroform. After separation, the combined chloroform extracts were washed four times with 200 mls of distilled water. The chloroform solution was evaporated to dryness and the resulting residue taken up in 0.5 ml pyridine. When the pyridine hemochromogen in this sample was chromatographed, a fluorescent spot typical of porphyrins was evident under ultra-violet light. In order to obtain radioactive heme with essentially constant specific activity, the pyridine hemochromogen was added to sufficient water to make a one percent solution and passed through a silicone-impregnated cellulose column. The procedure has been described in detail by Hulcher and Vishniac (51). The column was then washed several times with water and the heme was eluted with pyridine-propanol-water (1:3:12.5) v/v. The solvent was subsequently evaporated and the heme taken up in 1.0 ml pyridine for determination of radioactivity and chromatography.

The efficiency of this procedure for the extraction of heme from whole nodules was 50 to 55 percent and resulted in heme of high purity as evidenced by the data in Table 1. Successive chromatography through a column gave nearly constant specific activity.

Table 1. Purity of radioactive heme isolated from cowpea nodules.[a]

Times chromatographed (No.)	Heme isolated (μmoles)	Specific activity of heme (CPM/μmole)	Standard deviation
1	0.54	27,900	±280
2	0.29	27,800	±278
3	0.18	27,300	±273

[a] Thirty-nine day-old cowpea nodules were infiltrated with 5 μC of δ-aminolevulinic-4-C^{14} acid under the standard incubation conditions. Heme was isolated, purified and assayed by the procedures outline in the Materials and Methods section after 12 hours of incubation.

Identification of Heme

Heme isolated from soybean nodules was identified by both its absorption spectrum and R_f value following the chromatographic procedure of Chu and Chu (28). The absorption spectrum of heme isolated from nodule leghemoglobin was essentially identical with that of authentic recrystallized hemin. These spectra were recorded with a Cary model 11 spectrophotometer. The isolated pyridine hemochromogen described in the previous section was co-chromatographed with authentic heme in two different solvents. The

chromatography chamber consisted of a 1000 ml battery jar with an air tight cover and a 250 ml beaker served as the container for the solvent system. The battery jar was lined with wet filter paper and the atmosphere within each jar was saturated with 0.4 ml pyridine. Whatman No. 1 filter paper was cut into 14.5 × 14.5 cm sheets and dipped into a petroleum ether (b.p. 65-110° C) solution containing Dow-Corning silicone 550 (100 ml/12.5 gm). The sheets were dried for five minutes in an oven at 100° C. The chromatograms were developed at room temperature and dried at 105-110° C. Spots could be detected visually or by use of fluorescence after ultraviolet irradiation. In the solvent system consisting of water-n-propanol-pyridine (5.5:0.1:0.4) the R_f value of the standard was 0.59 while that of the sample was 0.56. When lutadine was used as the solvent, the R_f values were equal.

Determination of Heme

The concentration of the extracted heme was determined by measurement of the optical density of reduced pyridine hemochromogen at 555 mμ. The following procedure (49) was utilized. An aliquot of the unknown heme solution, in pyridine, was placed in a test tube containing 0.6 ml 1 N NaOH, 4.2 ml distilled water, and enough pyridine to make the final volume up to 6.0 ml. A blank was made without the addition of the heme. Twenty-eight mg of $Na_2S_2O_4$ were

added to each tube. After 30 minutes the optical density was read at 555 mμ in a 3.0 ml cuvette with a one cm light path. The concentration of heme was determined from a curve using recrystallized hemin as the standard.

Assay of Radioactivity

In one experiment, radioactive carbon dioxide was collected and converted into $BaCO_3$, then plated and assayed for radioactivity as described by Ormerod (71). In all other experiments radioactivity measurements of plated samples were made at infinite thinness. A self-absorption curve was prepared for radioactive heme which showed that the curve was linear to a concentration of 0.1 μmole heme. A sample aliquot containing 0.04 to 0.08 μmole heme was evaporated in a glass planchet by a warm jet of air. A Nuclear Chicago model 181 B gas flow detector with an efficiency of 12 to 15 percent was used for counting. The specific activity (CPM/μ mole heme) and background were determined for each sample. Most samples were counted to a standard deviation of 1.4 percent (5000 total counts). For the determination of heme purity and comparison of rates of incorporation into heme in whole nodules by different metabolites, samples were counted to a standard deviation of one percent.

In a typical experiment (Figure 7) the heme content of the four

gm nodule samples varied from 1.01 to 1.19 μ moles of heme. The total extracted heme of the samples in this experiment ranged from 0.42 to 0.58 μmole heme per sample. The percent incorporation of radioactivity into heme was from 0.8 to 1.0 percent. In order to prove that no appreciable radioactive contamination influenced the specific activity of the heme samples, a representative purified sample was chromatographed on paper and the dry strip of paper scanned with a Vanguard strip counter. From the data in Figure 1 it seems obvious that radioactivity was limited primarily to one spot corresponding to authentic heme.

Standard Incubation Procedure

Nodules were harvested into ice water, cleaned free of debris and weighed into 4.0 gm (wet weight) lots. Each sample was then placed into 6.0 ml of 0.1 M phosphate buffer at pH 5.6 or as indicated in the legends below appropriate Figures and Tables. With whole nodules, maximum incorporation of propionate was obtained at pH 5.6 and this pH was used as the standard. The 6.0 ml buffer solution contained 50 μmoles glutamate, 12 μmoles glycine, 5 μC isotope, and 12 μmoles of non-radioactive carrier to reduce the effect of the normal pool sizes. In order to introduce the isotope into the nodule tissues, each sample of nodules immersed in the incubation medium was evacuated at 70 mm Hg for three minutes. Since the rate of

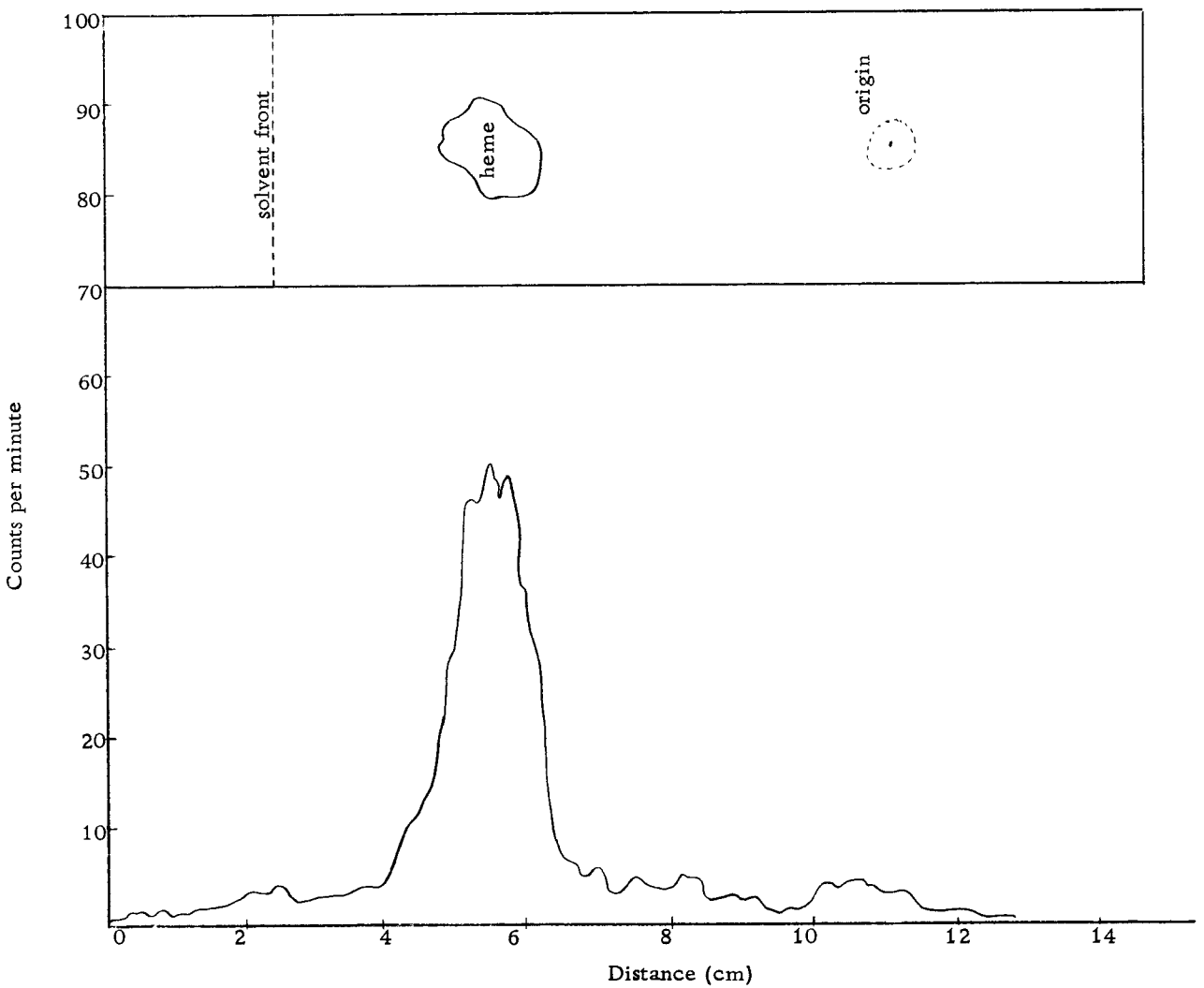

Figure 1. Radiogram of heme isolated from nodules. Four grams of soybean nodules were incubated with 5.0 μC of delta-aminolevulinic-4-C^{14} acid for nine hours following standard incubation and heme isolation procedures.

incorporation of propionate-2-C^{14} into the heme moiety of leghemoglobin varied somewhat with each sample of nodules, the rates of incorporation compared in each Figure and Table were determined from a single sample of nodules. An early experiment demonstrated that virtually no radioactivity was incorporated into heme from either propionate-2-C^{14} or succinate-2-C^{14} in a boiled nodule extract; therefore, a control of boiled nodules or nodule extract was not included in each experiment.

Separation and Identification of Organic Acids

The ether extraction method of Swim and Utter (89) was followed in detail in the preparation of nodule and bacteroid extracts for column chromatography. The homogenate from 10 gm fresh soybean nodules was acidified to pH 2.0 with 10 N H_2SO_4 and thoroughly mixed with 20 gm dry Celite. The mixture was then packed in a 20 mm (inside diameter) column and organic acids were eluted with ether. After collecting 500 ml eluate, small aliquots were collected at suitable intervals and titrated with 0.009 N NaOH to determine when extraction was complete. Phenol red was used as indicator. The ether was evaporated in the hood by the use of a warm jet of air and the residue was taken up in 1.5 ml of 0.2 N H_2SO_4 to be applied to a new column.

This method was modified somewhat for thin-layer

chromatography. After evaporation of the ether extract, the residue was taken up in 2.0 ml water which was further purified by passing it through a Dowex 50W-X8 cation exchange column. The eluate was evaporated and taken up in 0.5 ml of 95 percent ethanol. A 100 lambda aliquot was applied to glass plates covered with a 250 micron layer of Silica Gel G.

The volatile fatty acids were separated by steam distillation of acidified homogenates. Nodule homogenates were prepared by maceration of 10 gm fresh nodules in a mortar while bacteroid homogenates were prepared by breaking the cells in an Eaton press. The steam distillate was neutralized by titration with 0.2 N NaOH and evaporated in vacuo. The residue was taken up in 1.5 ml of 0.2 N H_2SO_4 and used for application to a Celite column.

The Celite column was prepared by mixing 10 gm of dry ether-washed Celite with 5 ml of 0.2 N H_2SO_4. The Celite was then saturated with chloroform (equilibrated with 0.2 N H_2SO_4) and packed in a 20 mm (inside diameter) column. The extract, in 1.5 ml of 0.2 N H_2SO_4, was mixed with 3 gm Celite and added to the top of the column. The acids were eluted from the column using chloroform-n-butanol solutions increasing the n-butanol content from 0 to 50 percent. One hundred ml of each solution was forced through the column under two pounds of air pressure. The effluent was collected in 18 × 150 mm tubes by a fraction collector equipped with a 10 ml

volume siphon. The organic acids were identified by comparison of the peak effluent values obtained from standard acids chromatographed on identical Celite columns. The concentration of the various acids was determined by the titration of each fraction with 0.009 N NaOH after the addition of five ml carbon dioxide free, distilled water. The peak effluent values of the volatile acids are shown in Figure 2. Butyrate and propionate were eluted with 100 percent chloroform, acetate with 95 percent chloroform-butanol and formate with 90 percent chloroform-butanol. Propionate was further identified by the use of C^{14}-labeled propionate. One μC propionate-2-C^{14} was added to a standard mixture and the radioactivity measured in each fraction coming from the column. The radioactive peak coincided with the titration peak. Succinate and malate isolated from a Celite column were confirmed by co-chromatography on thin-layer plates with standard acids.

Preparation of Cell-free Extracts

Cell-free extracts of soybean nodules for enzymatic studies were obtained by freezing the nodules in two volumes of 0.1 M phosphate buffer at pH 7.0 and breaking the cells in an Eaton press at a pressure of 8000 pounds per square inch. The cell debris was removed by centrifugation and the extract dialyzed under argon for five to six hours in two liters of 0.1 M phosphate buffer at pH 7.0.

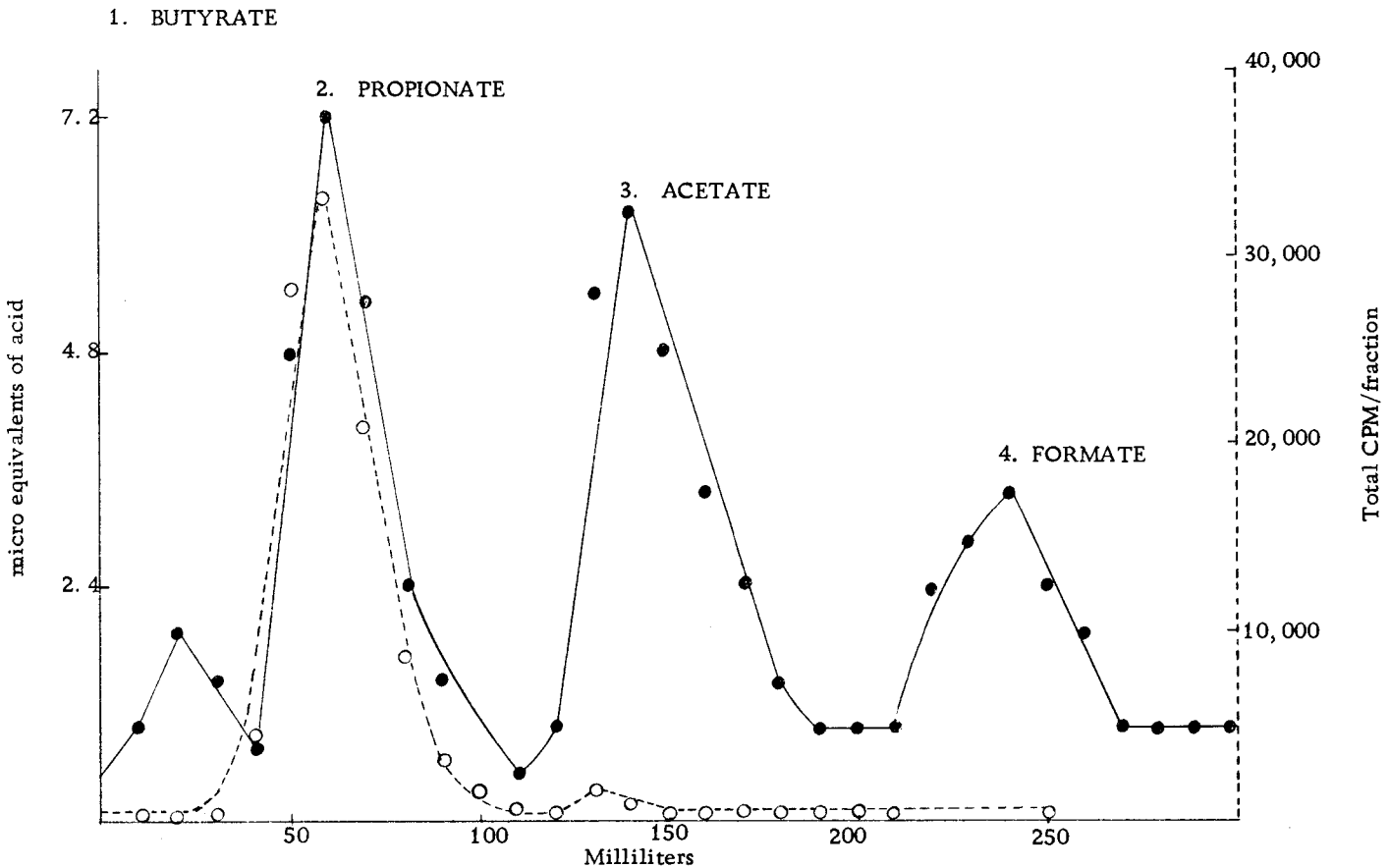

Figure 2. Chromatographic separation of a mixture of standard volatile fatty acids. The sample contained 3μ moles butyrate, 19μ moles propionate with 1 μ C propionate-2-C^{14}, 14 μ moles acetate and 8 μ moles formate. Acids were eluted from an acidified Celite column (20 x 75 mm) with chloroform and chloroform-butanol solutions in 10 ml fractions.

For the preparation of cell-free extracts of bacteroids, samples of bacteroids were collected by centrifugation from nodule homogenates (nodules macerated in an equal volume of 0.1 M phosphate buffer at pH 7.0) which had been squeezed through four layers of cheesecloth to remove the solid debris. The bacteroids were then washed successively with buffer until all traces of hemoglobin were removed. A slurry of the bacteroids (1:1 wt/v) was frozen in a pressure cell in dry ice and the cells broken in an Eaton press. After centrifugation, the cell-free extract was dialyzed as above and stored under argon at -70°C. Protein determination was made by the Biuret method (30).

RESULTS

Effect of Cobalt Deficiency on the Cytochrome Content of R. meliloti

As pointed out previously cobalt deficiency in soybean plants results in a decreased leghemoglobin content of nodules. Since the heme moiety of leghemoglobin and the prosthetic group of the b- and c-type cytochromes are chemically similar, if not identical, it was of interest to determine whether or not cobalt deficiency in pure cultures of Rhizobium had an effect on the content of the b- and c-type cytochromes in this organism. For this experiment R. meliloti cells were cultured in highly-purified liquid media some of which lacked cobalt and some of which contained one part per billion cobalt as indicated in Table 2. Both cultures were inoculated from a cobalt-deficient stock culture and harvested at the same time. From the data (Table 2) it is apparent that the cytochrome content of normal R. meliloti cells was almost double that in cobalt-deficient cells. These differences are statistically significant as indicated by the standard deviations calculated from four determinations of each sample. The results are therefore consistent with the observations that have been made in which the effect of cobalt deficiency on the leghemoglobin content of nodules has been investigated. Cobalt deficiency appears to result in a decreased biosynthesis of the heme

moiety of hemoproteins. This led to an investigation of the possible significance of the B_{12} coenzyme dependent pathway for propionate utilization which could supply precursors for heme biosynthesis in nodules.

Table 2. Effect of cobalt deficiency on the cytochrome content of pure cultured R. meliloti cells.

Condition of cells	Cobalt added to medium (ppb)	Cytochrome content[a] (μM/gm wet wt)	Standard deviation
Normal	1.0	0.12	±0.007
Deficient	0.0	0.07	±0.009

[a]Results expressed on the basis of crystalline cytochrome C measured by the Hartree microspectroscope method.

Incorporation of C^{14}-labeled Metabolites into Heme

Since cobalt deficiency reduced the rate of heme synthesis, experiments have been carried out using C^{14}-labeled metabolites to find out if propionate is actually converted into the heme moiety of leghemoglobin. In preliminary experiments it was shown that sliced, homogenized or whole nodules effectively incorporated labeled propionate into the heme moiety of leghemoglobin. The rate of incorporation of propionate-2-C^{14} into heme in whole nodules (Figure 3) is linear with time. The results of several replicate experiments confirmed the data in Figure 3. In experiments not reported here it was

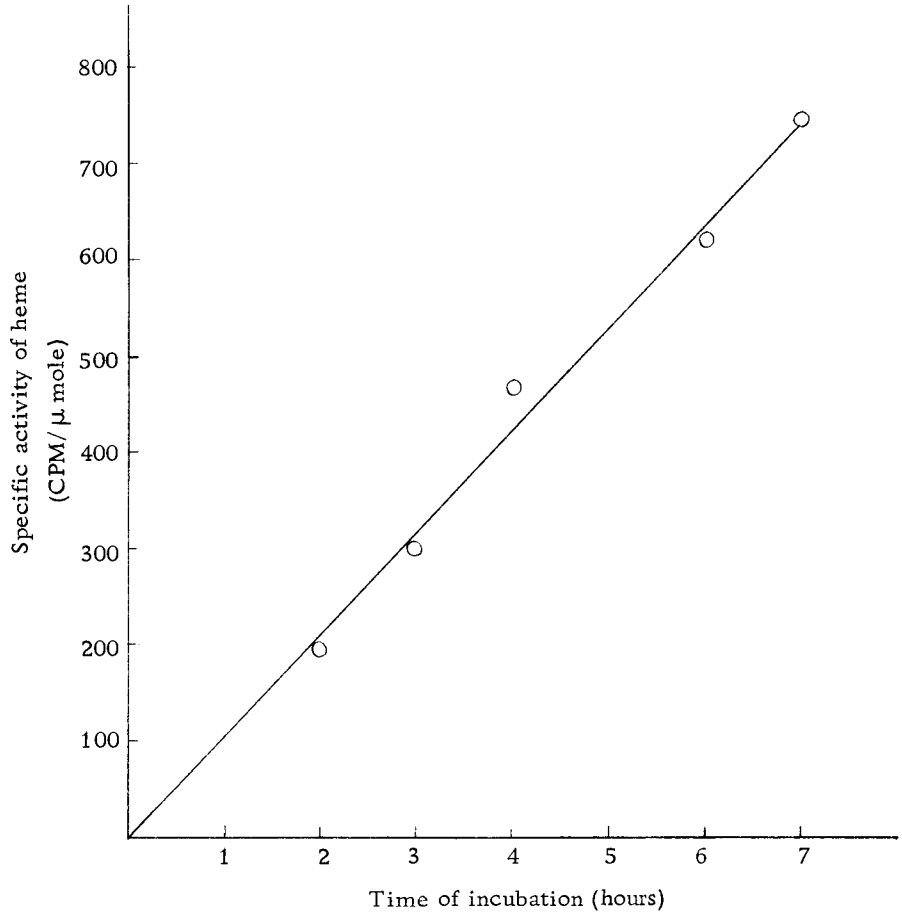

Figure 3. The rate of incorporation of propionate-2-C^{14} into the heme component of leghemoglobin. Whole nodules from 38 day-old soybean plants were utilized and incubation was conducted as described under Materials and Methods.

observed that propionate-C^{14} incorporation into the heme of leghemoglobin proceeded at a slower rate in nodules from plants that had begun to flower.

Since it was concluded that propionate could function as a heme precursor, additional experiments were needed to determine the relative rate of propionate incorporation in relation to the rate of incorporation of other metabolites. Because the pool size of the various organic acids in the nodules were unknown, non-radioactive carrier was added along with each radioactive compound in each incubation mixture. Soybean nodules from the greenhouse were harvested after 39 days and the time course (specific activity of heme vs. time of incubation) was determined for the incorporation of δ-aminolevulinate-4-C^{14}, acetate-2-C^{14}, propionate-2-C^{14}, citrate-1,5-C^{14}, and succinate-2-C^{14} (Figures 4 and 5). The nodules were incubated with 5 μC of each labeled compound under the incubation procedures described under Materials and Methods. In a series of replicate experiments the relative rates of incorporation of radioactive organic acids have been reproducible and have shown that propionate is incorporated into heme at a faster rate than most intermediates of the citric acid cycle (Figure 4). The results in Figure 5 indicate that δ-aminolevulinate-4-C^{14} is incorporated very rapidly into the heme component of leghemoglobin. Under all conditions tested acetate-2-C^{14} was incorporated into heme more

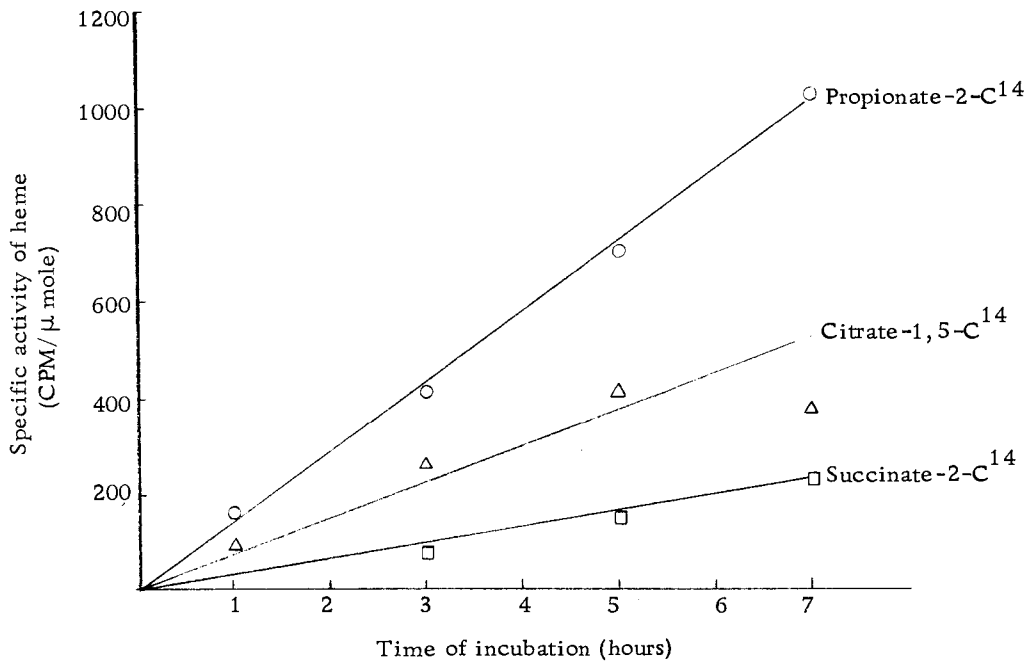

Figure 4. The rate of incorporation of propionate-2-C^{14} into the heme component of leghemoglobin compared to the rate of incorporation of citrate-1,5-C^{14} and succinate-2-C^{14}. Incubations were conducted as described under Materials and Methods utilizing 39 day-old soybean nodules.

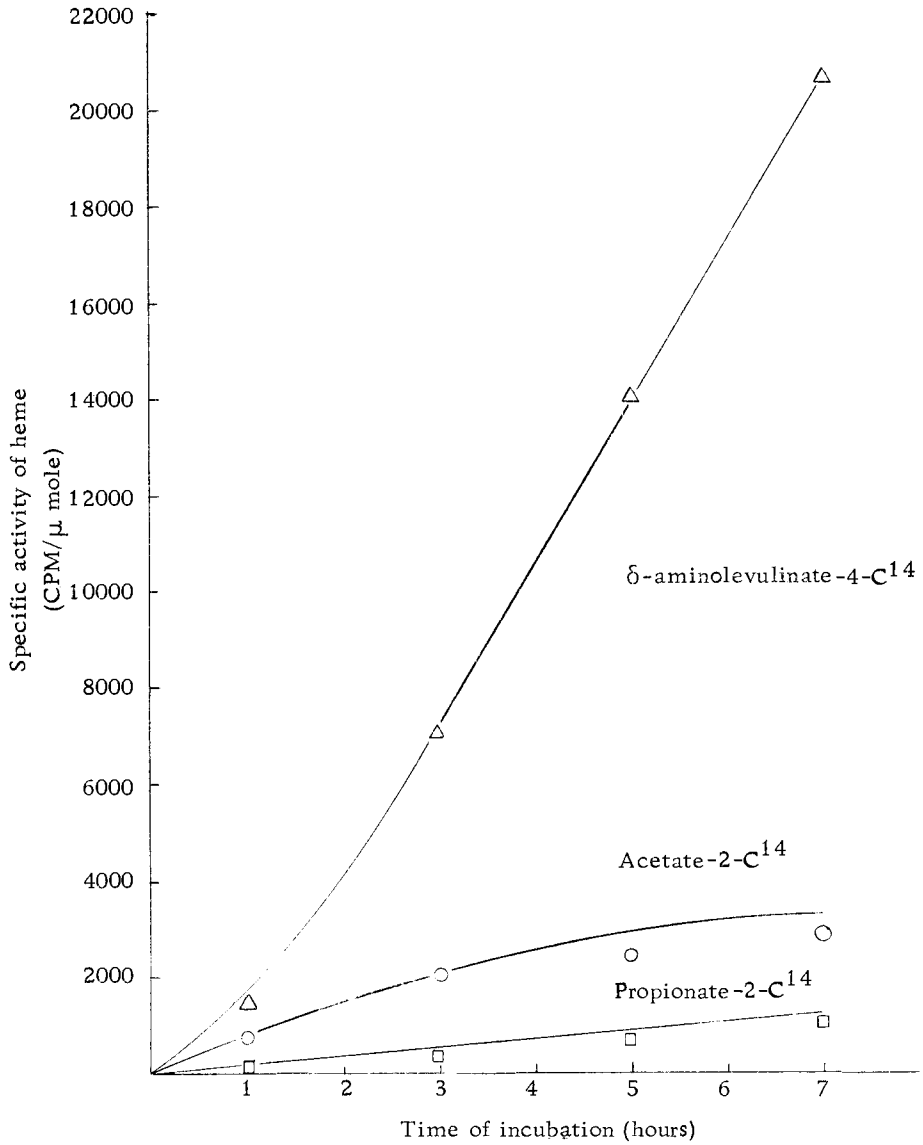

Figure 5. The rate of incorporation of propionate-2-C^{14} into the heme component of leghemoglobin compared to the rate of incorporation of acetate-2-C^{14} and δ-aminolevulinate-4-C^{14}. Incubations were conducted as described under Materials and Methods utilizing 39 day-old soybean nodules.

rapidly than radioactive propionate and intermediates of the citric acid cycle. After the experiments were completed it was established that 7.6 μC of acetate-2-C^{14} were added to the incubation mixture rather than 5 μC as indicated by the suppliers. This would account for the increased rate of incorporation.

Since the rhizobia initiating nodules on cowpea roots belong to the slow-growing group, the rate of propionate-2-C^{14} incorporation into heme was determined utilizing cowpea nodules. Forty-one day-old cowpea nodules were incubated with δ-aminolevulinate-4-C^{14}, propionate-2-C^{14} and succinate-2-C^{14} following the Standard Incubation Procedure described for soybean nodules. The rates of incorporation into heme of both δ-aminolevulinate-4-C^{14} and succinate-2-C^{14} were nearly the same as in 39 day-old soybean nodules (Figures 4 and 5). The rate of incorporation of propionate-2-C^{14} was still intermediate but was increased nearly 30 percent over the rate of incorporation of propionate-2-C^{14} in soybean nodules (Figure 4).

Identification of Non-volatile Organic Acids in Nodules

One parameter that would be expected to affect the rate of incorporation of isotopically labeled acids into heme is the concentration of the endogenous organic acids present in the nodules. It was deemed necessary therefore to isolate, identify and determine the concentration of the organic acids in nodules. Table 3 indicates

the micro equivalents of each acid present in 40 day-old soybean nodules. In this experiment, citrate was not detected, however, in other experiments this acid was present. The concentration of the various organic acids varied somewhat from one sample of nodules to another. From the analysis of the particular sample presented in Table 3 it is obvious that malate was present in the highest concentration and that α-ketoglutarate, succinate, fumarate and isocitrate were present in appreciable concentrations.

Table 3. Non-volatile organic acid content of soybean nodules.[a]

Acid	Concentration of acid (micro equivalents per gram nodule)
Fumaric acid	2.32
Succinic acid	3.77
Alpha-ketoglutaric acid	6.10
Malic acid	20.91
Citric acid	-----
Isocitric acid	2.90

[a]Acids were isolated from 40 day-old soybean nodules following the ether extraction procedure described in the Materials and Methods.

To identify the acids in soybean nodules a sample was prepared and subjected to thin-layer chromatography. The results of this experiment are presented in Figure 6. Again it is apparent that α-ketoglutarate, succinate, fumarate and malate are the principal

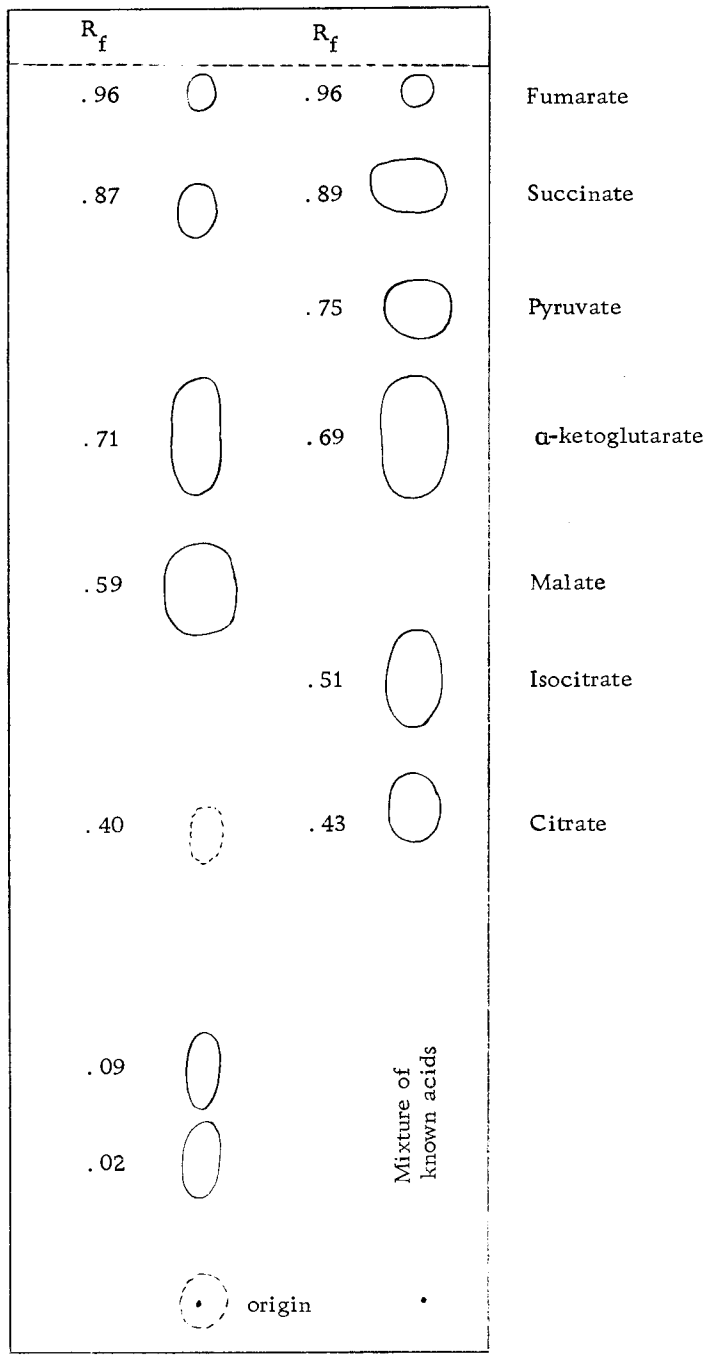

Figure 6. Comparison of the organic acids present in soybean nodules with a mixture of known acids by thin-layer chromatography. Four grams of 51 day-old nodules were incubated with 5 μC propionate-2-C^{14} under standard conditions. The acids were extracted following the procedure described in the Materials and Methods. A 100 lambda aliquot of the ethanol extract was applied to the plate.

acids in soybean nodules. Two additional acids with R_f values of 0.09 and 0.02 on the chromatogram were consistently observed, however, these were not identified. From these experiments it seems apparent that if citrate is present in nodules it occurs in only trace quantities insufficient in amount for detection in many experiments.

Incorporation of C^{14}-labeled Metabolites into Heme in Cell-free Extracts

In order to clarify the effect of the various pool sizes of the organic acids on the rate of incorporation of radioactive metabolites into heme, experiments were carried out using cell-free extracts of soybean nodules (Table 4). The incorporation of radioactive propionate into heme in cell-free extracts of nodules proceeded at an appreciable rate and it appears that dialysis failed to significantly influence the rate of incorporation. On the other hand, dialysis of the extract resulted in a two-fold increase in the rate of incorporation of α-ketoglutarate-5-C^{14} and lactate-1-C^{14} into heme. Dialysis increased the rate of incorporation of δ-aminolevulinate-4-C^{14} by approximately 23 percent. From this data it seems that α-ketoglutarate-5-C^{14} is converted into heme nearly four times faster than propionate-1-C^{14}. The possibility that the enzymes involved in propionate utilization are more labile than those involved in α-ketoglutarate

utilization should be considered.

Table 4. Incorporation of C^{14}-labeled precursors into heme by a cell-free extract from soybean nodules.[a]

Metabolite	Extract	Specific activity of heme (CPM/μmole)
Propionate-1-C^{14}	crude	99
Propionate-1-C^{14}	dialyzed	97
Alpha-ketoglutarate-5-C^{14}	crude	204
Alpha-ketoglutarate-5-C^{14}	dialyzed	394
Delta-aminolevulinate-4-C^{14}	crude	1785
Delta-aminolevulinate-4-C^{14}	dialyzed	2245
Lactate-1-C^{14}	crude	304
Lactate-1-C^{14}	dialyzed	681
Lactate-1-C^{14} plus 20 μmoles non-radioactive propionate	dialyzed	475

[a]Cell-free extract was prepared from 28 day-old soybean nodules. The reaction mixture in a final volume of 6 mls of 0.1 M phosphate buffer at pH 7.0 contained 12 μmoles metabolite containing 2 μC isotope; 10 μmoles of ATP and $MgCl_2$; 0.2 μmoles of CoA and NADH; and 0.260 μmole heme from leghemoglobin extract. Tubes were incubated 50 minutes at 38° C and reactions were stopped by the addition of 5 mls acetone at pH 3.0.

Effect of Acetate on the Rate of Incorporation of C^{14}-labeled Metabolites

Acetate enhances the rate of incorporation of C^{14}-labeled glycine into the heme moiety of leghemoglobin (76) and acetate-2-C^{14}

is more rapidly incorporated into heme than intermediates of the citric acid cycle. Thus, non-radioactive acetate as a sparker for the incorporation of propionate-2-C^{14} and other labeled organic acids into the heme moiety of leghemoglobin was examined. Figure 7 demonstrates that the rate of propionate-2-C^{14} incorporation into heme is still intermediate between the rates of incorporation of acetate-2-C^{14} and succinate-2-C^{14} in an experiment in which 12 μmoles acetate was added to each reaction mixture. With the addition of acetate as a sparker acid, the rate of incorporation of α-ketoglutarate-5-C^{14} was slightly more rapid than the rate of incorporation of propionate-2-C^{14}. The initial rate of incorporation of radioactive fumarate was nearly equal to that of succinate-2-C^{14} and citrate-1, 5-C^{14}. The effect of adding acetate to each reaction mixture on the rate of incorporation into heme of propionate-2-C^{14} and succinate-2-C^{14} is illustrated in Figure 8. The rate of incorporation of radioactive succinate was increased by approximately 50 percent, while the rate of incorporation of propionate-2-C^{14} into heme was reduced by approximately 33 percent. The rate of incorporation of citrate-1, 5-C^{14} was not greatly affected. As would be expected, doubling the concentration of non-radioactive acetate in the reaction mixture resulted in a dilution effect reducing the rate of incorporation of labeled acetate by nearly 59 percent. The rates of incorporation of α-ketoglutarate-5-C^{14} and fumarate-2, 3-C^{14} into heme without the addition of non-radioactive acetate were not determined.

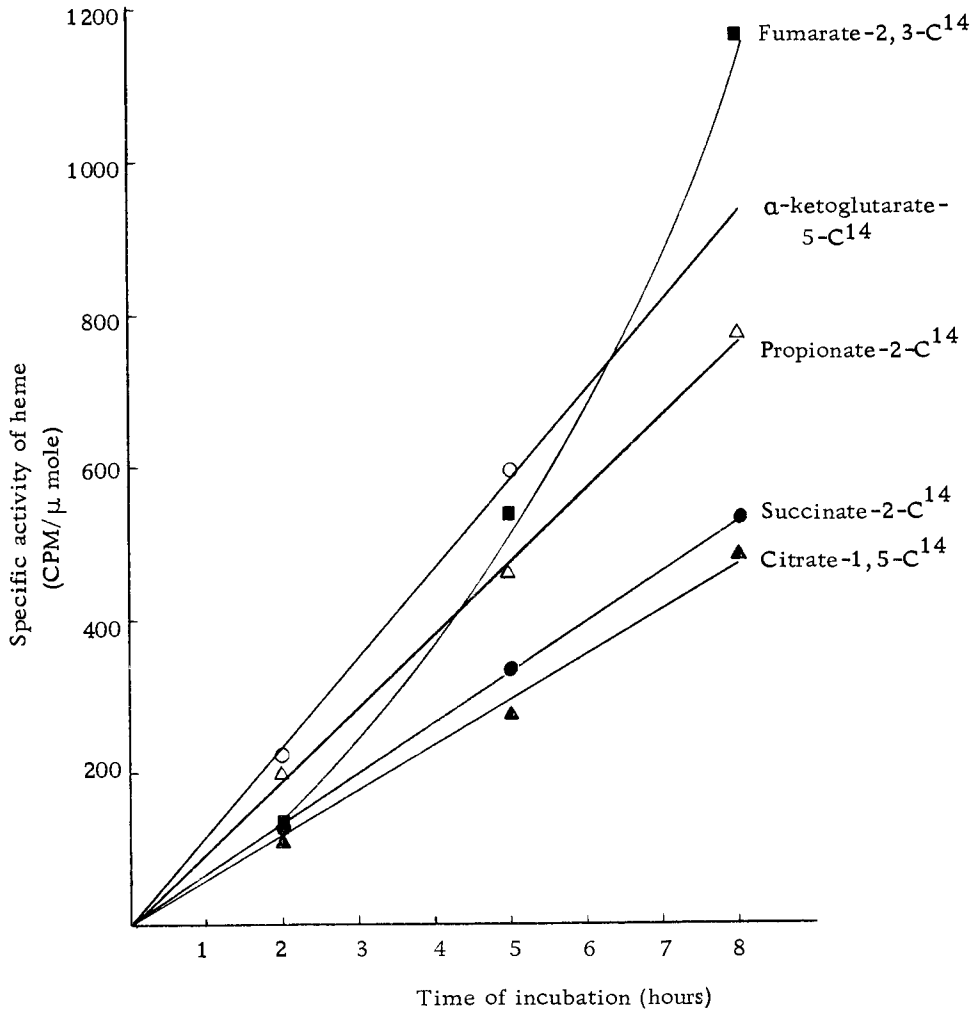

Figure 7. Relative rates of incorporation of labeled organic acids into heme in the presence of acetate. Intact nodules from 39 day-old soybean plants were utilized. The standard incubation mixture was used (Materials and Methods) to which was added 5μ C of the indicated acid and 12 μ moles of non-radioactive acetate.

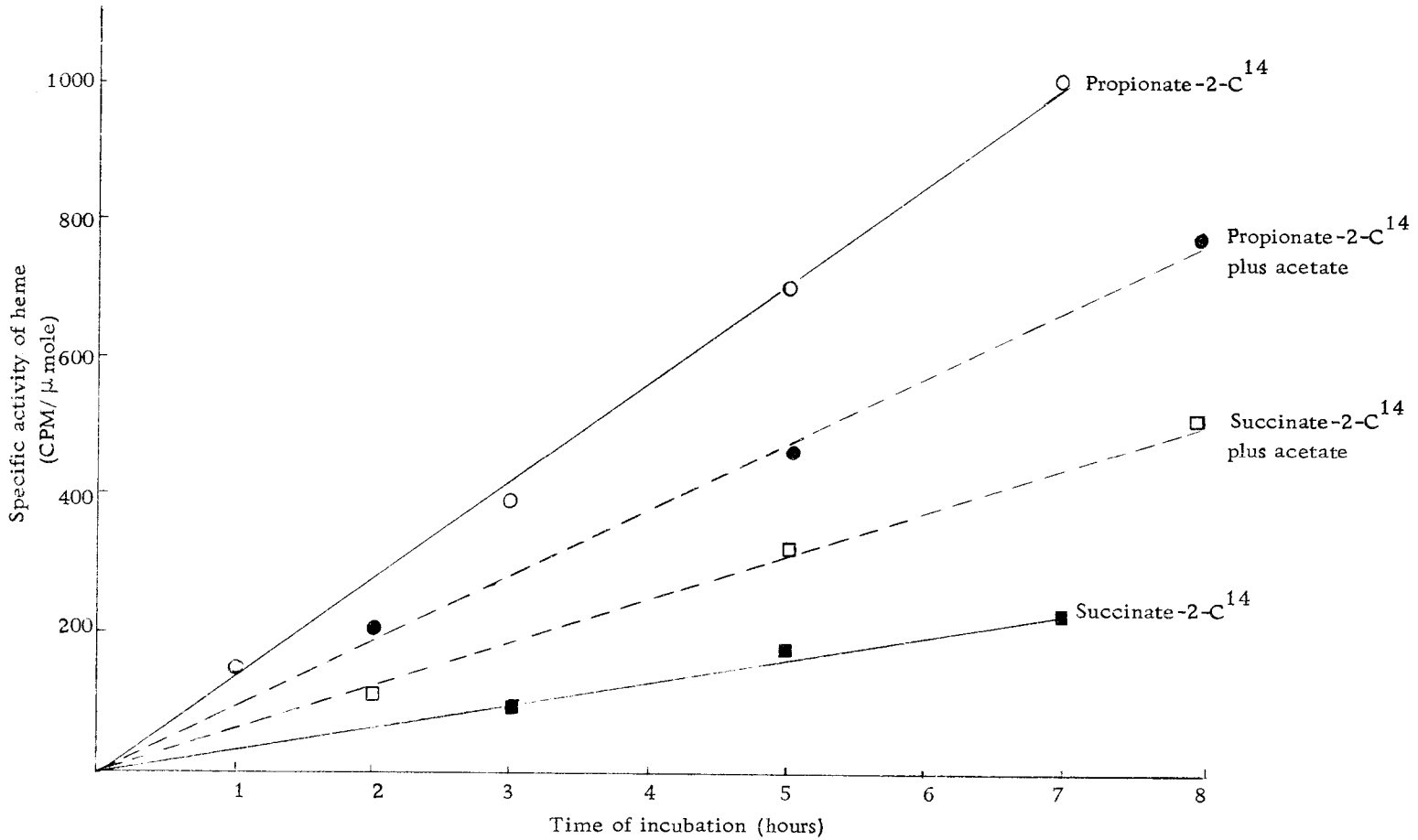

Figure 8. Effect of adding acetate on the rate of incorporation of propionate-2-C^{14} and succinate-2-C^{14} into heme. Rates of incorporation from Figures 4 and 7 are compared.

Competing Pathways for the Formation of Heme Precursors

From the data in Figures 7 and 8 it would appear that when the activity of the citric acid cycle is sparked by the addition of acetate, there is a decreased need for the formation of succinyl-CoA from propionate for citric acid cycle activity. To test this hypothesis, a series of competition experiments were carried out which show that an increased pool size of a component of one pathway reduces the rate of substrate utilization from the other pathway. Table 5 indicates that when 12 μmoles of non-radioactive propionate were added to the incubation mixture, the rate of incorporation of succinate-2-C^{14} into heme was substantially reduced. In all experiments carried out, trends in the results were similar.

The reverse of this experiment also has been conducted. As shown by the data in Table 6, the addition of non-radioactive succinate to either whole or macerated nodules resulted in a decreased rate of incorporation of propionate-2-C^{14} into heme. The incorporation rates using whole nodules were unusually low in this case. A likely explanation for this is that the plants had begun to flower.

Identification of Volatile Organic Acids in Nodules

The fact that propionate is readily oxidized by the bacteroids from nodules and the fact that the enzymes for propionate utilization do not appear to be adaptively formed suggest that propionate is a

Table 5. Effect of adding propionate on the rate of incorporation of succinate-2-C^{14} into heme in soybean nodules.[a]

Hours incubated	Propionate added (μ moles)	Specific activity of heme (CPM/μmole)
3	--	338
3	12	182
5	--	440
5	12	313

[a]Intact 39 day-old soybean nodules were utilized following the Standard Incubation Procedure. Incubation was carried out on a shaker in the hood at 26-28 ° C.

Table 6. Effect of adding succinate on the rate of incorporation of propionate-2-C^{14} into heme in soybean nodules.[a]

Nodule condition	Hours incubated	Succinate added (μ moles)	Specific activity of heme (CPM/μmole)
Macerated	2	--	527
Macerated	2	12	148
Intact	8	--	467
Intact	8	20	341

[a]Four gram samples of 48 day-old soybean nodules were macerated or left intact following the Standard Incubation Procedure except that the pH of the incubation mixture was 6. 2.

normal metabolite in nodules. Investigations were therefore carried out to determine the pool size of propionate in soybean nodules. Volatile fatty acids were determined but no detectable quantity of propionate was found either in the nodules (Table 7) or in the isolated bacteroids (Table 8). Acetate was present at a relatively high concentration while only traces of butyrate and formate were present. In some experiments 1.0 μC propionate-2-C^{14} (5.15 mC/m mole) was added to the acidified nodule breis before steam distillation was carried out. In these experiments, no titratable propionate was found in fractions from the Celite column, but the band of radioactivity was obtained in the proper fraction from the column. Further examination of the steam distillate by Dr. Te May Ching using gas chromatography failed to reveal a detectable quantity of propionate. The results presented in Table 4 are consistent with this conclusion. It is suggested therefore, that propionate, if present in nodules, is utilized as rapidly as it is formed.

Investigation of the Acrylate Pathway

Since considerable evidence is available showing that propionate is utilized by soybean nodules, it was of interest to attempt to determine possible precursors of propionate in nodules and a possible pathway whereby propionate may be synthesized. Experiments in which the rate of conversion of C^{14}-labeled lactate into heme was

Table 7. Volatile fatty acids in soybean nodules.[a]

Acid	Concentration of acid in fresh nodules (μmoles acid/gm nodules)	
	From plants 42 days-old	From plants 48 days-old
Formic	0.67	N.D.
Acetic	11.89	12.02
Propionic	-----	-----
Butyric	0.82	1.19

[a]Twenty grams of nodules were macerated in a mortar with 20 ml of water, acidified and steam distilled. The method for determination of acid concentration described under Materials and Methods was followed. Formic acid was not determined in the 48 day-old nodules.

Table 8. Volatile fatty acids in soybean bacteroids.[a]

Acid	Concentration of acid in fresh bacteroids (μmoles acid/gm bacteroids)
Formic	1.04
Acetic	12.52
Propionic	-----
Butyric	2.09

[a]Bacteroids were isolated from 40 grams of 33 day-old soybean nodules. The cells were broken in an Eaton press, acidified, and the acids isolated by steam distillation. The concentration of each acid was determined by the method outlined in Materials and Methods.

determined, provided an indication that propionate may be an intermediate in lactate utilization in nodule metabolism. Both lactate-2-C^{14} and lactate-1-C^{14} were converted into the heme moiety of leghemoglobin at nearly equal rates. As shown in Table 9 the addition of non-radioactive propionate to the reaction mixtures resulted in a reduction in the rate of C^{14} incorporation into heme from either lactate-1-C^{14} or lactate-2-C^{14}. Furthermore, when the remaining propionate from these experiments was isolated and assayed, it became apparent that C^{14} from lactate was incorporated into propionate. However, the label could have been converted into propionate via an indirect route since these experiments were run for five to eight hours.

By the use of cell-free extracts from nodule bacteroids, the enzymatic conversion of lactate into propionate was investigated. Data from Table 10 indicates that lactate utilization in nodules required adenosine-5'-triphosphate (ATP), coenzyme A (CoA), reduced nicotinamide-adenine dinucleotide (NADH) and magnesium ions as cofactors. A requirement for reduced nicotinamide-adenine dinucleotide phosphate (NADPH) or nicotinamide-adenine dinucleotide (NAD) was not investigated.

The time courses for the conversion of lactate-1-C^{14} and lactate-2-C^{14} were determined. From the data presented in Figure 9 the rate of propionate formed from lactate-1-C^{14} is linear with

Table 9. Effect of adding propionate on the rate of incorporation of lactate-C^{14} into heme in soybean nodules.[a]

Isotope	Age of nodules (days)	Hours incubated	Propionate added (μ moles)	Specific activity of heme (CPM/μ mole)
Lactate-2-C^{14}	44	5	--	841
Lactate-2-C^{14}	44	5	12	457
Lactate-1-C^{14}	39	8	--	1289
Lactate-1-C^{14}	39	8	12	529

[a]Nodules were incubated following the standard incubation procedure outlined in the Materials and Methods.

Table 10. Cofactor requirements for lactate utilization by a cell-free extract from nodule bacteroids.

System	Enzyme activity (total cpm incorporated into propionate in 20 min.)
Complete[a]	1480
-CoA	210
-NADH	330
-Mg^{++}	320
-ATP	400

[a]The reaction mixture in a final volume of 1.57 ml of 0.064 M phosphate buffer pH 7.0 contained 2 μ C lactate-1-C^{14} (5.47 μ C/μ mole) and the following in μ moles: unlabeled lactate, 20; unlabeled propionate, 10; ATP, 10; $MgCl_2$, 10; NADH, 0.2; CoA, 0.2 and 0.1 ml enzyme extract containing 14 mg protein. Each reaction mixture was incubated at 36° C for 20 min. Complete reaction mixture with enzyme extract omitted served as a negative control.

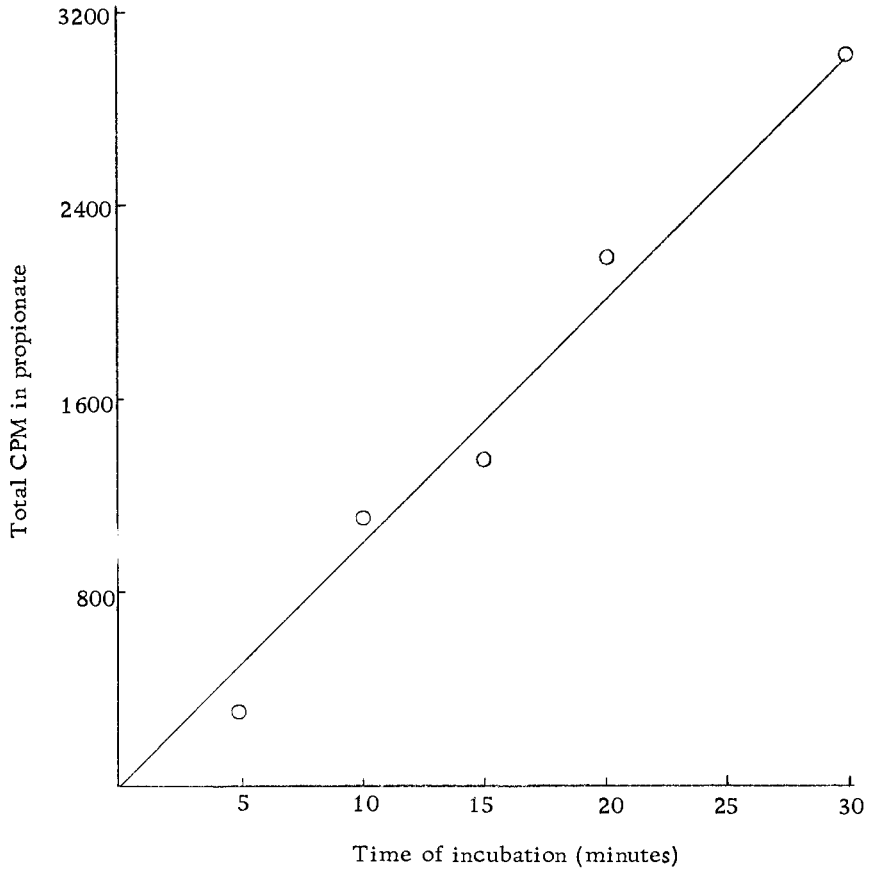

Figure 9. The rate of conversion of lactate-1-C^{14} into propionate by a cell-free extract from soybean bacteroids. Enzyme extract contained 14 mg protein per 0.1 ml and was prepared from 28 day-old bacteroids. The final volume of 1.5 ml 0.064 M phosphate buffer contained 0.1 ml extract; 2 μC lactate-1-C^{14} in 20 μmoles lactate; 10 μmoles propionate, ATP, and $MgCl_2$; 0.2 μmole CoA and NADH. Reactions were carried out under nitrogen at 36°C.

time. Other experiments (not reported) show that the rate of conversion of lactate-2-C^{14} into propionate is approximately equal to the rate of conversion of lactate-1-C^{14} into propionate. These results are in harmony with the data in Table 9. Further experiments (Table 11) show that the addition of acrylate to the incubation mixture significantly reduces the rate of formation of radioactive propionate from lactate-1-C^{14}. These results are consistent with the possibility that acrylate is an intermediate on the pathway.

Concurrently with the previous experiments, the rate of conversion of radioactive lactate into acetate and carbon dioxide was followed. The rate of conversion of either lactate-1-C^{14} or lactate-2-C^{14} into acetate was found to be linear (specific activity of acetate plotted against the time of incubation). The loss of radioactivity from lactate-1-C^{14} as $C^{14}O_2$ was appreciable (330 CPM per 80 mg sample $BaCO_3$ counted at saturation thickness) while very little CO_2 became labeled from lactate-2-C^{14} (17 CPM per 80 mg sample $BaCO_3$ counted at saturation thickness). As would be expected, the rate of C^{14} accumulation in acetate from lactate-2-C^{14} (1565 CPM per μmole acetate) was much more rapid than from lactate-1-C^{14} (227 CPM per μmole acetate). This difference in the rate of label accumulation was not apparent when specifically labeled lactate-C^{14} was converted into propionate. In addition, when 10 μmoles pyruvate were added to the incubation medium, the rate of conversion of lactate-1-C^{14} into propionate was stimulated (Table 11).

Table 11. Effect of adding pyruvate and acrylate on the rate of conversion of lactate-1-C^{14} into propionate by a cell-free extract from soybean bacteroids.[a]

Conditions	Specific activity of propionate (CPM/μmole)
Control	428
Control + 10 μ moles pyruvate	656
Control + 10 μ moles acrylate	232

[a] The control incubation mixture consisted of 20 μmoles lactate containing 2 μC lactate-1-C^{14}, 5 μmoles propionate and other constituents as outlined in the Materials and Methods. Pyruvate and acrylate were added in other treatments as indicated. Extract was obtained from 20 gm of 34 day-old bacteroids and was dialyzed for six hours at pH 7.4 under argon. Reactions were carried out at 35°C for 20 minutes using 0.1 ml extract containing 20 mg protein.

DISCUSSION

Propionate Utilization in Relation to Heme Biosynthesis

Bacteroids from the nodules of symbiotically grown soybean plants contain the enzymes necessary for the conversion of propionate to succinyl-CoA (34). Cobalt is essential for Rhizobium and its deficiency blocks propionate utilization by reducing the synthesis of B_{12} coenzyme which is essential for the function of the methylmalonyl-CoA mutase step of propionate utilization. Experiments have been carried out therefore to provide evidence concerning the role that propionate plays in the metabolism of soybean nodules. Many of these experiments were designed to test the hypothesis that propionate utilization makes possible a second mechanism for the formation of succinyl-CoA that may be utilized for heme synthesis as well as for the maintenance of the citric acid cycle.

This hypothesis is in harmony with present knowledge concerning the metabolism of free living R. japonicum or bacteroids from nodules (Figure 10). Ammonia formed from nitrogen fixation initially enters the metabolism of the cells by the rapid formation of glutamate and glutamine (24). Because effective nodules would be expected to form large quantities of glutamic acid, it would seem logical to expect that a large portion of the α-ketoglutarate from the citric

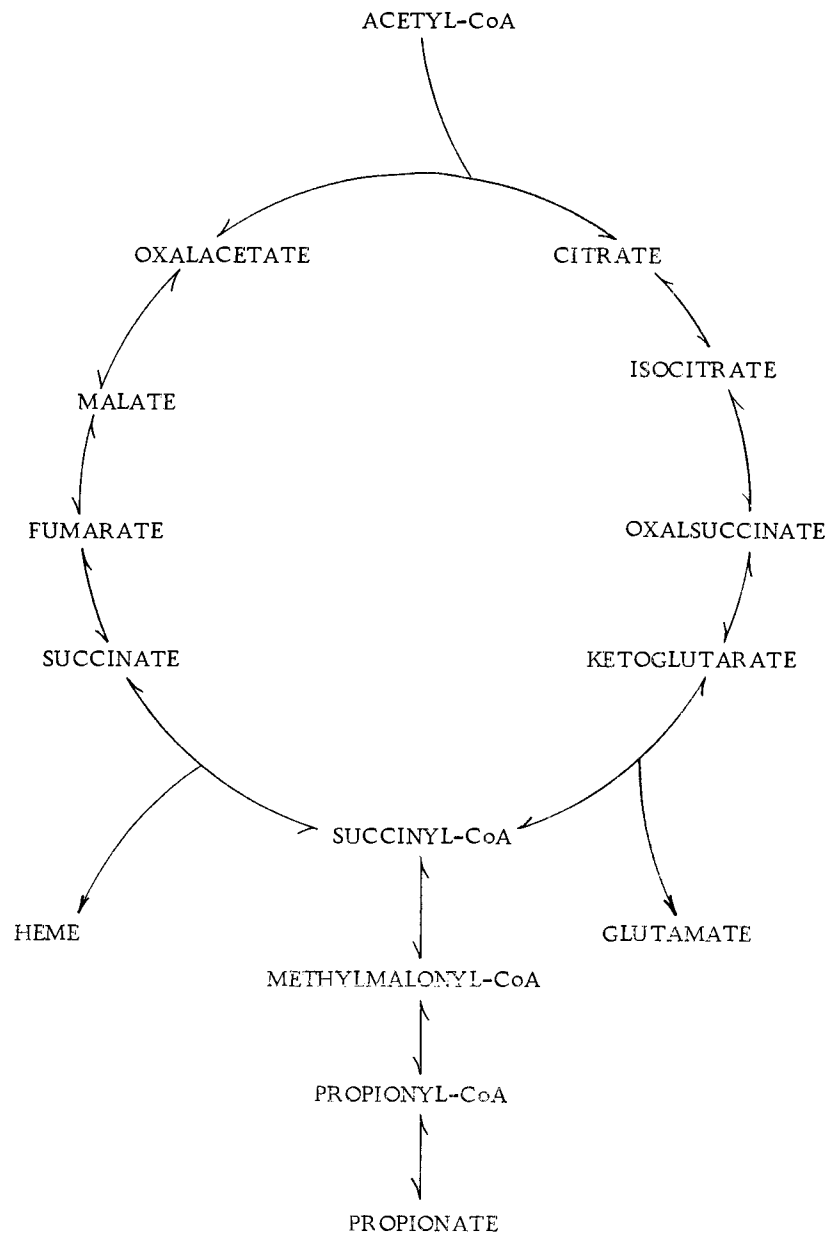

Figure 10. General pathway of propionate utilization and its possible relationship to the citric acid cycle and heme biosynthesis.

acid cycle may be utilized in the nitrogen fixing process. If this is true, a deficiency in the synthesis of succinyl-CoA would be expected. In addition, succinyl-CoA would also be utilized for heme and porphyrin synthesis. The supply of succinyl-CoA from propionate may also be important in supplying intermediates of the citric acid cycle in soybean nodules since the enzymes of the glycolytic sequence appear to be adaptive (94) and the glyoxylate cycle does not function (53). The research of De Hertogh (34) and results reported in this thesis (Figures 3 and 6) show that propionate is utilized by free living Rhizobium and bacteroids in nodules and is incorporated into intermediates of the citric acid cycle. A radiogram made by scanning the thin-layer plate of Figure 6 showed that fumarate, succinate, α-ketoglutarate and malate became radioactive when nodules were incubated with propionate-2-C^{14}. These results are consistent with the hypothesis that propionate serves as an alternative carbon source for the maintenance of the citric acid cycle.

In 1961 Ahmed and Evans (3) demonstrated that cobalt deficiency in soybean plants resulted in a marked reduction in the leghemoglobin content of nodules. Experiments reported in this thesis (Table 2) demonstrate that a cobalt deficiency in R. meliloti results in a decreased cytochrome content of bacterial cells. Since succinyl-CoA is a direct precursor of the heme component of leghemoglobin and cytochromes, isotopic experiments were carried out in order to

determine if propionate was actually converted into the heme moiety of leghemoglobin. As illustrated by the data in Figure 3, radioactivity from C^{14}-labeled propionate was incorporated into heme. This incorporation occurred in cell-free extracts (Table 4) as well as in macerated, sliced, and whole nodules. It appears that propionate is utilized without a lag period (Figure 3) and that the rate of C^{14} incorporation into heme is linear with time. The fact that C^{14}-labeled propionate is incorporated into intermediates of the citric acid cycle (Figure 6) and also serves as a precursor of the heme moiety of leghemoglobin (Figure 3) is consistent with the hypothesis that this compound serves as an alternate carbon source for the supply of succinyl-CoA. Furthermore, the series of events outlined in Figure 10 would offer a feasible explanation for the observation that cobalt deficiency, which results in an insufficiency of B_{12} coenzyme, blocks the synthesis of succinyl-CoA from propionate. This lesion in propionate metabolism may interfere therefore, with heme biosynthesis which involves succinyl-CoA as a precursor.

Incorporation of Propionate-2-C^{14} into Heme

Isotopic experiments have been carried out to determine the relative rate of propionate incorporation into heme. The rates of incorporation into heme of C^{14}-labeled intermediates of the citric acid cycle (Figure 4) have been compared. The rate of incorporation

into heme of propionate-2-C^{14} was compared with that of succinate-2-C^{14} and citrate-1, 5-C^{14}. Propionate-2-C^{14} incorporation into heme always proceeded more rapidly than the incorporation of succinate-2-C^{14} and citrate-1, 5-C^{14}. It can be argued that the rate of incorporation of label from succinate-2-C^{14} (Figure 4) was reduced because of dilution by a larger pool of succinate (Table 3) than of propionate (Table 9). It is also possible that the C^{14} from succinate-2-C^{14} was diluted by the large pools of malate and α-ketoglutarate assuming that succinate was first oxidized by the enzymes of the citric acid cycle. Unpublished data, however, show that succinate can be converted directly to succinyl-CoA via the phosphorylating enzyme which was shown to be present in soybean nodules. The significance of relative rates of incorporation of metabolites into heme is difficult to determine because of the different sizes of endogenous organic acid pools that are present and the lack of information regarding equilibration of internal pools with external additions. Since little citrate accumulates in the nodules, however, pool size does not offer a good explanation for its sluggish use for the synthesis of the heme of leghemoglobin.

No data was obtained proving net synthesis of heme during the incubation periods in either whole nodules or in cell-free extracts of nodules. Due to the concentration of leghemoglobin and the methods used, it was not feasible to calculate heme concentration at the

beginning and end of an experiment to demonstrate a significant increase in the heme content. It is known that incorporation of radioactive propionate and other metabolites into heme proceeds enzymatically since no C^{14} was incorporated into heme when nodules or nodule extracts were boiled. The possibility that C^{14} accumulation in the heme moiety of leghemoglobin is from exchange reactions mediated by the reversible enzymes of the system and not by the net synthesis of heme has not been ruled out. Acetate is rapidly utilized as a heme precursor (Figure 5). The reason for this is not clearly established; however, it is interesting to note that C. sporogenes and C. botulinum as well as Diplococcus glycinophitus are able to use glycine as a hydrogen acceptor and convert it to acetic acid and ammonia (27). It may be possible in this system that acetate is converted into glycine or directly condensed to form succinyl-CoA. As a result of a recent examination of the radioactivity of acetate-C^{14} samples, it may be possible that more than 5 μC of acetate-2-C^{14} was present in the incubation mixture resulting from a labeling error. In a recent experiment (not reported) using a new supply of isotopes, the determination of radioactivity in the incubation mixture after the addition of what was calculated to be 5μC of each metabolite showed 1.68×10^6 CPM from propionate-2-C^{14}, 1.62×10^6 CPM from succinate-2-C^{14} and 2.51×10^6 CPM from acetate-2-C^{14}. The activity of this sample of acetate-2-C^{14}

therefore was much higher than indicated by the manufacturer.

The addition of non-radioactive acetate as a sparker to the reaction mixture significantly increased the rate of incorporation of succinate-2-C^{14} into heme (Figure 7). This would be expected since the addition of acetate would provide more readily available acetyl-CoA for the synthesis of a six-carbon tricarboxylic acid. The addition of acetate as well as the addition of succinate to incubation mixtures decreased the rate of incorporation of propionate-2-C^{14} into heme (Figure 7). It has been established also that the addition of propionate to the incubation mixture reduces the rate of incorporation of succinate-2-C^{14} into heme (Table 6). This is in harmony with the hypothesis that propionate makes possible a second mechanism for the synthesis of succinyl-CoA.

Possible Pathways of Propionate Formation

It can be concluded from Tables 7 and 8 that propionate was not present in detectable amounts in nodules. If this acid is present in nodules, it must be utilized as rapidly as it is formed. A search was initiated therefore, to identify possible precursors of propionate. Considering the fact that the interior of the nodules is nearly anaerobic (81) and that lactate can be fermented by bacteroids (25) the pathway where lactate is converted to propionate via acrylate (13) was investigated initially. Results indicate (Tables 10 and 11) that

this pathway is probably functional in soybean nodules. Experiments utilizing cell-free extracts of bacteroids demonstrated the enzymatic conversion of lactate to propionate (Table 10). Furthermore, the rate of conversion of lactate-1-C^{14} into propionate is approximately equal to the rate of conversion of lactate-2-C^{14} into propionate. This would not be expected if the major pathway involved a conversion of lactate to pyruvate and then to propionate after decarboxylation. The addition of the possible intermediate, acrylate, to the reaction mixture reduced by 46 percent the rate of conversion of lactate-1-C^{14} into propionate (Table 11). The possibility that acrylate was inhibitory because of its chemical properties has not been ruled out. The hypothesis that propionate may be an intermediate in the utilization of lactate in nodule metabolism is supported by the fact that both lactate-1-C^{14} and lactate-2-C^{14} were incorporated into the heme moiety of leghemoglobin at approximately equal rates (Table 9). Furthermore, the addition of non-radioactive propionate to the incubation mixture significantly reduced the rate of incorporation of these labeled compounds into heme. When the remaining propionate from this experiment was isolated and assayed, it had become radioactive (365 CPM per μmole propionate).

It is possible that some fermentation of lactate into pyruvate occurs since the rate of incorporation of specifically labeled lactate-C^{14} into heme was more rapid than the rate of incorporation of

propionate-2-C^{14} in both whole nodules and cell-free extracts. The partial conversion of lactate to pyruvate is supported by the fact that lactate-1-C^{14} and lactate-2-C^{14} can be converted to acetate and CO_2 under anaerobic conditions by a cell-free extract. Much of the label from lactate-1-C^{14} was lost as $C^{14}O_2$ thus causing a great difference in the rate of accumulation of C^{14} in acetate from the two isotopes.

No evidence in this dissertation is presented concerning the relative importance of lactate as a precursor of propionate. There may be other pathways of propionate formation that provide a source of this compound. For example, the catabolism of isoleucine (77) is reported to produce propionate. The importance of this and other pathways of propionate formation in nodules remains for future investigation.

SUMMARY

An investigation was conducted to obtain information concerning propionate metabolism and its relationship to leghemoglobin formation in the R. japonicum-soybean symbiotic relationship. The results of these experiments are summarized as follows:

1. Our investigations showed that the cytochrome content of cobalt deficient R. meliloti cells grown in pure culture was reduced to approximately one-half of that in normal cells. It is concluded that the effect of cobalt deficiency on cytochrome content of Rhizobium cells and on the leghemoglobin content of nodules possibly may be explained by an effect of cobalt deficiency on the utilization of propionate which has been shown to serve as a precursor of the heme moiety of leghemoglobin.

2. A chromatographic procedure was developed for the isolation and assay of the heme moiety of leghemoglobin from legume nodules.

3. Radioactivity from C^{14}-labeled propionate was incorporated into the heme moiety of leghemoglobin in cell-free extracts of nodules and in macerated or whole nodules. The rate of incorporation of propionate-2-C^{14} into heme proved to be linear with time.

4. The relative rate of propionate-2-C^{14} incorporation into heme of the leghemoglobin of soybean nodules was determined by

comparison of the rates of incorporation of several intermediates of the citric acid cycle. Propionate-2-C^{14} was incorporated into heme at a faster rate than succinate-2-C^{14}, citrate-1, 5-C^{14} and fumarate-2, 3-C^{14}. The rate of incorporation of α-ketoglutarate-5-C^{14} was slightly more rapid than the rate of incorporation of propionate-2-C^{14}. The effect of the endogenous organic acids on these rates of incorporation however, complicates the interpretation of the data.

5. The data showing propionate-2-C^{14} incorporation into the heme component of leghemoglobin of cowpea nodules exhibited trends similar to those obtained with soybean nodules.

6. Acetate-2-C^{14} and δ-aminolevulinate-4-C^{14} were incorporated into heme at rapid rates. The addition of acetate to the incubation mixture was found to stimulate the incorporation of succinate-2-C^{14} into heme while it inhibited the rate of incorporation of propionate-2-C^{14}.

7. The addition of non-radioactive propionate to the incubation mixture inhibited the rate of incorporation of succinate-2-C^{14} into heme. Furthermore, when a large amount of substrate which can be utilized via the citric acid cycle is added to the reaction mixture (succinate or acetate), the rate of incorporation of propionate-2-C^{14} into heme is decreased. It was concluded, therefore, that the supply of succinyl-CoA from the utilization of propionate is competitive with the supply of succinyl-CoA from the citric acid cycle.

8. Further proof of an active citric acid cycle in the

bacteroids was obtained by the isolation and identification of citric acid cycle intermediates from soybean nodules. Malate and α-ketoglutarate were generally present at high concentration. Succinate, fumarate and isocitrate were also present in appreciable quantities. Organic acids of the citric acid cycle in soybean nodules became radioactive after they were incubated with propionate-2-C^{14}.

9. No significant quantity of propionic acid was found either in nodules or in isolated bacteroids. Acetic acid was found in rather high concentration but only traces of butyrate and formate were found.

10. Isotope incorporation studies indicated that lactate-C^{14} could be a precursor of heme. The time course of incorporation was linear with time. The rate of incorporation into heme of lactate-1-C^{14} was approximately the same as that of lactate-2-C^{14}. The addition of propionate inhibited the rate of incorporation into heme of lactate-C^{14} labeled either in the first or in the second carbon. At the conclusion of the experiment, the non-radioactive propionate had become radioactive.

11. Experiments using cell-free extracts from soybean bacteroids demonstrated the direct conversion of lactate to propionate. Lactate utilized by this system required the cofactors NADH, Mg^{++}, ATP, and coenzyme A. The addition of acrylate to the incubation mixture was inhibitory.

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