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Utilization of Aldehydes and Alcohols by Soybean Bacteroids

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ABSTRACT

Aldehydes, alcohols and acids were tested for their ability to support acetylene reduction and oxygen consumption by *Rhizobium japonicum* bacteroids isolated from soybean nodules. Several alcohols and aldehydes increased acetylene reduction and oxygen uptake. This is consistent with the concept that the plant nodule cytosol can metabolize carbohydrate via anaerobic fermentative pathways.

Symbiotic nitrogen fixation requires carbon compounds for at least three functions. Their metabolism provides the ATP required by nitrogenase; they are the ultimate source of electrons for reduction; they provide carbon skeletons for the incorporation and transport of fixed NH_3 to the shoot. How the plant and bacteroids share the metabolism of carbon compounds is hardly known.

Phloem analyses (14) indicate that sucrose is the major carbon source for nodules. There is a neutral invertase in the nodule cytosol but not in the bacteroid (12). The soybean host cell cytosol contains sucrose and glucose, and cyclitols such as pinitol and inositol (20). It is generally assumed that the Embden-Myerhof and tricarboxylic acid pathways are operative in the host cell, but this has never been firmly established. The isolated soybean bacteroid can use pyruvate, fumarate and succinate, but not sucrose, to support respiration and nitrogen fixation (2). It is not known what carbon compound(s) the bacteroid obtains from plant cytosol.

Determination of enzyme activity and respirometric measurements indicate that *Rhizobium japonicum* bacteroids from soybean nodules have a functional tricarboxylic acid cycle (16). The existence of the anapleurotic glyoxylate cycle is in doubt because isocitrate lyase could not be detected in either bacteroids or plant cytosol of functioning nodules (11). However, that enzyme could be detected in bacteroids from senesced nodules (24) and a partial glyoxylate cycle was indicated from utilization of [^{14}C]acetate by bacteroid suspensions (19).

The outer layer of nodule tissue is a barrier to O_2 diffusion (22). Transport of O_2 to support oxidative phosphorylation by bacteroids is mediated by leghemoglobin. Because that protein binds O_2 firmly, the O_2 in the plant cytosol is about 10 nM (1). The plant cytosol is thus microaerobic. De Vries (6) suggested that the nodule cytosol should therefore have a fermentative carbon metabolism similar to that observed in roots of "flood tolerant" plants. Oxaloacetate and malate, acetaldehyde and ethanol, lactate, alanine, or other compounds (18, and references cited therein) might be produced fermentatively as alternatives to oxidative metabolism of PEP[1] and pyruvate. DeVries examined the role of malate, which can be synthesized via cytosol PEP carboxylase (E.C. 4.1.1.31) and malate dehydrogenase (E.C. 1.1.1.37) (5, 6, 13, 15) reactions. Like previous researchers, he found that nodule cytosol had higher activities of both of these enzymes than did root cytoplasm. Nodule malate levels have been estimated at about 3.4 mM in peas (6) and 4.2 mM in soybeans (21).

An alternative fermentative pathway from pyruvate is via acetaldehyde to alcohol. We have found that the nodule cytosol contains pyruvic decarboxylase (E.C. 4.1.1.1) and its product acetaldehyde (S. Tajima and T. LaRue, unpublished data). The purpose of this study was to determine whether aldehydes and alcohols can be utilized by the isolated bacteroid.

MATERIALS AND METHODS

Chemicals. Sodium formate was obtained from Baker Chemical Co., Phillipsburg, NJ. Acetaldehyde, propionaldehyde and pyruvaldehyde were obtained from Eastman Kodak Co. Sodium acetate, acetone, 1-butanol and sodium succinate were obtained from Mallinckrodt Chemical Co. Ethanol was obtained from U.S. Industries Chemical Co., Louisville, KY. Sodium glyoxylate, sodium pyruvate, sodium DL-lactate, L-alanine, butyric acid, sodium DL-α-hydroxybutyrate, sodium DL-β-hydroxybutyrate, sodium L-malate, succinic semialdehyde, sodium benzoate, hypoxanthine, and MOPS were obtained from Sigma Chemical Co. Butyraldehyde, 2-butanol, 2-butanone, benzaldehyde, and benzyl alcohol were obtained from Aldrich Chemical Co., Metuchen, NJ. Acetylene was a product of Union Carbide Corp., Linde Division, New York, NY. Acetaldehyde, ethanol, propionaldehyde, pyruvaldehyde, acetone, butyraldehyde, butyric acid, 1-butanol, 2-butanol, 2-butanone, benzaldehyde, and benzyl alcohol were distilled prior to use.

Biological Materials. Soybean seeds (*Glycine max.*, var Wilkin) were obtained from William Boyd Seed Co., Phelps, NY. Plants were grown in greenhouses in a solid medium of Turface (IMC Corp., Havelock, Ontario) with $CaCO_3$ added to keep the pH near neutral. They were supplied with nutrient solution three times weekly. The nutrient was that described by Evans *et al.* (9), except iron sequestrine was increased ten-fold. Seeds were inoculated with commercial soybean inoculum (Nitragin Co., Milwaukee, WI). Nodules were collected from 5- to 6-week-old plants which were flowering or in early stages of pod fill.

Bacteroids were prepared from root nodules essentially as described by Emerich *et al.*, (8). For preparation of bacteroids, all buffers were 0-4 C and thoroughly sparged with Ar. All operations were performed under a continuous stream of Ar and centrifugations were in sealed, Ar-flushed tubes. Nodules were rinsed extensively with deionized water. Ten-g batches were ground in a chilled mortar and pestle with three volumes grinding medium [50 mM K-phosphate (pH 6.8) made 200 mM with sodium ascorbate] and 1/3 weight acid-washed polyvinylpolypyrollidone. The macerate was filtered through four layers of cheesecloth and the filtrates were centrifuged 10 min at 5000*g*. The supernatant was discarded. The pellet was resuspended and centrifuged as above three times with assay buffer (50 mM MOPS, 2.5 mM $MgCl_2$, 1 mM K-phosphate [pH 7.5 with KOH)]. The final pellet was resus-

[1] Abbreviation: PEP, phosphoenolpyruvate; MOPS, morpholinopropane sulfonic acid.

Table I. *The Effects of Substrates on Acetylene Reduction and O_2 Uptake by Isolated R. japonicum Bacteroids*

Acetylene reduction. Assays were performed in triplicate over the same O_2 range as in Figure 3 except with highly stimulatory substrates. Each assay contained 3.4–4.2 mg dry wt bacteroids, depending on the preparation used. The ratios of acetylene reduction were calculated from the highest mean values obtained with and without added substrate. Some experiments were repeated with different bacteroid preparations and replicate values are presented. Data from Figs. 1 to 4 are incorporated in the table. In most cases the pO_2 of maximum activity with added substrate was estimated by interpolation (e.g. Figs. 3–6).

Oxygen uptake. Assays were as described in "Materials and Methods" with 6.4 to 7.0 mg dry wt bacteroids. Initial rates of O_2 uptake ranged from 0.092–0.264 μmol/ml min. Values are means of four determinations. Substrates were added when O_2 concentration was between 190 and 150 μM (254 μM saturating concentration) and O_2 uptake was followed to zero detectable O_2. Treatments giving definite rate increases also gave increases in rate at the lowest detectable (about 10 μM) concentration.

Compound Class	Compound	Concentration	Effect on Acetylene Reduction		Effect on O_2 Uptake
			Peak height +subst./endog.	O_2 optimum +subst./endog.	+subst./endog. ± SE
1 Carbon	formate (Na)	0.20 mM	0.69; 1.00	0.94; 1.0	1.00 ± 0.01
		2.0 mM	0.71; 1.00	0.92; 1.1	1.01 ± 0.01
2 Carbon	acetaldehyde	0.00020%, 46 μM	1.2	1.2	
		0.0010%, 0.23 mM	1.72; 2.75	1.5; 1.4	2.21 ± 0.09
		0.010%, 2.3 mM	1.53; 2.12; 1.45	2.0; 1.7; 1.9	3.33 ± 0.24
	acetate (Na)	0.20 mM	1.37	1.6	1.20 ± 0.06
		2.0 mM	1.23	1.7	1.25 ± 0.08
	ethanol	0.0010%, 0.22 mM	1.23	1.3	1.14 ± 0.04
		0.010%, 2.2 mM	1.32	1.4	1.22 ± 0.04
	glyoxylate (Na)	0.20 mM	1.33	1.2	1.14 ± 0.06
		2.0 mM	1.07	1.0	1.13 ± 0.05
3 Carbon	propionaldehyde	0.0010%, 0.17 mM	1.33	1.3	2.75 ± 0.14[a]
		0.010%, 1.7 mM	1.47	1.7	3.51 ± 0.30
	pyruvate (Na)	0.20 mM	1.08	1.1	1.04 ± 0.02
		2.0 mM	1.24	1.3	1.15 ± 0.08
	pyruvaldehyde	0.0010%, 0.14 mM	1.00	1.2	1.37 ± 0.08
		0.010%, 1.4 mM	0.45	1.2	1.72 ± 0.15
	acetone	0.0010%, 0.17 mM	0.75	0.84	1.00 ± 0.01
		0.010%, 1.7 mM	0.97	1.0	0.97 ± 0.01
	DL-lactate	0.20 mM	1.10	1.0	
		2.0 mM	1.09	1.3	
	L-alanine	0.20 mM	1.11	1.1	
		2.0 mM	0.85	1.5	
4 Carbon	butyraldehyde	0.0010%, 0.14 mM	1.22	1.3	2.66 ± 0.13[a]
		0.010%, 1.4 mM	1.51	2.1	3.15 ± 0.22
	butyric acid	0.0010%, 0.11 mM	1.24	1.2	
		0.010%, 1.1 mM	1.31	1.3	
	1-butanol	0.0010%, 0.13 mM	1.69	1.3	
		0.010%, 1.3 mM	1.55	1.3	
	2-butanol	0.0010%, 0.13 mM	1.04	1.0	
		0.010%, 1.3 mM	1.00	1.0	
	2-butanone	0.0010%, 0.14 mM	0.89	1.0	
		0.010%, 1.4 mM	1.12	1.0	
	DL-β-hydroxy-butyrate (Na)	0.20 mM	1.29	1.1	
		2.0 mM	1.41	1.3	
	DL-α-hydroxy-butyrate (Na)	0.20 mM	1.20	1.3	
		2.0 mM	1.18	1.3	
	L-malate (Na)	0.20 mM	1.46	2.0	
		2.0 mM	1.60	2.0	
	succinate (Na_2)	0.2 mM	1.76	2.1	3.35 ± 0.31
		2.0 mM	≥1.61	≥2.9	5.11 ± 0.71
		10.0 mM	≥1.40; 1.58	≥2.5; 2.7	
	succinic semi-aldehyde	0.0010%, 86 μM	1.42	1.7	2.14 ± 0.06
		0.010%, 0.86 mM	1.26	2.4	2.83 ± 0.21
Benzene derivatives	benzaldehyde	0.0010%, 94 μM	1.15	1.0	3.24 ± 0.11[a]
		0.010%, 0.94 mM	1.34; 1.46	1.8; 1.7	3.28 ± 0.22
	benzoate (Na)	0.20 mM	0.93; 0.98	1.0; 1.0	0.99 ± 0.01
		2.0 mM	0.59; 0.71	1.0; 1.0	1.05 ± 0.03
	benzyl alcohol	0.0010%, 92 μM	1.21; 1.00	1.1; 1.0	1.01 ± 0.02
		0.010%, 0.92 mM	1.13; 1.03	1.1; 1.1	1.00 ± 0.01
Misc.	hypoxanthine	0.20 mM	1.12	1.0	
		2.0 mM	0.89	1.0	
	NaCl	0.20 mM	1.18	1.0	
		2.0 mM	1.02	1.0	
		20.0 mM	0.92	1.2	0.93 ± 0.08

[a] Treatments giving transient (1–4 min) rate increases.

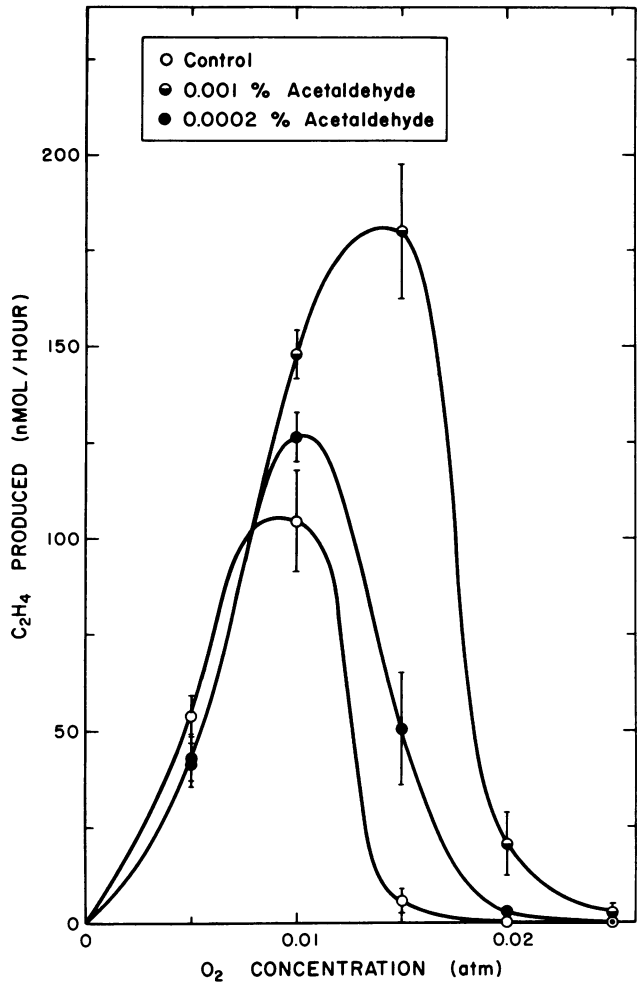

FIG. 1. Effect of acetaldehyde on acetylene reduction in *R. japonicum* bacteroids. Assays were in triplicate. Standard errors are indicated by error bars. The O_2 concentration is that in the vials at the beginning of the incubation.

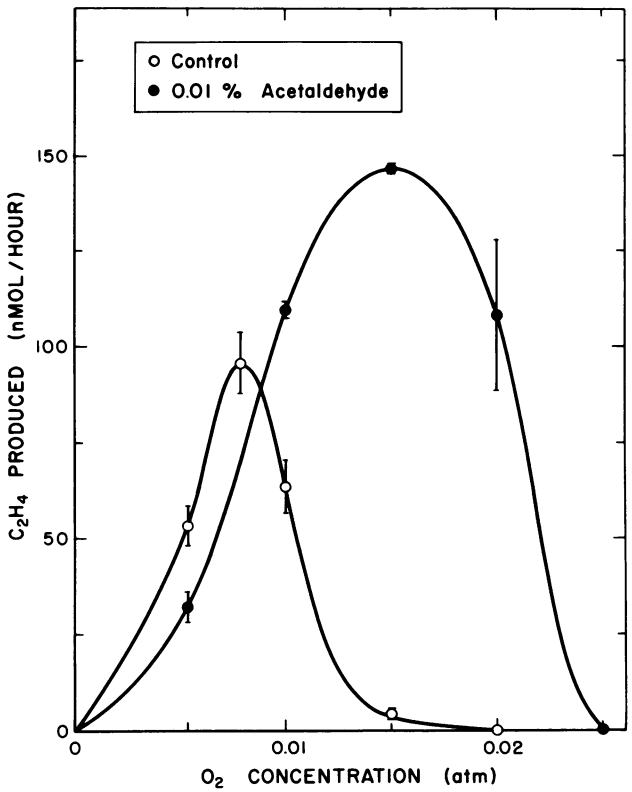

FIG. 2. Effect of acetaldehyde on acetylene reduction in *R. japonicum* bacteroids.

pended in assay buffer (1 ml/g nodules used) and frozen in 7- to 8-ml aliquots by dropping into liquid N_2. Bacteroids stored at −80 C retained approximately 70% of their initial acetylene-reducing activity. Bacteroids prepared with hypotonic buffers in this fashion have been shown to be free of mitochondria (4, 8).

Assays for Nitrogenase Activity. The method was similar to that described by Emerich *et al.*, (8). Assays were performed in 22-ml serum vials in a water bath-shaker at 23.5 C and 150 rpm. Reaction mixtures were prepared by flushing the vials with Ar followed by addition of 2.4 ml (Ar-flushed) assay buffer (see above), acetylene to make 0.1 atm, and O_2. Substrate was usually added in a volume of 25 μl or less and the reactions were initiated with addition of 0.10 ml bacteroid suspension. Samples (0.5 ml) of the gas phase were taken at 20 and 40 min intervals and assayed for ethylene by GC.

O_2 Uptake Assays. Bacteroids were prepared as for nitrogenase assays except the washing procedures were done under air instead of under Ar. The nitrogenase is destroyed by O_2. Assays were performed in 2.0 ml of the assay buffer described above. Each assay contained 0.20 ml bacteroid suspension and substrates were usually added in a volume of 20 μl. O_2 was measured with a YSI 5331 O_2 probe (Yellow Springs Instrument Co., Yellow Springs, OH) in a water-jacked cuvette (23.5 C) equipped with a stir bar for rapid equilibrations.

RESULTS AND DISCUSSION

A series of alcohols, aldehydes and acids have been tested for their effects on acetylene reduction, O_2 optimum for acetylene reduction, and O_2 uptake. Results are summarized in Table I.

Bacteroids have an endogenous metabolism which permits reduction of acetylene; the activity varies with O_2 concentration. Acetaldehyde, and to a lesser extent ethanol, supported an increased C_2H_2 reduction when supplied to bacteroids at low concentrations (Figs. 1–3). The effect is similar to that of succinate (Fig. 5) and malate. Our results with succinate and malate are similar to observations reported by others (2, 8, 23).

Acetaldehyde and ethanol also cause an upward shift in the level of O_2 at which maximum acetylene reduction is observed though not as much as malate and succinate. This effect has been reported for several carbon compounds (8, 23) and for H_2 (8). It is attributed to an apparent respiratory protection: compounds supporting oxidative phosphorylation reduce the intercellular concentration of O_2 which would otherwise denature nitrogenase. Stimulation of soybean bacteroid O_2 uptake by a wide range of carbon substrates, including ethanol, has been observed (17).

Ethanol and acetaldehyde are interconverted by alcohol dehydrogenase (E.C. 1.1.1.71). Acetaldehyde may be converted to acetate which then can enter the tricarboxylic acid cycle via acetyl CoA synthetase (E.C. 6.2.1.1). Acetate also supports acetylene reduction (Fig. 4). How acetaldehyde could be converted to acetate in bacteroids is unknown. In mammalian or microbial systems, the reaction may be catalyzed by aldehyde oxidase (E.C. 1.2.3.1), aldehyde dehydrogenase (E.C. 1.2.1.3), xanthine oxidase (E.C. 1.2.3.2) or xanthine dehydrogenase (E.C.1.2.1.37) (7).

To determine if metabolism was specific for acetaldehyde, structurally related compounds were tested (Table I). The ketones acetone and 2-butanone did not increase nitrogenase activity nor offer apparent respiratory protection. Acetylene reduction and apparent respiratory protection was increased by 1-butanol. Ben-

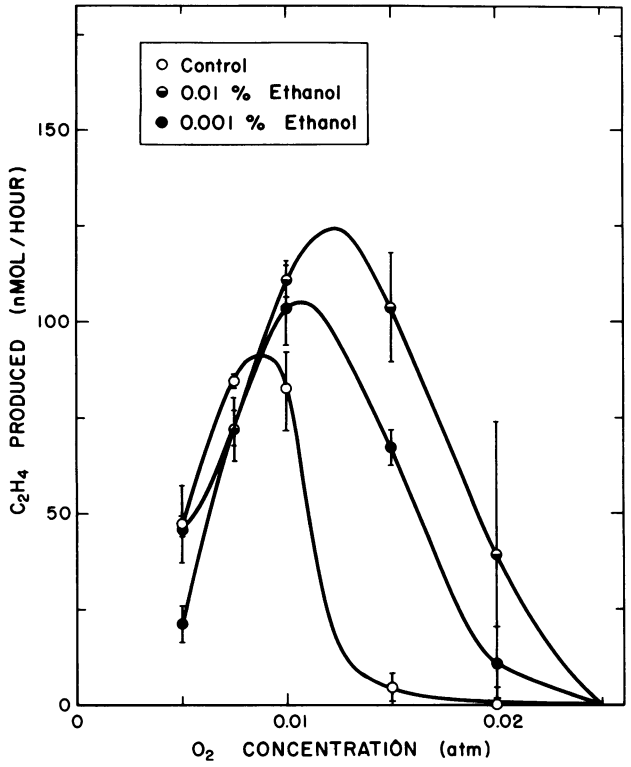

FIG. 3. Effect of ethanol on acetylene reduction in *R. japonicum* bacteroids.

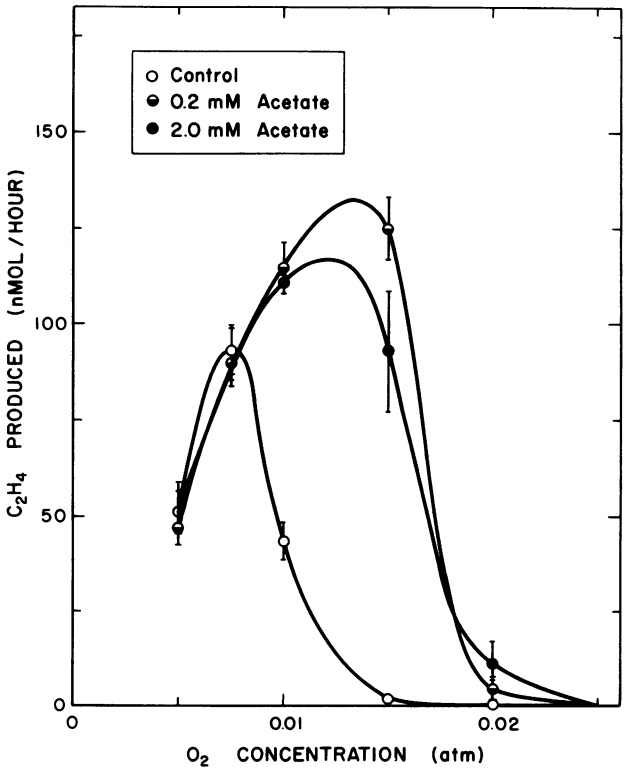

FIG. 4. Effect of sodium acetate on acetylene reduction in *R. japonicum* bacteroids.

zyl alcohol produced a slight increase and 2-butanol was without effect; presumably the bulky substitutions prevent enzyme action.

The aldehydes glyoxylate, acetaldehyde, propionaldehyde, butyraldehyde, succinic semialdehyde and benzaldehyde all increased acetylene reduction above endogenous levels. Each of these increased O_2 uptake and apparently provided at least some respiratory protection. Hypoxanthine was without effect, suggesting that acetaldehyde was not oxidized via xanthine oxidase or xanthine dehydrogenase.

Alanine and lactate, possible anaerobic products of pyruvate, did not increase the level of acetylene reduction. At 2.0 mM, they gave a significant increase in apparent respiratory protection. Among the carboxylic acids, succinate and malate were most effective at supporting acetylene reduction and raising the optimum O_2 for activity. Acetate, pyruvate, butyrate, β-hydroxybutyrate and α-hydroxybutyrate were less effective while formate and benzoate were inactive.

O_2 uptake was measured for bacteroids prepared aerobically. Their nitrogenase was therefore inactive. Measurements were made at O_2 concentrations higher than exist in the nodule. The stimulations of O_2 uptake (Table I) correlated well with apparent respiratory protection, except in the case of 0.0010% benzaldehyde. Benzaldehyde, as well as propionaldehyde and butyraldehyde at this concentration gave a temporary increase in respiration rate. The brief period of effectiveness may have been due to their being used up in the experiment. Benzaldehyde at 0.0010% stimulated O_2 uptake as well as at the ten-fold higher concentration. Therefore, it may be that under the conditions of acetylene reduction assay, the benzaldehyde is metabolized before it can have a noticeable effect on the assay. The same situation may exist with propionaldehyde and butyraldehyde but their products might be further metabolized. Alternatively, affinities for these compounds may change when the O_2 concentration is lowered. The presence of various terminal oxidases with greatly different O_2 affinities has been established (2). Nevertheless, it is clear that correlations of O_2-uptake and apparent respiratory protection were good.

The effects of aldehydes added with tricarboxylic acid cycle precursors were examined. Acetaldehyde did not increase acetylene reduction over the values obtained with succinate (Fig. 5); and benzaldehyde did not increase values over those for acetate (Fig. 6). There was an increase in the O_2 optimum. This suggests that electrons from the aldehydes and citric acid cycle precursors

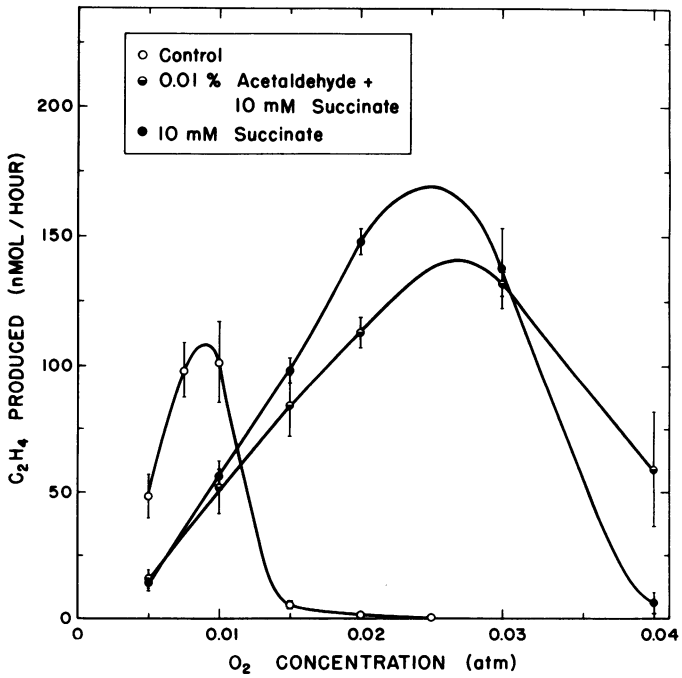

FIG. 5. Effect of acetaldehyde on succinate-stimulated acetylene reduction in *R. japonicum* bacteroids.

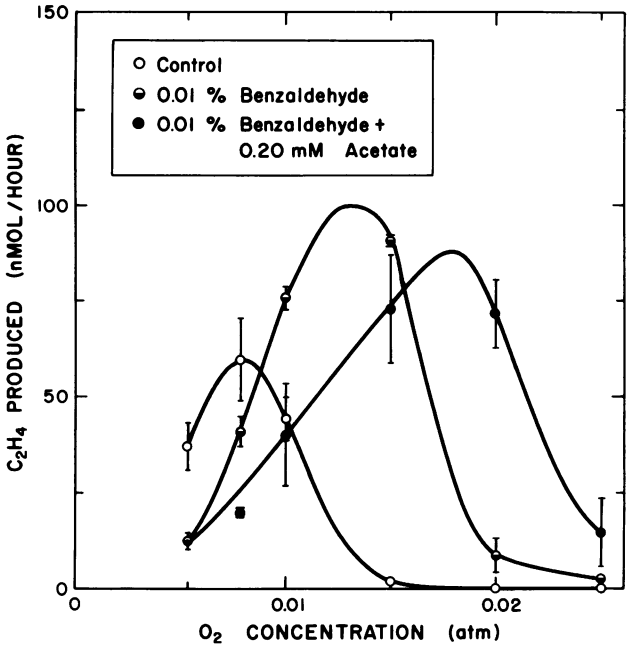

FIG. 6. Effect of sodium acetate on benzaldehyde-stimulated acetylene reduction in *R. japonicum* bacteroids.

entered a common pool from which reductant for nitrogenase and oxidative phosphorylation was drawn. This concept is consistent with extensive data, summarized by Evans and Phillips (10), that reduced nicotinamide nucleotides can support nitrogenase activity in reconstituted electron transport systems. Interestingly, Emerich *et al.*, (8) obtained similar results with H_2 and succinate. Addition of H_2 was shown to elevate ATP levels in their system. Other interpretations of our data are possible, especially since the source of endogenous reductant is not known.

The data show that acetaldehyde and ethanol may be utilized by soybean bacteroids. The metabolism of acetaldehyde seems to be via enzymes of broad specificity, since other aldehydes behave similarly. Acetaldehyde and ethanol are more lipophilic than carboxylic acids, and their movement across the bacteroid membrane may require less energy than the transport of substrates such as succinate and malate. This is consistent with the relative lack of inhibition of acetylene reduction at low O_2 concentrations with acetaldehyde and ethanol (Figs. 1-3). Succinate at 10 mM (Fig. 5) and lower concentrations gave a much greater inhibition than acetaldehyde and ethanol. At the suboptimal O_2 concentrations, ATP is apparently limiting to nitrogenase activity. Therefore, energy required for transport or activation of carbon compounds might be reflected in this inhibition. The inhibitory effect was also noted for H_2 and succinate by Emerich *et al.* (8).

This is the first report of a wide range of compounds supporting acetylene reduction by bacteroids. It was already known that isolated bacteroids have a broad capacity to utilize various carbon substrates in oxidative metabolism (3, 16, 17, 19). Assignment of a physiological significance to any compound requires demonstration of its presence, synthesis, and utilization. For compounds to be sources of carbon for bacteroids, synthesis must occur in plant cells. Malate is one such compound. It has been detected in nodules (6, 21) at mM concentrations. It can be synthesized fermentatively with plant cytoplasm enzymes (5, 6, 13, 15) and can support nitrogenase activity in bacteroids (Table I). Acetaldehyde and ethanol have been detected in nodules (24, Tajima and LaRue, unpublished data). Our studies have demonstrated that acetaldehyde and ethanol can support acetylene reduction and O_2 uptake. Their biosynthesis in plant cells as well as pathways of energy coupling to bacteroid oxidative phosphorylation and nitrogenase are under current investigation.

Our experiments support the concept of De Vries *et al.* (6) of an anaerobic plant cytosol with a fermentative metabolism providing substrate for oxidation by bacteroids. De Vries demonstrated the involvement of malic acid in bacteroids. Our data indicate that ethanol and acetaldehyde also could be involved as substrates for bacteroid metabolism.

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