Enzymes of the Glyoxylate Cycle in Rhizobia and Nodules of Legumes[1]

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Received April 25, 1966.

Summary. The relatively high level of fatty acids in soybean nodules and rhizobia from soybean nodules suggested that the glyoxylate cycle might have a role in nodule metabolism. Several species of rhizobia in pure culture were found to have malate synthetase activity when grown on a number of different carbon sources. Significant isocitrate lyase activity was induced when oleate, which presumably may act as an acetyl CoA precursor, was utilized as the principle carbon source. Malate synthetase was active in extracts of rhizobia from nodules of bush bean (*Phaseolus vulgaris* L.), cowpea (*Vigna sinensis* L.), *lupine* (*Lupinus angustifolius* L.) and soybean (*Glycine max* L. Merr.). Activity of malate synthetase was, however, barely detectable in rhizobia from alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) and pea (*Pisum sativum* L.) nodules. Appreciable isocitrate lyase activity was not detected in rhizobia from nodules nor was it induced by depletion of endogenous substrates by incubation of excised bush bean nodules. Although rhizobia has the potential for the formation of the key enzymes of the glyoxylate cycle, the absence of isocitrate lyase activity in bacteria isolated from nodules indicated that the glyoxylate cycle does not operate in the symbiotic growth of rhizobia and that the observed high content of fatty acids in nodules and nodule bacteria probably is related to a structural role.

Marked metabolic differences exist between rhizobia collected from legume nodules and those cultured on synthetic media. Nitrogen fixation by rhizobia occurs only under conditions of symbiotic growth. If more information on the activity of various metabolic pathways could be procured from the symbiotic and cultured rhizobia, a better understanding of symbiosis possibly could be achieved. Evidence for the function of the tricarboxylic acid cycle, the oxidative pentose phosphate pathway and the Embden-Meyerhof glycolytic pathway in rhizobia has been provided (13), but the glyoxylate cycle in rhizobia has not been investigated.

The key enzymes of the glyoxylate cycle are: isocitrate lyase (isocitratase) which catalyzes the cleavage of isocitrate to glyoxylate and succinate, and malate synthetase catalyzing the formation of malate from glyoxylate and acetyl CoA. Isocitrate lyase and malate synthetase provide a mechanism for synthesis of C-4 intermediates of the tricarboxylic acid cycle from acetyl CoA derived from acetate, ethanol or fatty acids. By operation of the glyoxylate cycle and reversal of glycolysis all cellular constituents may be derived from C-2 precursors.

Examination of rhizobia for the key enzymes of the glyoxylate cycle was further encouraged by the observation that soybean nodules and bacteria extracted from soybean nodules contained relatively large amounts of fatty acids. Poly-β-hydroxybutyrate has been suggested as an important energy storage form in leguminous nodule bacteria (12). Utilization of this polymer would be expected to result in formation of acetoacetate and then acetyl CoA (9).

Isocitrate lyase and malate synthetase occur widely in bacteria, yeast, fungi and algae (10, 16). The formation of these enzymes in many microorganisms is known to be greatly influenced by the nature of the carbon source utilized for growth (16, 20). In higher plants appreciable isocitrate lyase and malate synthetase activities have been detected only in tissues in which fats are being utilized (3, 22).

Materials and Methods

Cultural Methods. Pure cultures of effective strains of *Rhizobium meliloti* F29, *R. leguminosarum* C56, *R. phaseoli* K11, *R. trifolii* P28 and *R. japonicum* 61A76 were investigated. These species of rhizobia are the endophytes of alfalfa, pea, bean,

[1] Technical paper No. 2099 Oregon Agricultural Experiment Station. This research was supported in part by grants from the Hill Foundation and the National Science Foundation (GB2518).

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clover and soybean associations respectively. The cultures were grown in 400 ml of medium in 1 liter flasks with continuous shaking at 25 to 30°. The basic culture medium for all species of rhizobia contained the following components in g/liter: K_2HPO_4, 1.00; KH_2PO_4, 1.00; $MgSO_4{\cdot}7H_2O$, 0.36; $CaSO_4$, 0.13; $FeCl_3{\cdot}6H_2O$, 0.004 and yeast extract (Difco Bacto), 1.00. Carbon sources were added to the basic medium as indicated in the tables. For the cultures of *R. meliloti* 60 μg/liter Co as $CoCl_2$ was added to the basic medium and the pH was adjusted to 6.5 to 7.0. For culture of *R. japonicum*, *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* 0.7 g/liter KNO_3 was added to the basic medium and the pH was adjusted to 6.3. Cultures were inoculated with 1 % by volume of log phase cultures of *R. meliloti* supplied with mannitol or the other species of rhizobia supplied with arabinose and glycerol.

Alfalfa (*Medicago sativa* L., var. Dupre), bush bean (*Phaseolus vulgaris* L., var. Top Crop), red clover (*Trifolium pratense* L., Common var.), cowpea (*Vigna sinensis* L., var. Iron Clay), lupine (*Lupinus angustifolius* L., var. Borre Blue), pea (*Pisum sativum* L., var. Little Marvel) and soybean (*Glycine max* L. Merr., var. Chippewa) seeds were inoculated with commercial inoculum and planted in pots containing Perlite. The legumes, except soybeans, were supplied throughout their growth with a nutrient solution lacking nitrogen and containing the following macronutrient salts in g/liter: K_2SO_4, 0.28; $MgSO_4{\cdot}7H_2O$, 0.49; KH_2PO_4, 0.02; K_2HPO_4, 0.15; $CaSO_4{\cdot}2H_2O$, 1.03; and $CaCl_2{\cdot}2H_2O$, 0.06. The nutrient solution supplied to soybeans contained in g/liter: K_2SO_4, 0.34; KH_2PO_4, 0.12; and K_2HPO_4, 0.01 and the other macronutrient salts at the concentrations previously indicated. Micronutrients were supplied at the following concentrations in mg/liter: Fe, 1.0 as FeEDDHA; B, 0.25 as H_3BO_3; Mn, 0.25 as $MnSO_4{\cdot}7H_2O$; Zn, 0.05 as $ZnSO_4{\cdot}7H_2O$; Cu, 0.02 as $CuSO_4{\cdot}5H_2O$; Mo, 0.01 as $Na_2MoO_4{\cdot}2H_2O$ and Co, 0.05 as $CoCl_2{\cdot}6H_2O$. After emergence the cultures were irrigated with nutrient solution daily for 2 days followed by tap water every third day. The legumes, except soybeans which were cultured in a growth chamber, were grown in a greenhouse with sunlight supplemented by fluorescent lights during the winter months.

Chemical Constituents of Nodules and Bacteria. The procedure for lipid extraction and analysis was that of Ching (5). Five g of soybean nodules were ground in 75 ml of an ether:ethanol solution (2:1, v/v) for 2 minutes in an Omnimixer. Homogenized nodule material or bacteria from 5 g of nodules was then extracted 3 times with 75 ml of ether:ethanol at room temperature for a total of 18 hours. The residue was washed with the solvent, dried in a vacuum oven overnight at 50° and then weighed. The filtered crude lipid extracts were separated from aqueous material by 3 extractions with 2 % K_2CO_3. The washed lipids were dried in a vacuum evaporator and further dried overnight in a vacuum oven at 50°. Dried lipids were saponified by 10 % NaOH in 75 % ethanol for 20 hours. After saponification, the aqueous phase was acidified and fatty acids were extracted with petroleum ether:ether (1:1, v/v). The fatty acids were methylated with diazomethane in ether and concentrated under N_2. Fatty acids were determined using a 12 foot column containing 14 % diethylene glycol succinate on Chromosorb W, 80/100 mesh, with a F & M gas chromatograph (thermal conductivity detector). The unsaponifiable fraction was dried and weighed.

Lipids were extracted from 2.9 g samples of bush bean nodules with 3 different 20 ml volumes of chloroform:methanol (2:1, v/v) for 3 hours at room temperature. The lipids were washed with distilled water and dried in a vacuum oven at 60° for 24 hours prior to weighing (11). Soluble sugars were determined in washings of the lipid extract and the water soluble portion of the 80 % ethanol extract by the anthrone method (23). Starch was determined as sugar by the anthrone method after hydrolysis of the sugar-free residue with perchloric acid (14).

Preparation of Cell-free Extracts. Cultures of rhizobia were harvested in the log phase of growth, at the times indicated in the tables. Cells were collected by centrifugation at 10,500 $\times$ g for 10 minutes. Subsequent operations were carried out at 0 to 4°. After harvesting, the cells were washed twice by suspension in cold 0.01 M potassium phosphate buffer at pH 6.8 followed by centrifugation at 35,000 $\times$ g for 10 minutes. The yield of the cultures was estimated from the fresh weight of the washed cells.

Nodules were removed from the roots of actively growing legumes and washed to remove Perlite. Subsequent operations were carried out at 0 to 4°. Bacteria were isolated from the nodules by crushing the nodules with a cold mortar and pestle in 3 volumes of potassium phosphate buffer (0.01 M at a pH of 6.8) and then squeezing the crushed nodules through 4 layers of cheese cloth (4). The bacteria were separated from the hemoglobin and other soluble components by centrifugation at 35,000 $\times$ g for 10 minutes. The bacteria from nodules were washed and weighed in the same manner as were the pure culture bacteria.

Washed bacterial cells from either pure cultures or nodules were suspended in 4 volumes of 0.02 M potassium phosphate buffer at pH 7.4 containing 0.01 M 2-mercaptoethanol and 10^{-4} M Na_2EDTA. This suspension, surrounded by an ice bath, was sonicated in an MSE sonicator after addition of 0.2 g of cold levigated alumina for each g of cells. In order to minimize heating during sonication, the cell suspensions were sonicated for 2 minutes, then cooled to 1° and sonicated for an additional 2 minutes. Cell-free extracts were obtained by centrifugation at 35,000 $\times$ g for 20 minutes.

Extracts of rhizobia were stirred with 5 mg/ml of activated charcoal (Darco, 5–60 mesh) for 5

minutes in order to remove pyridine nucleotides that may have been present in the crude extract (3). Charcoal was removed by centrifugation.

For the characterization of isocitrate lyase and malate synthetase from *R. meliloti* grown on oleate, ice cold saturated $(NH_4)_2SO_4$ at pH 7.4 and containing 0.01 M 2-mercaptoethanol was added drop wise with stirring to the charcoal treated cell-free extract to precipitate the enzymes. Precipitated protein was recovered by centrifugation at 35,000 × *g* for 10 minutes, and resuspended in 0.02 M potassium phosphate buffer at pH 7.4 containing 10^{-4} M Na_2EDTA and 0.5 mg/ml GSH.

The roots of soybeans and cowpeas after removal of nodules were homogenized for 1 minute in an ice cold Omnimixer in 1.4 volumes of the buffer used in preparation of cell-free extracts of rhizobia. The homogenate was strained through 4 layers of cheese cloth followed by centrifugation at 35,000 × *g* for 20 minutes. Only malate synthetase activity was determined in root extracts.

Enzyme Assays. Isocitrate lyase and malate synthetase activities were determined using charcoal treated crude extracts of bacteria from pure cultures and nodules. Isocitrate lyase activity was determined by the procedure of Daron and Gunsalus (6). The complete reaction mixture consisted of: 100 μmoles TrisCl pH 7.9, 3.0 μmoles $MgCl_2$, 2.0 μmoles cysteine-HCl, 7.5 μmoles Na isocitrate, cell-free extract containing 0.25 to 10.0 mg of protein, and water to a final volume of 1.5 ml. Sodium isocitrate was prepared from DL-isocitric acid lactone (Sigma, type II). After 10 minutes incubation with the substrate at 30° the reaction was terminated by the addition of 0.1 ml of 80 % trichloroacetic acid. After centrifugation to remove precipitated protein, glyoxylate was determined colorimetrically as the 2,4 dinitrophenylhydrazone. The optical density of the complete reaction mixture was corrected for the optical density of the reaction mixture lacking substrate. Glyoxylate formation was found to be proportional to enzyme concentration up to 0.9 μmole glyoxylate in the reaction mixture and isocitrate lyase activity was measured within the proportional range.

Malate synthetase activity was estimated from the decrease in optical density at 240 mμ upon cleavage of the thioester bond of acetyl CoA in the presence of glyoxylate using a Cary model 11 spectrophotometer (8). The complete reaction mixture consisted of: 273 μmoles TrisCl pH 8.0, 10 μmoles $MgCl_2$, 0.14 μmole acetyl CoA, 1.4 μmoles Na glyoxylate and cell-free extract containing 0.1 to 0.6 mg of protein in a final volume of 3.0 ml. Acetyl CoA was prepared daily from Coenzyme A (Sigma) and redistilled acetic anhydride (Merck) as described by Stadtman (21). A weak deacylase system, as indicated by a gradual decrease in optical density prior to the addition of glyoxylate, was detected in only a few extracts. When deacylase activity was present, the rate of decrease in optical density after the addition of glyoxylate was corrected for the rate of decrease prior to addition of this substrate. Although malate synthetase activity was estimated from the change in optical density during the first 1.0 or 2.0 minutes of the reaction, these results have been expressed on the basis of 10 minutes in order to facilitate comparison with the values for isocitrate lyase activity.

The protein content of cell-free extracts was determined by the method of Lowry et al. (17).

Identification of Products of Enzymatic Reactions. The fraction obtained by 50 to 75 % $(NH_4)_2SO_4$ saturation of a cell-free extract of *R. meliloti* grown on oleate was used for the identification of the products of the isocitrate lyase and malate synthetase reactions. The 2,4 dinitrophenylhydrazones of keto-acids from the complete reaction mixture, from the reaction mixture without substrate, and from a standard sample of Na glyoxylate were formed and extracted as described by Ranson (19, method 1). The hydrazones of glyoxylate were identified by paper chromatography with 3 solvent systems: A) methanol:benzene:*n*-butanol:H_2O (4:2:2:2); B) water saturated *n*-butanol; C) n-butanol:0.5 N NH_4OH:ethanol (7:2:1):

The deproteinized supernatant solution from a complete malate synthetase reaction mixture and a reaction mixture lacking acetyl CoA were passed through a column of 50 to 100 mesh Dowex 50W-×4. The effluent and washings were concentrated using a flash evaporator. The reaction product was identified by paper chromatography with malic acid using isoamyl alcohol saturated with 4 N formic acid as a solvent system (7).

Results

Fatty Acid Composition of Soybean Nodules and Symbiotic Bacteria. In table I the composition of esterified fatty acids from a sample of intact soybean nodules and the bacteria isolated from soybean nodules is shown. In the whole nodules, palmitic, oleic, linoleic and linolenic are the predominant fatty acids. Only small amounts of other fatty acids are present. Oleic acid makes up 42 % by weight of the esterified fatty acids in the nodule bacteria. Palmitic and linoleic acids also are major constituents. A smaller weight percent of linolenic acid is found in the bacteria than in whole nodules. On a fresh weight basis the lipid content of nodules and bacteria from the nodules was 20 % and 17 % respectively.

Enzymes of the Glyoxylate Cycle in Rhizobia in Pure Culture. The influence of the carbon source on the activity of the key enzymes of the glyoxylate cycle in cell-free extracts of *R. meliloti* grown in pure culture is shown in table II. In experiment I it is apparent that while appreciable malate synthetase activity was detectable in all extracts, isocitrate lyase activity was virtually absent except when oleate was supplied as a carbon source.

Table I. *Fatty Acid Composition of Saponifiable Material from Intact Soybean Nodules and Bacteria from Soybean Nodules*

Five g of fresh nodules from approximately 7-week-old soybean plants contained 1.00 g of lipids of which 0.57 was saponifiable. The bacteria from 5.0 g of nodules contained 0.16 g of lipids of which 0.12 g was saponifiable.

Fatty acid	Percentage by weight of total fatty acids in: Nodules	Bacteria
Butyric, valeric, caproic, C4,5,6	2.7	3 8
Heptoic, caprylic, C7,8	0.4	0.4
Capric, C10	0.6	0.2
Lauric, C12	1.0	0.3
Myristic, C14	1.4	0.6
Palmitic, C16	15.3	17.9
Palmitoleic, C16=1	1.0	1.0
Stearic, C18	2.7	1.9
Oleic, C18=1	23.3	42.2
Linoleic, C18=2	26.2	19.7
Linolenic, C18=3	17.3	7.6
Arachidic, C22	0.4	0.0
Eicosadienoic, C20=2	4.1	0 2
Behenic, C22	1.7	0.0
Unidentified fatty acid	2.3	4.3

Since the basic medium includes 0.1 % (w/v) yeast extract, experiment II was conducted to determine the influence of yeast extract alone on the activity of glyoxylate cycle enzymes. When yeast extract was supplied at a level of 0.3 % (w/v) isocitrate lyase activity was negligible. Malate synthetase activity increased 2 to 3-fold with oleate as the carbon source compared to the activity with other carbon sources which failed to induce isocitrate lyase.

Ammonium sulfate fractionation of cell-free extracts of *R. meliloti* cultured with oleate as the carbon source resulted in a concentration of isocitrate lyase in the 50 to 75 % $(NH_4)_2SO_4$ saturation fraction. This fraction was used for identification of the product, glyoxylate, and characterization of isocitrate lyase. That glyoxylate was formed enzymatically from isocitrate was confirmed by paper chromatography. The major hydrazones of authentic glyoxylate and complete reaction mixtures had comparable R_F values in solvent systems A, B and C, while hydrazones were not observed in reaction mixtures without isocitrate.

Malate synthetase activity was distributed in several of the $(NH_4)_2SO_4$ precipitated fractions; however, the enzyme was characterized in the 50 to 75 % $(NH_4)_2SO_4$ saturation fraction prepared from oleate cultured *R. meliloti.* Malate was identified as the product of the reaction of glyoxylate and acetyl CoA by chromatographic comparison of the acids separated from the complete reaction mixture with a malic acid standard. When acetyl CoA was omitted from the reaction mixture the spot corresponding to malate did not appear.

Isocitrate lyase from oleate cultured *R. meliloti* was found to require isocitrate, magnesium, and cysteine-HCl for maximum activity and to exhibit a pH optimum between pH 8.0 and pH 8.5. Activity of malate synthetase from *R. meliloti* was found to be dependent on glyoxylate, acetyl CoA, and magnesium with an optimum pH range between pH 8.0 and pH 9.0. The requirements and pH optimums for isocitrate lyase and malate synthetase from *R. meliloti* are similar to those reported for other species of microorganisms (6, 8) and higher plants (3, 22).

The influence of the carbon source in the growth medium on the specific activity of the key enzymes of the glyoxylate cycle in several other species of rhizobia is shown in table III. When *R. leguminosarum, R. phaseoli,* and *R. trifolii* were cultured with oleate as the principle carbon source, active isocitrate lyase systems were detected while isocitrate lyase activity was negligible when these species

Table II. *Influence of Carbon Source on Activity of Key Enzymes of the Glyoxylate Cycle in* R. meliloti
Cultures were harvested after 18 to 20 hours of growth.

Carbon source**	Fr wt of cells (g/800 ml)	Specific activity* Isocitrate lyase	Malate synthetase
Experiment I			
D-Mannitol	2.22	0.01	0.31
Succinate	2.23	0.00	0.26
Glycerol	2.39	0.00	0.37
L-Glutamate	2.07	0.02	0.37
Oleate	1.47	2.17	0.93
Experiment II			
Yeast extract	2.64	0.02	0.50
D-Mannitol + oleate	2.01	0.01	0.75
Oleate	1.39	2.40	1.28

* Specific activities of isocitrate lyase and malate synthetase are expressed as µmoles of glyoxylate formed/10 min/mg protein and µmoles acetyl CoA utilized/10 min/mg protein respectively.
** All carbon sources were supplied at a level of 0.3 % (w/v) in medium.

Table III. *Key Enzymes of the Glyoxylate Cycle in* R. leguminosarum, R. phaseoli, R. trifolii, *and* R. japonicum
R. leguminosarum, R. phaseoli and *R. trifolii* were harvested after 24 hours of growth. *R. japonicum* was harvested after the following growth periods on the various carbon sources: L-arabinose + glycerol, 34 hours; L-glutamate, 49 hours; succinate, 39 hours.

Species	Carbon source**	Specific activity*	
		Isocitrate lyase	Malate synthetase
R. leguminosarum	L-arabinose + glycerol	0.00	1.43
	Oleate	3.11	2.16
R. phaseoli	L-arabinose + glycerol	0.01	1.77
	Oleate	1.58	1.66
R. trifolii	L-arabinose + glycerol	0.02	1.37
	Oleate	2.01	2.41
R. japonicum	L-arabinose + glycerol	0.01	0.70
	L-glutamate	0.01	0.45
	Succinate	0.02	0.99
	Oleate***	...	...

* Specific activities of isocitrate lyase and malate synthetase are expressed as μmoles glyoxylate formed/10 min/mg protein and μmoles acetyl CoA utilized/10 min/mg protein respectively.
** Carbon sources were added to the basic medium at the following levels (w/v): L-arabinose, 0.1 %, glycerol, 0.5 % oleate acid, 0.3 %; L-glutamate, 0.3 %; succinate, 0.3 %.
*** *R. japonicum* failed to grow when oleate was supplied.

were cultured on the medium containing arabinose and glycerol. Malate synthetase was active in all extracts and was influenced only slightly by the nature of the carbon source. *R. japonicum* failed to grow with oleate as the principle carbon source. Appreciable malate synthetase activity was found in *R. japonicum* cultured on glutamate, succinate or arabinose and glycerol; however, the isocitrate lyase activity of these extracts was negligible.

Enzymes of the Glyoxylate Cycle in Symbiotic Rhizobia. The results of enzymatic assays on extracts from symbiotic rhizobia from nodules of several species of legumes are reported in table IV. An active malate synthetase system was present in extracts of bacteria from bush bean, cowpea, lupine and soybean nodules but bacteria from alfalfa, clover and pea nodules exhibited very weak malate synthetase activity. Significant isocitrate lyase activity was not found in any of the symbiotically cultured bacteria investigated. Malate synthetase activity in bacteria from cowpea nodules decreased between 33 and 62 days of growth as the nodules and host plants neared senescence. Little or no isocitrate lyase or malate synthetase activity could be detected in several supernatants of nodule homogenates tested. Extracts prepared from soybean and cowpea roots after the removal of nodules had negligible malate synthetase activity.

Table IV. *Key Enzymes of the Glyoxylate Cycle in Bacteria from Nodules of Leguminous Plants*

Species	Age (days)	Specific activity*	
		Isocitrate lyase	Malate synthetase
Alfalfa	40	0.01	0.06
Bush bean	27	0.00	0.42
Clover	62	0.00	0.02
Cowpea	33	0.01	0.52
Cowpea	62	0.01	0.34
Lupine	33	0.00	0.41
Pea	32	0.00	0.06
Soybean	43	0.01	0.57

* Specific activities of isocitrate lyase and malate synthetase are expressed as μmoles glyoxylate formed/10 min/mg protein and μmoles acetyl CoA utilized/10 min/mg protein respectively.

An experiment was conducted to determine if, under conditions of an inadequate energy supply to nodules from the host plant, symbiotic rhizobia would utilize fatty acids present in the nodules via the glyoxylate pathway. When excised bush bean nodules were incubated aerobically in a buffer solution for 0, 10, 20, 36 and 60 hours, appreciable induction of isocitrate lyase did not occur. Little if any change in the activity of malate synthetase was detectable in these bacterial extracts even after 60 hours of incubation. In table V the analysis of the whole bush bean nodules initially and after 60 hours of incubation shows that while the lipid content changed negligibly, the sugar and starch content of the nodules decreased by 70 % and 40 % respectively. Semiquantitative analysis of the components of the lipid fraction failed to reveal appreciable changes in the relative amounts of various classes of lipids.

Discussion

The data in table II indicated that the alfalfa endophyte, *R. meliloti*, had malate synthetase regardless of the growth substrate supplied to pure cultures. Appreciable isocitrate lyase was induced only when oleate was supplied as the principal carbon source. With this medium the specific activities

Table V. *Influence of Time of Incubation of Excised Bush Bean Nodules on Some Nodule Constituents*

Ten g samples of nodules excised from 31 day old bush bean plants were suspended in 200 ml of 1 fifth-strength nitrogen-free nutrient solution buffered with 0.008 M KH_2PO_4-K_2HPO_4 at a final pH of 6.7. The suspended nodules were incubated on a rotary shaker at 26° for times up to 60 hours.

Constituent	Time of incubation (hrs) 0	60
	% of dry wt	
Lipids*	9.9	10.7
Residue**	53.2	42.1
Free sugars	4.07	1.31
Starch	2.13	1.40

* Lipids were separated into the following classes by thin layer chromatography: pigments and sterol esters, triglycerides, free fatty acids, sterols, diglycerides and glycolipids, and phospholipids and aminolipids (18). The relative amounts of the various classes of lipids as indicated by densitometry did not change appreciably between 0 and 60 hrs of incubation.

** Residue remaining after extraction of lipids and methanol soluble materials.

of isocitrate lyase and malate synthetase were of the same order. Oleate would be expected to be degraded by β-oxidation to acetyl CoA which might then be utilized in the synthesis of carbon skeletons of cellular constituents via the glyoxylate cycle and reversal of glycolysis. When oleate and mannitol were both supplied, significant induction of isocitrate lyase was not detectable indicating that mannitol was utilized in the formation of carbon skeletons of cellular constituents. In this medium oleate may have been degraded to acetyl CoA by the β-oxidation pathway; however, acetyl CoA apparently was not then utilized via the glyoxylate cycle.

The role of isocitrate lyase in the growth of *R. leguminosarum*, *R. phaseoli*, and *R. trifolii* on an acetyl CoA precursor seems to be very similar to its role in *R. meliloti* (table III). Acetate has been used as a growth substrate to induce isocitrate lyase in many microorganisms (16), however, the species of rhizobia investigated failed to make satisfactory growth on Na acetate in our laboratory.

The operation of the glyoxylate cycle in bacteria has been proposed to be controlled largely by intracellular regulation of the synthesis and activity of isocitrate lyase (16). Kornberg (15) has proposed that induction of isocitrate lyase in *Escherichia coli* is controlled by an intracellular repressor closely related metabolically to oxalacetate or malate. Acetyl CoA acts to reduce the level of oxalacetate and malate by formation of citrate thus permitting the synthesis of isocitrate lyase. Phosphoenolpyruvate and isocitrate have been reported as intracellular inhibitors of isocitrate lyase in *E. coli* B (1).

While an appreciable level of isocitrate lyase has been detected in several species of bacteria when not utilizing an acetyl CoA precursor as the carbon source, an investigation with mutants of *E. coli* lacking this enzyme indicates that isocitrate lyase is not essential for the ultilization of glutamate or glycolate (2). Thus isocitrate lyase may not play an essential role in rhizobia except when an acetyl CoA precursor is the principal carbon source. This is consistent with the virtual absence of isocitrate lyase activity in extracts of rhizobia from nodules (table IV) or bacteria cultured on carbon sources other than oleate.

Rhizobia from nodules of bush bean, cowpea, lupine and soybean were found to have active malate synthetase systems, however, this enzyme was barely detectable in bacteria from alfalfa, clover or pea nodules. While malate synthetase has been considered by some investigators to be a constitutive enzyme, Reeves and Ajl (20) report that *E. coli* cultured anaerobically on a glucose-citrate medium exhibit neither isocitrate lyase nor malate synthetase activity and that both enzymes are adaptive. The detection of appreciable levels of malate synthetase in bacteria from nodules is of interest in view of the partially anaerobic environment believed to be maintained in nodules (13).

The occurrence of malate synthetase in aerobically cultured bacteria supplied with a variety of carbon sources has led to the suggestion that glyoxylate may arise metabolically from sources other than isocitrate via isocitrate lyase. While glyoxylate may be derived from glycolate, glycine or oxalate (16) by known metabolic pathways, an actual pathway of formation of glyoxylate from longer chain carbon compounds in bacteria is not known. Translocation of glyoxylate from host tissue or formation of glyoxylate from other sources, possibly glycolate, may be responsible for the appreciable activity of malate synthetase in bacteria from nodules of certain species of legumes.

The relatively high content of lipids in soybean and bush bean nodules (tables I and V) apparently does not under normal conditions provide raw material for growth or energy via the glyoxylate cycle since negligible activity of isocitrate lyase was found in nodules. The differential composition of fatty acids in soybean nodules and bacteria is of interest as it is the first attempt to characterize the fatty acids in these materials. A detailed study of the composition and function of these lipids would probably provide an explanation of their high level in nodules.

When excised bush bean nodules were incubated aerobically for as long as 60 hours, lipids from nodules were neither utilized for the synthesis of cellular constituents nor oxidized for energy. Failure of lipids to be utilized or isocitrate lyase to be induced in the excised nodules may perhaps be attributed to the presence of adequate endogenous sugar and starch. By 60 hours of incubation the condition of the nodules had begun to deteriorate as evidenced by the disappearance of the hemoglobin.

Acknowledgment

The authors express their appreciation to Dr. George Sorger for his review of this manuscript.

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