METABOLISM OF L-ARABINOSE IN *AZOSPIRILLUM BRASILENSE*

BY

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By

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Chairman: Arnold S. Bleiweis
Major Department: Microbiology and Cell Science

Under dinitrogen fixing conditions *Azospirillum brasilense* showed comparable generation times using L-arabinose or malate as sole carbon and energy sources. This root associated bacterium was found to metabolize L-arabinose through an oxidative pathway that has also been found in certain species of *Pseudomonas* and fast-growing *Rhizobium*. L-Arabinose is converted to L-arabono-γ-lactone by an NAD(P) dependent dehydrogenase, hydrolyzed to L-arabonic acid by a lactonase, and dehydrated to L-2-keto-3-deoxyarabonate (L-KDA) by dehydratase activity. In crude extracts NAD is rapidly reduced if potassium L-arabonate or DL-KDA is added as substrate. DL-KDA has been found to reduce NAD at 3 times the rate of L-arabonate. Alpha-ketoglutarate accumulated in crude extracts to which L-arabonate and NAD were added. It is proposed that L-KDA is dehydrated to alpha-ketoglutaric semialdehyde which is then oxidized to alpha-ketoglutaric acid by an NAD dependent dehydrogenation reaction. L-Arabinose dehydrogenase and L-arabonate dehydratase have been partially purified and characterized. The NAD(P) dependent

dehydrogenase, which has been found to be specific for the L-arabono-configuration, has been purified 59 fold. No enhancement of activity in the presence of any divalent cation or reducing agent tested has been found. The K_m values of 75 μM and 140 μM were found with NADP and NAD as cofactors, respectively. The enzyme has a pH optimum of 9.5 in glycine buffer and was stable when heated to 55°C for 5 minutes. The enzyme product has been identified as L-arabono-γ-lactone. L-Arabonate dehydratase has been purified 38 fold. The presence of $MgCl_2$ and $MnCl_2$ has been found to significantly increase enzyme activity. The pH optimum for the dehydratase has been found to be 7.8. The enzyme product was identified as L-2-keto-3-deoxyarabonate. In further studies TCA cycle dehydrogenases have been assayed and indicate an active TCA cycle. The presence of b and c type cytochromes has been confirmed and their distribution between membrane and soluble fractions determined.

SECTION I

IDENTIFICATION OF ENZYMES INVOLVED IN L-ARABINOSE METABOLISM IN *AZOSPIRILLUM BRASILENSE*. ASSAY OF TCA CYCLE DEHYDROGENASES AND IDENTIFICATION OF COMPONENTS INVOLVED IN ELECTRON TRANSPORT

Introduction

Azospirillum brasilense (ATCC 29145) grows and fixes molecular nitrogen best on organic acids such as succinate, malate, and pyruvate (13, 28, 29, 38). Variable results have been reported for *A. brasilense* growth on sugars. Day and Dobereiner (13) reported that several sugars including glucose could be used for growth under nitrogen fixing conditions but only if "starter" organic acid was added to the medium. Okon et al. (28) found growth and acetylene reduction rates were less on galactose than on several organic acids tested and found little or no growth under nitrogen fixing conditions with glucose as a carbon and energy source. Tarrand and Krieg (38) have also found *A. brasilense* unable to use glucose as a sole carbon and energy source under nitrogen fixing conditions. Okon et al. (29) found little or no increase in oxygen uptake rates above endogenous levels in cell free extracts if glucose, fructose, galactose, or several phosphorylated metabolic intermediates were added as substrates.

Child and Kurz (6) reported that in certain cases nitrogenase activity in *A. brasilense* could be enhanced by supplementing the organic acid containing medium with arabinose. In examining *A. brasilense* growth under nitrogen fixing conditions on a variety of

substrates, I found that L-arabinose served as an excellent sole carbon and energy source. I therefore decided to investigate the mode of L-arabinose catabolism in this root associated bacterium.

My initial failure to find a pathway involving phosphorylated intermediates led me to look at other possible pathways. There are two well documented pathways of L-arabinose metabolism not involving phosphorylated intermediates. In the first pathway L-arabinose is oxidized to L-arabono-γ-lactone by an NAD(P) dependent dehydrogenase, the lactone is cleaved by lactonase to L-arabonic acid, followed by two successive dehydration reactions forming L-2-keto-3-deoxyarabonate and alpha-ketoglutaric semialdehyde, respectively. The last step involves the NAD dependent dehydrogenation of the semialdehyde to alpha-ketoglutaric acid. The second pathway has the same initial steps but the L-2-keto-3-deoxyarabonate is cleaved through an aldolase reaction to glycolaldehyde and pyruvate. The first pathway has been demonstrated in *Pseudomonas saccharophila* (42), *Pseudomonas fragi* (7, 41), and the fast growing rhizobia (15, 16), *R. meliloti, R. trifolii, R. phaeseoli,* and *R. leguminosarum*. The second pathway has been demonstrated in *Pseudomonas* strain MSU-1 (8), and slow growing rhizobia; *R. japonicum* (15, 31) and *Rhizobium* sp. 32H1 (15). In addition to these L-arabinose dehydrogenase has also been demonstrated in *Aquaspirillum gracile* (23) although the subsequent pathway is unknown.

In this paper evidence is presented indicating the first pathway (i.e. L-arabinose ⟶ alpha-ketoglutaric acid) is present in *A. brasilense*. The presence of TCA cycle dehydrogenases and cytochromes is also demonstrated, indicating that the alpha-ketoglutarate formed

provides energy to the cell through electron transport coupled to TCA cycle dehydrogenases.

Materials and Methods

Bacterial Strain

Azospirillum brasilense (ATCC 29145) was obtained from the American Type culture collection. For daily use cultures were maintained on Trypticase soy agar (BBL) with biweekly transfer. Stock cultures were frozen in liquid nitrogen.

Media

The growth medium was that of Nelson and Knowles (27) with twice the concentration of potassium phosphate buffer (65 mM, pH 7.1). Malate or L-arabinose (37 mM) was filter sterilized and added to the autoclaved and cooled medium. Ammonium chloride, 0.1%, was added to ammonia grown cells.

Growth and Nitrogen Fixation

Two and one-half liters of the nitrogen free medium were added to a vessel consisting of a 2.8 L Fernbach flask tightly capped with a rubber stopper through which had been placed a gas inlet port (ending in a sparging stone), a gas outlet port, a sampling port, and an oxygen probe (2). The oxygen probe and air line were connected to an oxygen stat (see Appendix 1). The flask containing a stirring disc was immersed in a 25°C water bath set on a magnetic stirrer. Air and nitrogen were mixed to maintain the O_2 concentration at 0.5% $\pm$ 0.05%.

For NH_4Cl grown cells, 1.5 L of medium were placed in a cotton stoppered Fernbach flask and rapidly shaken at room temperature. In both N_2 and ammonia grown cells a 10% inoculum containing 0.05% NH_4Cl was grown aerobically.

Acetylene Reduction Assay

Six milliliters of cells were removed anaerobically from the growth vessel and placed in a 125 ml flask that had previously been sparged with argon and capped with a serum stopper. Oxygen, 0.5%, was added as air back to the flask. The samples were shaken on a New Brunswick rotary shaker for 1 h at 150 rpm and 25°C. One-tenth milliliter gas volume was removed and injected into a Varian 2400 series gas chromatograph with Poropak Q columns and flame ionization detectors.

Growth was followed by reading optical density at 560 nm and protein determined by the method of Lowry (24).

Determination of Arabinose Pathway Enzymes

Both NH_4Cl and N_2 grown cells gave similar specific activities for enzymes involved in arabinose metabolism. Due to this and the ease of growing large batches of cells with a fixed nitrogen source, ammonium chloride grown cells were used for the enzyme studies. Crude extracts were prepared in the following manner. Cells, near the middle or end of exponential growth, were collected by centrifugation and washed once in 0.1M sodium-potassium phosphate buffer, pH 7.5, resuspended to ca. 0.25 g per ml in the same buffer, passed twice through an Aminco french pressure cell at 20,000 p.s.i., and centrifuged at 10,000 xg for 30 min. The supernate, crude extract, could be stored for several months at

-80°C without significant loss of enzymatic activity. L-arabinose dehydrogenase was assayed in a reaction mixture containing 125 μmoles glycine/NaOH buffer, pH 9.0, 0.5 μmoles NAD, 0.5 μmoles L-arabinose, 1 μmole NaCN, and enzyme plus water to 1 ml. The change in optical density at 340 nm was followed on a Zeiss spectrophotometer at 25°C. Lactonase activity was measured by the method of Dahms and Anderson (10) using alkaline hydroxylamine reagent (18) to quantitate the disappearance of L-arabonolactone. Arabonate dehydratase assay was that of Pedrosa and Zancan (31) with L-2-keto-3-deoxyarabonate (L-KDA) accumulation being measured with semicarbazide reagent (25). KDA aldolase was assayed in the reverse direction (31) with KDA formation being followed by the thiobarbituric-periodate method (43). The following assay mixture was used to determine NAD dependent KDA oxidation: 60 μmoles potassium phosphate buffer pH 7.2, 10 μmoles NAD, 10 μmoles potassium arabonate, 1 μmole NaCN, and water plus enzyme to 1 ml. In all cases 1 unit of enzyme activity is that amount of enzyme which produces 1 μmole of product per minute at 25°C.

Identification of Pathway End Product

The accumulation of alpha-ketoglutaric acid in crude extracts, after the addition of potassium arabonate (10 μmoles), was determined as follows. The arabonate oxidation mix was the same as that used to follow KDA oxidation. In some experiments water or 10 μmoles sodium arsenite replaced NaCN. The reaction mix was incubated up to six hours and alpha-ketoglutarate was enzymatically quantitated in a reaction mixture containing potassium phosphate buffer, 80 μmoles, pH 7.2, 0.32 μmoles NADH, 40 μmoles NH_4Cl, 3.3 units glutamate dehydrogenase (Sigma)

and 0.05 ml of the above arabonate oxidation mixture in a total of 1 ml. The amount of NADH oxidized was determined against controls without NADH and without both glutamate dehydrogenase and NH_4Cl to correct for NADH oxidase activity. Controls were also run using the arabonate oxidation mixture to which arabonate had not been added. Pyruvate was enzymatically determined in a reaction mixture containing potassium phosphate buffer, 50 µmoles, pH 7.2, 1 unit lactic dehydrogenase (Sigma), 0.32 µmoles NADH, 0.05 ml arabonate oxidation mix, and water to 1 ml. Controls were run without lactic dehydrogenase to correct for endogenous NADH oxidase activity and with arabonate oxidation mixture to which arabonate had not been added. Glycolaldehyde was quantitated with diphenylamine reagent (14).

Chromatography

Dehydrogenase and dehydratase reaction products, using partially purified enzyme, as well as identification of the arabinose pathway terminal product, were determined by thin layer and paper chromatography. The samples were processed as follows. The reaction mixture was passed through a small Dowex-X8 (H+ form) column, 0.5 ml bed volume, to remove cations and precipitate proteins, then passed through a 0.45 µ membrane filter, and concentrated 20 fold by lyophilization. KDA was further processed by the method of Weimberg (40) before spotting. Samples were spotted on Whatman #1 paper and resolved in one of the following solvent systems: pyridine: 1-butanol:water (6:4:3), 1-propanol:formic acid: water (6:3:1); or 1-butanol:1-propanol:water (10:7:5). Compounds were detected with alkaline silver nitrate reagent (36) or aniline-xylose

reagent (36). Dinitrophenylhydrazones of alpha-keto acids, produced by the method of Cavallini et al. (5), were spotted on Silica G gel plates and resolved in 1-butanol saturated with 3% NH_4OH. Spots were accentuated by spraying with 0.5N NaOH.

TCA Cycle Enzymes

Isocitrate and malate dehydrogenases were assayed by the method of Reeves et al. (33). One unit of activity was the amount of enzyme which reduced 1 µmole of NAD(P) per minute at 25°C. Succinate dehydrogenase was assayed by a modification of the method of Veeger et al. (39). The 1 ml reaction mixture contained 50 µmoles potassium phosphate buffer, pH 7.6, 40 µmoles sodium succinate, 1 µmole NaCN, 1 mg phenazine methosulfate, and 0.25 µmoles 2,4 dichlorophenolindolphenol (DCPIP). One unit of activity was the amount of enzyme which reduced 1 µmole of DCPIP per min. at 25°C. The dye had an extinction coefficient at 600 nm of 19,100 (35). Alpha-ketoglutarate activity was assayed by following the reduction of ferricyanide. The assay was the same as that for pyruvate decarboxylase (32). One unit of activity was the amount of enzyme necessary to reduce 2 µmoles of ferricyanide per hour.

Cytochrome Determinations

Crude extracts were separated into membrane and soluble fractions by the method of Jones and Redfearn (19). Reduced minus oxidized spectra were determined on a Beckman model 25 recording spectrophotometer at room temperature. Samples were reduced by the addition of a few grains of sodium dithionite to a 1 ml quartz cuvette containing the fraction to be assayed. Samples were oxidized by rapidly shaking the cuvette

before insertion into the spectrophotometer. Cytochrome b was quantitated using a difference spectrum of 560-538 nm and an estimated extinction coefficient of 17,300. Cytochrome c was quantitated using a difference spectrum of 549-575 and an estimated extinction coefficient of 17,500. Flavoprotein was quantitated using a difference spectrum of 465-510 and an extinction coefficient of 11,000. All values for difference spectra and extinction coefficients are those of Jones and Redfearn (19).

Potassium arabonate was prepared by the hypoiodite oxidation of L-arabinose (26). DL-2-keto-3-deoxyarabonate was chemically synthesized by the method of Stoolmiller and Abeles (37) and purified by the method of Weimberg (40). L-Arabonolactone was produced by boiling potassium arabonate in 0.2N HCl for 5 min.

Results

Growth and Nitrogen Fixation

Azospirillum brasilense showed a doubling time of 16-20 h when grown on either malate (Fig. 1) or L-arabinose (Fig. 2) at 0.5% O_2. Malate showed a 40% higher specific activity of acetylene reduction than did L-arabinose grown cells (average of 3 experiments). The pH of the medium dropped only slightly after 72 h growth on L-arabinose (7.1-7.05) while pH rose sharply in malate grown cells (7.1 to 8.6).

Arabinose Pathway Enzymes

Table 1 gives the specific activities of L-arabinose dehydrogenase, L-arabonolactonase, L-arabonate dehydratase, and the DL-KDA reduction

Figure 1. Growth and acetylene reduction on L-malate by *Azospirillum brasilense*. Each acetylene reduction point average of 3 samples. Standard deviation given by width of bar through point. ■ Optical density, O acetylene reduction

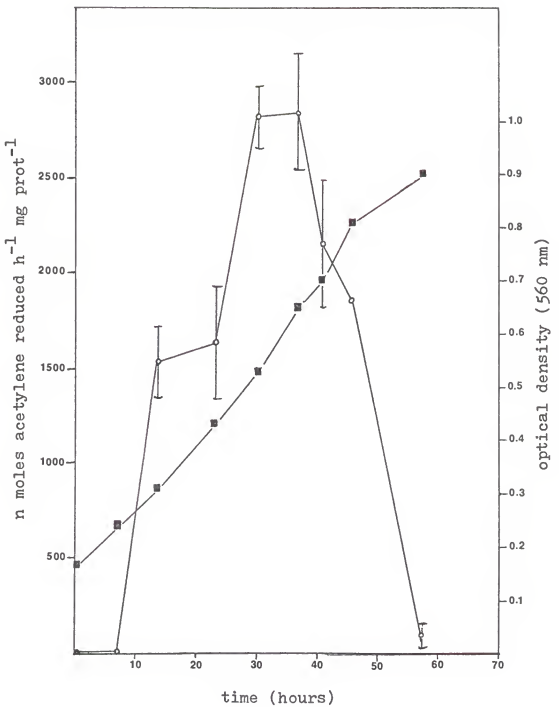

n moles acetylene reduced h-1 mg prot-1
optical density (560 nm)
time (hours)
3000
2500
2000
1500
1000
500
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70

Figure 2. Growth and acetylene reduction on L-arabinose by *Azospirillum brasilense*. Each acetylene reduction point average of 3 samples. Standard deviation given by width of bar through point.
■ Optical density, O acetylene reduction

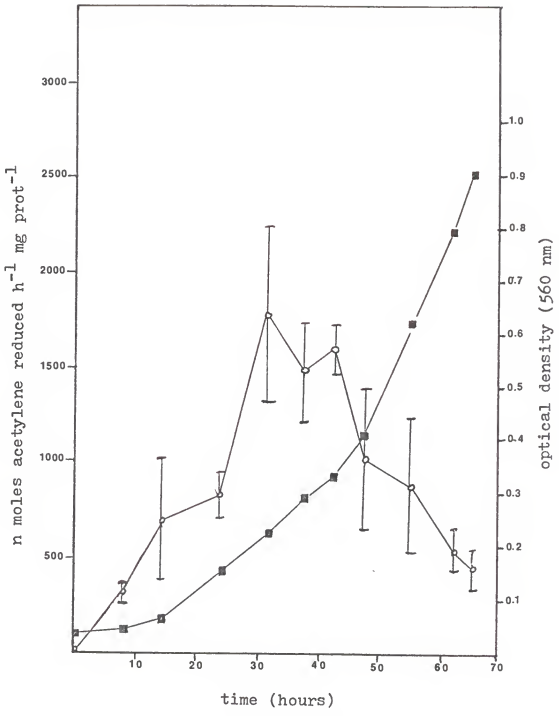

n moles acetylene reduced h-1 mg prot-1
optical density (560 nm)
time (hours)
3000
2500
2000
1500
1000
500
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70

Table 1-1. L-arabinose pathway enzymes. Average of 3 crude extract preparations.

Enzyme	Cofactor	Activity (nmol/min/ mg protein)
L-arabinose dehydrogenase	NAD	70
	NADP	30
Arabonolactonase		13
L-Arabonate dehydratase		17
DL-KDA oxidation	NAD	62
L-KDA aldolase		0

of NAD in crude extracts of L-arabinose grown cells. Figure 3 shows the reduction of NAD in a crude extract to which L-arabonate or DL-KDA was added as substrate. DL-KDA exhibited 3 times the rate of NAD reduction than did potassium L-arabonate. No KDA aldolase activity could be found in any crude extract of L-arabinose grown cells.

End Product Accumulation

Table 2 indicates that crude extracts of L-arabinose grown cells accumulate alpha-ketoglutaric acid when L-arabonate is added as substrate. The largest accumulation is seen if sodium arsenite, an inhibitor of alpha-ketoglutarate and pyruvate dehydrogenase activity (21, 41), is added to the reaction mixture. No accumulation of either pyruvate or glycolaldehyde has been found in crude extracts to which L-arabonate had been added as substrate. This lent further credence to the likely absence of L-KDA aldolase in these cells.

Identification of Reaction Products

At pH 6.5 a major product of the L-arabinose dehydrogenase reaction is arabono-γ-lactone as identified on paper chromatograms run against authentic lactone. The sample comigrates with standard in all 3 solvent systems given in *Methods* and *Materials*. Even at this low pH, however, L-arabonic acid appears as a significant part of the product. At pH 9.0 the sample comigrates with L-arabonic acid in all three solvent systems. The product of the L-arabonate dehydratase reaction comigrates with DL-KDA in all three solvent systems. In addition the 2,4 dinitrophenylhydrazone of the reaction product comigrated with the derivatized standard DL-KDA on Silica G gel plates in 1-butanol

Figure 3. Reduction of NAD in crude extracts of L-arabinose grown cells with 1 μmole potassium L-arabonate o; or DL-KDA ■; as substrate.

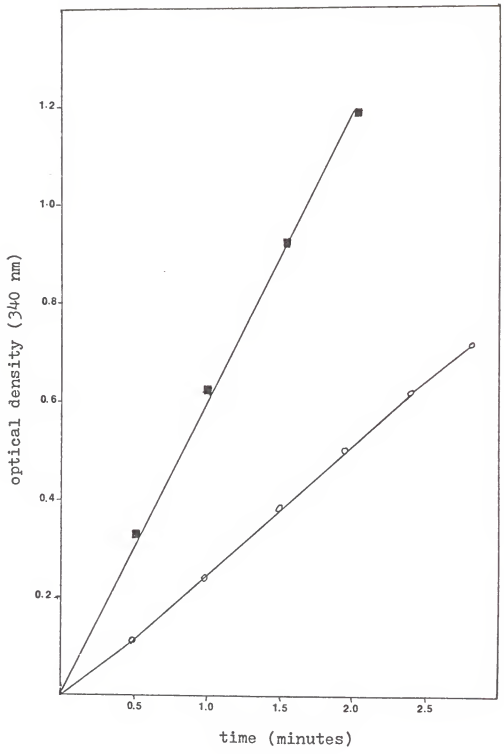

optical density (340 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.5
1.0
1.5
2.0
2.5
time (minutes)

Table 1-2. Accumulation of alpha-ketoglutarate in crude extracts of L-arabinose grown cells with L-arabonate as substrate.

Rxn. Mix Addition	Alphaketoglutarate Accumulation (µmoles)
Sodium arsenite	7.6
Sodium cyanide	4.2
none	4.4
control*	0.49

*No arabonate was added to arabonate oxidation mix. See Materials and Methods.

saturated with 3% NH_4OH. On spraying with 0.5N NaOH the spot gave the reddish brown color reported by Weimberg (40). In addition to enzymatic assay alpha-ketoglutaric acid was identified chromatographically in crude extracts to which potassium L-arabonate had been added as substrate in the presence of NAD. The reaction product comigrated with alpha-ketoglutarate (Sigma) in all three solvents. The 2,4 dinitrophenyl-hydrazone likewise comigrated with the derivitized alpha-ketoglutarate in 1-butanol saturated with 3% NH_4OH.

TCA Cycle Dehydrogenases and Cytochrome Content

Table 3 shows the specific activity of isocitrate, malate, alpha-ketoglutarate, and succinate dehydrogenases in crude extracts of L-arabinose grown cells. Clearly cells grown with L-arabinose as substrate possess an active TCA cycle for the oxidation of alpha-ketoglutarate.

Membrane and soluble fractions clearly contain b and c type cytochrome and a probable cytochrome oxidase (Fig. 4-6). Cytochrome b shows an alpha band at 556 nm which appears as a shoulder on the c cytochrome alpha band at 549. The c cytochrome shows a beta band at 520 nm. Soret bands at 429 (cytochrome b) and 416 (cytochrome c) are also seen. The cytochrome oxidase shows a broad peak around 600 nm. The 105,000 xg supernatant contains only c type cytochrome (Table 4), but c cytochrome is also found in the 35,000 xg and 105,000 xg pellets. All the b cytochrome is found in the 35,000 xg and 105,000 xg pellets. The 105,000 xg supernate also exhibited a deep trough at 451 nm (Fig. 4) which is probably a flavoprotein. This trough only appears in the soluble fraction. A broad peak seen at 600 nm in the soluble fraction and in the 105,000 xg pellet probably represents a cytochrome oxidase.

Table I-3. TCA cycle dehydrogenases.

Enzyme	Electron Acceptor	Activity (nmoles/min/mg protein)
Malate dehydrogenase	NAD	2,080
Isocitrate dehydrogenase	NADP	230
Succinate dehydrogenase	PMS DCPIP	150
Alpha-ketoglutarate dehydrogenase	Ferricyanide	540*

* nmoles/hr/mg protein

Figure 4. Dithionite reduced minus oxidized spectra of membrane and soluble fractions from L-arabinose grown cells. 105,000 xg supernatant.

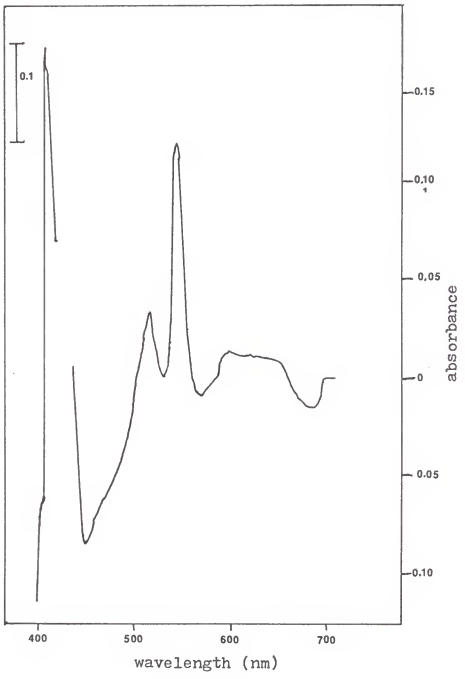

0.1
0.15
0.10
0.05
0
0.05
0.10
absorbance
400
500
600
700
wavelength (nm)

Figure 5. Dithionite reduced minus oxidized spectra of membrane and soluble fractions from L-arabinose grown cells. 35,000 xg pellet.

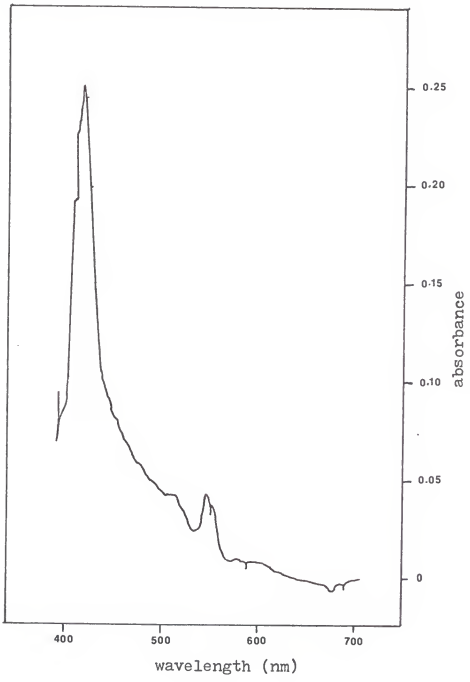

0.25
0.20
0.15
0.10
0.05
0
absorbance
400
500
600
700
wavelength (nm)

Figure 6. Dithionite reduced minus oxidized spectra of membrane and soluble fractions from L-arabinose grown cells. 105,000 xg pellet.

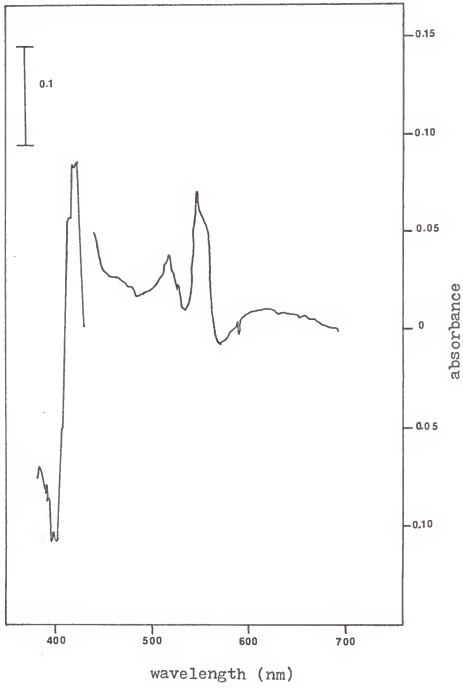

0.1
0.15
0.10
0.05
0
0.05
0.10
absorbance
400
500
600
700
wavelength (nm)

Table 1-4. Cytochrome content of membrane and soluble fractions. μmoles/g protein.

	Cytochrome c	Cytochrome b	Flavoprotein
105,000 xg supernatant	0.64	0	0.79
105,000 xg pellet	0.56	0.83	0
35,000 xg supernatant	0.20	0.31	0

Discussion

Azospirillum brasilense grew at approximately the same rate on both malate and L-arabinose. Malate grown cells have shown a consistently higher specific activity of acetylene reduction than have L-arabinose grown cells, although both rates are high and compare closely with the rate reported by Nelson and Knowles (27) for malate grown cells at 0.5% O_2. The doubling time of 16-20 h is much slower than the 5.5 to 7 h reported by Okon et al. (30) at 0.5% O_2 (with malate as substrate), but approximately the same as the 20 h generation time reported for stagnantly grown cultures (28). It should be noted in my study, that cells grown in malate or L-arabinose were cultured under identical conditions. The sharp rise in pH of malate grown cells may have accounted for a similar growth rate compared to that of L-arabinose grown cells despite the higher acetylene reduction rates shown by malate grown cells.

Initial attempts in our laboratory to demonstrate an L-arabinose catabolic pathway involving phosphorylated intermediates were unsuccessful. No L-arabinose isomerase or phosphotransferase activity could be found. I have since found transketolase and transaldolase activity, in crude and soluble fractions of L-arabinose grown cells, with about one-tenth the specific activity of L-arabinose dehydrogenase. The means by which L-arabinose might enter an oxidative pentose cycle is still unknown. Some ability to metabolize phosphorylated compounds has been indicated in O_2 uptake studies (29).

In examining the possibility of a pathway without phosphorylated intermediates I found high NAD(P) dependent L-arabinose dehydrogenase

activity. In addition L-arabono-γ-lactonase, L-arabonate dehydratase and NAD dependent 2-keto-3-deoxyarabonate oxidation activity were found. Alpha-ketoglutarate semialdehyde has been shown to be the substrate of the NAD dependent dehydrogenation reaction (37). I did not look for this intermediate nor did I assay for the L-KDA dehydratase activity, which produces the alpha-ketoglutarate semialdehyde. The rapid reduction of NAD with DL-KDA as substrate, which has also been reported by Weimberg (40) in *Pseudomonas saccharophila*, would seem to be evidence of significant L-KDA dehydratase activity. Weimberg and Doudoroff (42) reported a more rapid reduction of NAD if crude extracts of *Ps. saccharophila* were first preincubated with L-arabonate before the addition of NAD. This preincubation probably allowed time for the accumulation of L-KDA and perhaps alpha-ketoglutarate semialdehyde in the reaction mixture.

An average of 75% of the potassium arabonate added to the crude extract, in the presence of NAD, accumulated as alpha-ketoglutaric acid if sodium arsenite was added to the crude extract. Sodium arsenite is an inhibitor of alpha-ketoglutarate (41) and pyruvate dehydrogenases (21), probably by inhibiting the decarboxylation step. Significant amounts of alpha-ketoglutarate still were found in the absence of sodium arsenite. Sodium cyanide had no effect on alpha-ketoglutarate accumulation.

The presence of L-arabinose dehydrogenase, L-arabono-γ-lactonase, and L-arabonate dehydratase activity, along with the rapid reduction of NAD in crude extracts with DL-KDA as substrate, and the accumulation of alpha-ketoglutaric acid in crude extracts, is clear indication of the following pathway of L-arabinose metabolism in *A. brasilense*.

$$\text{L-arabinose} \xrightarrow{\text{NAD(P)}} \text{L-arabino-}\gamma\text{-lactone} \xrightarrow{+H_2O} \text{L-arabonic acid} \xrightarrow{-H_2O} \text{L-2-keto-3-deoxyarabonic acid} \xrightarrow{-H_2O} \text{alpha-keto-glutaric semialdehyde} \xrightarrow{\text{NAD}} \text{alpha-ketoglutaric acid}$$

Oxygen uptake studies (29) have indicated a very active TCA cycle in *A. brasilense*, although the specific enzymes have not been previously assayed. My assays of TCA cycle dehydrogenases would seem to confirm high TCA cycle activity and these activities were comparable to that shown by *Rhizobium meliloti* (16). The significance of the very high activity of malate dehydrogenase relative to the other dehydrogenases is unknown.

The presence of b and c type cytochromes and a cytochrome oxidase in deoxycholate extracts of *Azospirillum brasilense* has previously been shown (4). For the first time the distribution of the cytochromes between particulate and soluble fractions has been demonstrated. The distribution is similar to that found in *A. vinelandii* (19). There also appears to be a large flavoprotein factor. The possible pathway of electron transport in *A. brasilense* may be the following. The X represents a possible ubiquinone type electron carrier.

$$\text{NADH} \longrightarrow \text{Flavoprotein} \longrightarrow \text{X} \longrightarrow \underset{\text{b}}{\text{cyt}} \longrightarrow \underset{\text{c}}{\text{cyt}} \longrightarrow \underset{\text{oxidase}}{\text{cyt}} \longrightarrow O_2.$$

It has been suggested by Eskew et al. (17) that the pink pigment found in some strains of *Azospirillum* is due to a c type cytochrome. The reduced minus oxidized spectrum published by Eskew et al. (17) of the concentrated pigment is virtually identical to the spectrum I found in the soluble fraction (105,000 xg supernatant) of the L-arabinose

grown cells. This, along with the significant amount of c cytochrome I found in the soluble fraction, is further evidence that the pink pigment produced by this strain of *Azospirillum* is in fact a c cytochrome.

We have demonstrated a totally oxidative pathway by which L-arabinose is metabolized in *Azospirillum brasilense*. This ability may offer some distinct advantages to this soil bacterium. Plant cell walls are rich in compounds with the L-arabono-configuration (i.e. L-arabinose, D-Fucose, D-galactose)(1). L-Arabinose is one of the few isomers found in plant cell walls. The cellulose fibers in the walls are cemented together by xyloglucans, arabinogalactans, and rhamnogalacturonan (1). It is possible that root cell death and the sloughing off of the cells provides root associated bacteria, like *Azospirillum* which is able to metabolize these sugars, a plentiful carbon substrate. The advantage provided to these bacteria in the highly competitive soil environment is obvious.

Another benefit of this pathway is that there is a ready pool of alpha-ketoglutarate which can act as substrate for glutamate dehydrogenase and glutamate synthase activity (29). Both enzymes are important in nitrogen assimilation under fixed nitrogen and dinitrogen fixing conditions, respectively. Finally there is the increasing similarities demonstrated between *Azospirillum brasilense* and some *Rhizobium* species. Included in these is the oxygen sensitivity of their nitrogenase (20, 30), high dissimilatory nitrate reductase activity (34, 38), accumulation of PHB granules in cells (3, 29), the enhancement of nitrogenase activity by inclusion of a pentose with the organic acid substrate (6), and similar GC content (3, 38). The L-arabinose pathway described in this paper is one that had previously been found only in some species of *Pseudomonas* (7, 41, 42) and fast growing rhizobia (15, 16). The presence of this pathway is another indication of a close relationship between *Azospirillum* and *Rhizobium* species.

SECTION II
PURIFICATION AND CHARACTERIZATION OF L-ARABINOSE DEHYDROGENASE AND L-ARABONATE DEHYDRATASE FROM L-ARABINOSE GROWN CELLS OF *AZOSPIRILLUM BRASILENSE*

Introduction

Azospirillum brasilense (ATCC 29145) can metabolize L-arabinose by the following series of reactions

$$\text{L-arabinose} \xrightarrow{\text{NAD(P)}} \text{L-arabono-}\gamma\text{-lactone} \xrightarrow{+H_2O} \text{L-arabonic acid} \xrightarrow{-H_2O} \text{L-2-keto-3 deoxyarabonic acid} \xrightarrow{-H_2O} \text{alpha-keto-glutaric semialdehyde} \xrightarrow{\text{NAD}} \text{alpha-ketoglutaric acid}$$

This pathway has also been demonstrated in *Pseudomonas saccharophila* (42), *Pseudomonas fragi* (7, 41), and the fast growing rhizobia (15, 16) *R. meliloti, R. trifolii, R. phaseoli,* and *R. leguminosarum.* A second pathway which carries through the first 3 steps of the alpha-keto-glutarate pathway but cleaves L-2-keto-3-deoxyarabonate (L-KDA) by an aldolase reaction to glycolaldehyde and pyruvate has been found in *Pseudomonas* strain MSU-1 (8) and the slow growing rhizobia: *R. japonicum* (15, 31) and *Rhizobium* sp. 32H1 (15).

The enzymes of these pathways have not been extensively studied. L-arabinose dehydrogenase and the L-arabonate oxidation system (L-arabonate ——→ alpha-ketoglutaric acid) have been partially purified in *Pseudomonas saccharophila* (42). L-2-keto-3-deoxyarabonate dehydratase has been purified and characterized by Stoolmiller and Abeles (37). Dahms and Anderson have partially purified L-KDA aldolase from *Ps.* MSU-1 (8).

D-Fucose metabolism in *Ps.* MSU-1 is very similar to that found for L-arabinose. In the D-fucose pathway, D-2-keto-3-deoxyfuconate is cleaved to pyruvate and lactaldehyde. All the enzymes in this pathway have been purified and characterized by Dahms and Anderson (9-12).

I have partially purified and characterized L-arabinose dehydrogenase and L-arabonate dehydratase from L-arabinose grown cells of *Azospirillum brasilense*.

Materials and Methods

Bacterial Strain

Azospirillum brasilense (ATCC 29145) was obtained from the American Type Culture Collection. For daily use cultures were maintained on Tryticase soy agar (BBL) slants with biweekly transfer. Stock cultures were frozen in liquid nitrogen.

Enzyme Purification

Crude extracts were prepared as stated in Section I. All steps in the purification of enzymes were carried out at 4°C unless otherwise stated.

Nucleic acid precipitation

A 2% solution of protamine sulfate (Sigma, Grade II) in 0.1M sodium-potassium phosphate buffer, pH 7.5 was slowly added to the crude extract to give a final concentration of 0.33%, stirred in the cold for 30 min., and centrifuged at 20,000 xg for 1 hr. The pellet was discarded.

Ammonium sulfate fractionation

Solid ammonium sulfate (Sigma, Grade III) was slowly added to the protamine sulfate supernatant while maintaining the pH at 7.5 with 0.1N NaOH. The sample was stirred in the cold for 1 hr then centrifuged at 20,000 xg for 1 hr. The pellets were resuspended in 0.1M sodium-potassium phosphate buffer pH 7.5. Ninety-two percent of the dehydratase activity was found in the 30% and 40% ammonium sulfate precipitate fraction. These fractions were pooled before being placed on Biogel A column. Eighty-nine percent of the dehydrogenase was found in the 50% ammonium sulfate precipitate fraction. This fraction was carried through a heat treatment step before being loaded onto the Biogel column.

Heat treatment (dehydrogenase only)

The resuspended 50% ammonium sulfate fraction was heated to 55°C for 5 min, immediately cooled in ice to 4°C, and centrifuged at 20,000 xg for 1 hr.

Gel filtration

Sample was added to a 2 x 150 cm Biogel A 1.5M column equilibrated with 25 mM sodium-potassium phosphate buffer pH 7.4. Flow rate was 25 ml per hr and 5 to 20 ml fractions were collected.

Ion exchange chromatography

A 2 x 10 cm DEAE cellulose (fine) column was equilibrated with 3 column volumes of 25 mM sodium-potassium phosphate buffer, pH 7.4. Samples were either placed directly on the column from active, pooled Biogel A fractions or Biogel fractions were concentrated first in an

Amicon pressure cell with UM30 filter. Enzyme was eluted in a 0 to 0.2M (dehydrogenase) or 0 to 0.3M (dehydratase) 300 ml linear NaCl gradient. All NaCl solutions were prepared in 25 mM sodium-potassium phosphate buffer, pH 7.4. Active DEAE fractions were immediately desalted on a G-25 column, equilibrated with 25 mM sodium-potassium phosphate buffer, pH 7.4. Flow rate was 50 ml per hr and 20 ml fractions were collected. Active fractions were concentrated by lyophilization.

Protein Determination

Protein was determined by the method of Lowry et al. (24) with BSA as a standard.

Enzyme Assays

L-arabinose dehydrogenase was assayed in 125 µmoles glycine/NaOH buffer, pH 9.0, 0.5 µmoles NAD, 0.5 µmoles L-arabinose, and enzyme plus water to 1 ml. In crude extracts 1 µmole NaCN was added to the reaction mix. The change in optical density at 340 nm was followed on a Zeiss spectrophotometer at 25°C. One unit of L-arabinose dehydrogenase activity was the amount of enzyme which reduced 1 µmole NAD(P) per min. Arabonate dehydratase activity was assayed by the method of Pedrosa and Zancan (31) with 2-keto-3-deoxyarabonate accumulation being measured with semi-carbazide reagent (25). One unit of activity was the amount of enzyme which produces 1 µmole of L-KDA per min.

Molecular Weight Determination

The 2 x 150 cm Biogel A column was equilibrated with 25mM sodium-potassium phosphate buffer, pH 7.4, and 2 mg of each standard (Boehringer Calibration Proteins II), including

catalase (240,000 daltons), aldolase (158,000), albumin (68,000), and albumin (45,000), were dissolved in 20 ml of buffer and placed on the column. The column was run at 25 ml per hour and 7.5 ml fractions were collected. Standard peaks were located by A_{280}. L-Arabinose dehydrogenase and L-arabonate dehydratase were located by enzyme activity. A plot of the molecular weight of the standards versus the logarithm of the volume of the half-height of the leading edge of each compound was made. The standards generated a straight line from which unknown molecular weights were determined.

Product Identification

Products of L-arabinose dehydrogenase and L-arabonate dehydratase were identified as previously stated in Section I.

Polyacrylamide Gel Electrophoresis

Slab gels were prepared by the method of Laemmli (22). A 5-15% exponential gel was cooled to 4°C and run at 32 ma. Enzyme activity was located by slicing the gel in 8 mm sections, placing the sections in 13 x 100 mm tubes, and eluting the enzyme in 1 ml of 25 mM potassium phosphate buffer, pH 7.2. Enzyme activity was assayed in the standard manner.

Results

The purification of L-arabinose dehydrogenase and L-arabonate dehydratase are outlined in Tables 1 and 2, respectively. The dehydrogenase was purified 59 fold with 1.2% yield and L-arabonate dehydratase was purified 38 fold with 9% yield. Attempts were made to improve

Table 2-1. Purification of L-arabinose dehydrogenase.

Fraction	Protein (mg)	Sp. Act. Units/mg Protein	Total Activity Units	Purifi-cation	Recovery %
Crude	1484	0.07	103.8		
Protamine sulfate supernatant	1280	0.07	89.6	0	86
Ammonium sulfate (50% prec.)	733	0.08	58.6	1.1	56
Heat treatment	288	0.20	57.6	2.9	55
Biogel A 1.5M (fractions pooled)	20.6	1.43	29.4	20.4	28
DEAE G-25 washed conc. 10X	0.3	4.12	1.2	58.9	1.2

Table 2-2. Purification of L-arabonate dehydratase.

Fraction	Protein (mg)	Sp. Act. Units/mg Protein	Total Activity Units	Purification	Recovery %
Crude	2497	0.011	27.5		
Protamine sulfate supernatant	1783	0.015	26.7	1.4	97
Ammonium sulfate prec. (30 & 40% fractions)	635	0.007	4.4		16
Biogel A 1.5M (pooled fractions)	19	0.19	3.6	17.2	13
DEAE G-25 washed	6	0.42	2.5	38.2	9

the purification and yield of L-arabinose dehydrogenase. Calcium phosphate gels, affinity chromatography (Affi-gel Blue), and DEAE Sephadex ion exchange columns failed to improve either of the above factors. The elution profiles of L-arabinose dehydrogenase and L-arabonate dehydratase activities from a Biogel A 1.5M column are shown in Fig. 7. It is clear that only partial resolution of these two enzymes was achieved by gel filtration. Therefore it was necessary to further chromatograph the Biogel fractions on DEAE cellulose eluted with a linear NaCl gradient. Figure 8 demonstrates preliminary results obtained from crude 30% and 40% ammonium sulfate fractions. Excellent resolution of the two enzymes was achieved.

Dehydrogenase activity started to elute from the DEAE column at 0.08M NaCl while the dehydratase did not start to elute from the column until 0.19M NaCl was applied. The dehydrogenase came off in unstable condition and it was necessary to desalt the eluate immediately on a G-25 column. Thus ion exchange chromatography allowed excellent separation of enzymes from each other.

Characteristics of L-Arabinose Dehydrogenase

L-Arabinose dehydrogenase showed no enhancement of activity in the presence of any of the divalent cations listed in Table 3. The presence of $CaCl_2$, $FeSO_4$, $MnCl_2$ and $CoCl_2$ severely inhibited enzyme activity. The presence of EDTA has a slight negative effect on activity and the presence of the reducing agents 2-mercaptoethanol, glutathione (reduced), and dithiothreitol also had a small negative effect on activity.

Only those substrates with the L-arabono configuration (i.e. D-galactose, D-fucose) were good substrates for the L-arabinose

Figure 7. Elution from a Biogel A 1.5M (2 x 150 cm) column of L-arabinose dehydrogenase and L-arabonate dehydratase. The resuspended 30% and 40% ammonium sulfate precipitate fractions were pooled and placed directly on the column. Assays are as stated in Materials and Methods. Fractions (20 ml) were collected and protein determined by A_{280}.

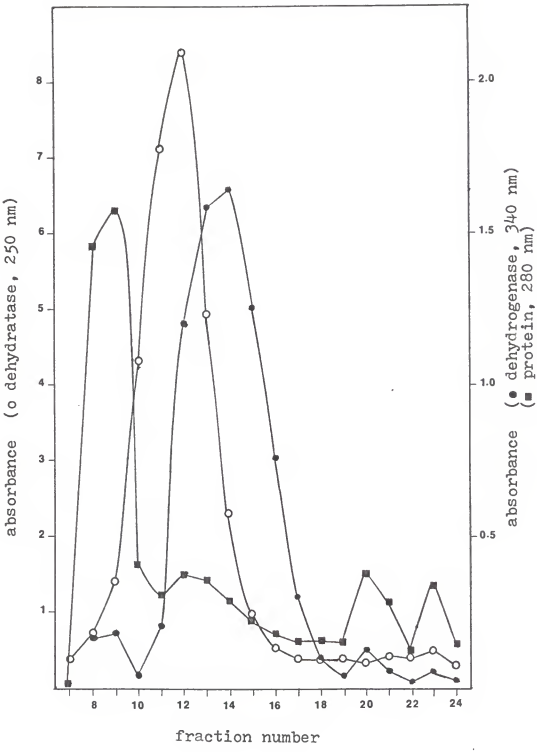

absorbance (o dehydratase, 250 nm)
absorbance (● dehydrogenase, 340 nm)
(■ protein, 280 nm)
fraction number
1
2
3
4
5
6
7
8
0.5
1.0
1.5
2.0
8
10
12
14
16
18
20
22
24

Figure 8. Elution from a DEAE cellulose column (2 x 10 cm) of L-arabinose dehydrogenase and L-arabonate dehydratase. The resuspended 30% and 40% ammonium sulfate precipitate fractions were pooled and placed on the column. Enzyme was eluted from the column with a 300 ml, 0 to 0.3M NaCl linear gradient. Fractions (5 ml) were collected and protein determined by A_{280}. Enzyme assays were as in Materials and Methods.

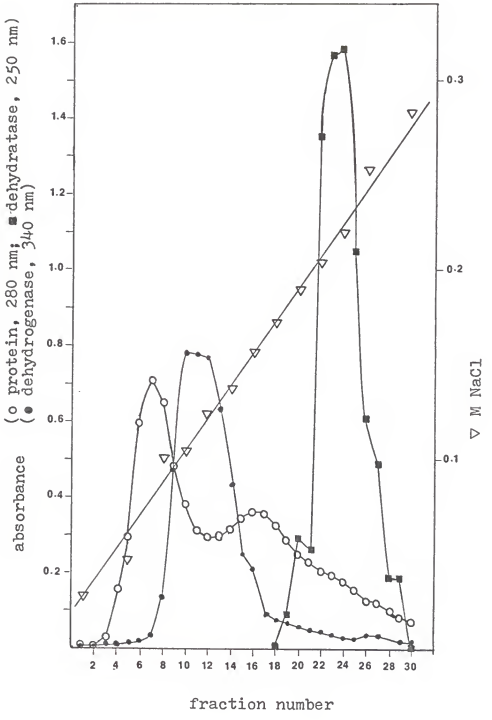

absorbance
(o protein, 280 nm; ■ dehydratase, 250 nm)
(● dehydrogenase, 340 nm)
▽ M NaCl
fraction number
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.3
0.2
0.1
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Table 2-3. Effect of divalent cations and reducing agents on L-arabinose dehydrogenase activity.

Reagent	Concentration	Relative Activity %
None		100
$MgSO_4$	10 mM	100
NH_4SO_4	10 mM	100
$CaCl_2$	10 mM	70
$FeSO_4$	10 mM	0
$MnCl_2$	10 mM	55
$CoCl_2$	10 mM	46
EDTA	2.5 mM	92
2-mercapto-ethanol	1 mM	95
Glutathione (reduced)	1 mM	93
Dithiothreitol	1 mM	90

dehydrogenase (Table 4). Lineweaver-Burk plots (Fig. 9) show a K_m value of 140 µM with 0.5 µmole NAD as cofactor and 72 µM with 0.5 µmole NADP as cofactor. The heat stable dehydrogenase (no loss of activity when heated to 55°C for 5 min) had a pH optimum of 9.5 in glycine/NaOH buffer (Fig. 10)

Characteristics of L-arabonate Dehydratase

L-Arabonate showed a pH optimum of 7.8-8.0 in Hepes/NaOH buffer (Fig. 11). Magnesium sulfate and manganese chloride (Table 5) enhanced enzyme activity 35% and 56%, respectively. The presence of $ZnCl_2$, $CaCl_2$, $FeSO_4$, and $CoCl_2$ severely inhibited activity. The addition of EDTA (2.5 mM) resulted in a 95% inhibition of activity. Reducing agents 2-mercaptoethanol and glutathione (reduced) had a small negative effect on activity while dithiothreitol greatly reduced activity.

Molecular Weight Determination

L-Arabinose dehydrogenase had a molecular weight of 175,000 and L-arabonate dehydratase had a molecular weight of 225,000 according to gel filtration determinations (Fig. 12).

Enzyme Product Identification

At pH 6.6 the product of the L-arabinose dehydrogenase reaction was found to be L-arabono-γ-lactone, although, L-arabonic acid was also found in the reaction mixture. At pH 9.0 L-arabonic acid was the only product found. The product of the dehydratase reaction was identified as L-2-keto-3-deoxyarabonate. See Section I for a complete description of enzyme product identification.

Table 2-4. Substrate specificity of L-arabinose dehydrogenase. The standard assay was used except substrate was varied.

Substrate 10 mM	Relative Velocity %
L-arabinose	100
D-ribose	0
D-galactose	63
D-xylose	4
D-fucose	100
L-fucose	0
L-rhamnose	0
D-mannose	0
D-glucose	0

Figure 9. Lineweaver-Burk plot showing the effect of substrate concentration on reaction velocity. NAD o and NADP■ (both 10 mM) were used as cofactors. The standard assay was used except substrate concentration was varied.

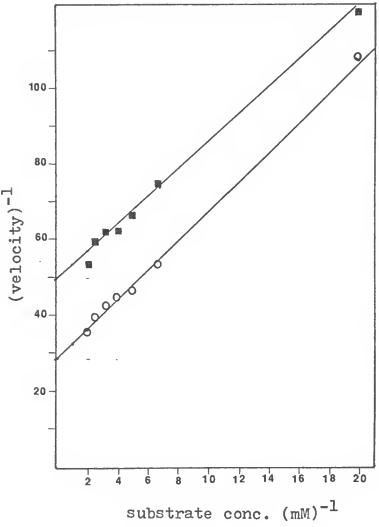

100
80
60
40
20
(velocity)$^{-1}$
2
4
6
8
10
12
14
16
18
20
substrate conc. (mM)$^{-1}$

Figure 10. Effect of pH on reaction velocity of L-arabinose dehydrogenase. Standard assay was used except pH of the glycine/NaOH buffer was varied.

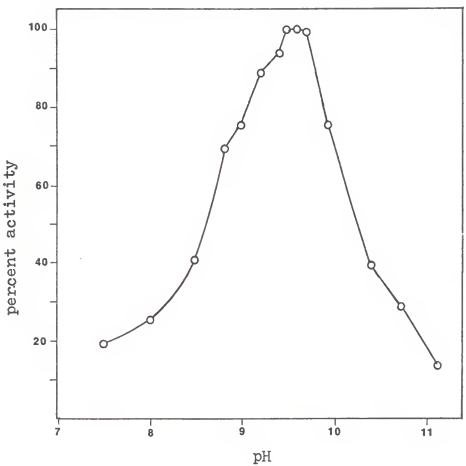

100
80
60
40
20
percent activity
7
8
9
10
11
pH

Figure 11. Effect of pH on reaction velocity of L-arabonate dehydratase. Standard assay was used except pH of the HEPES/NaOH buffer was varied.

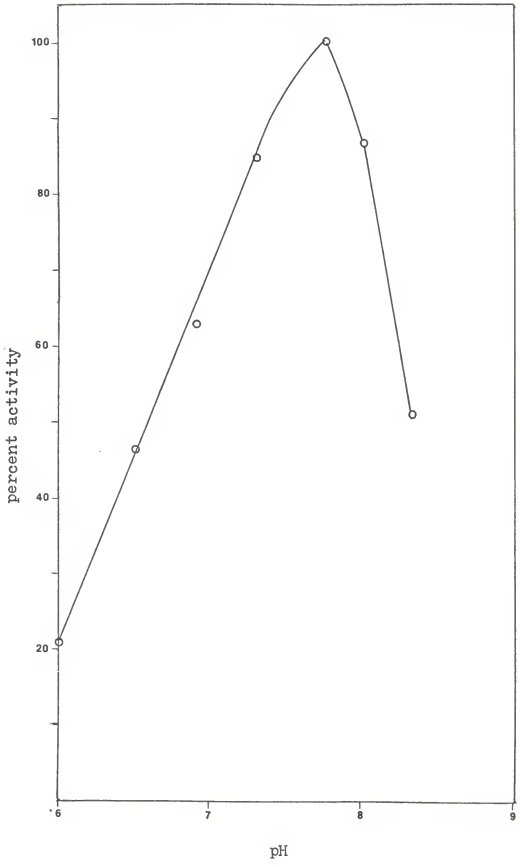
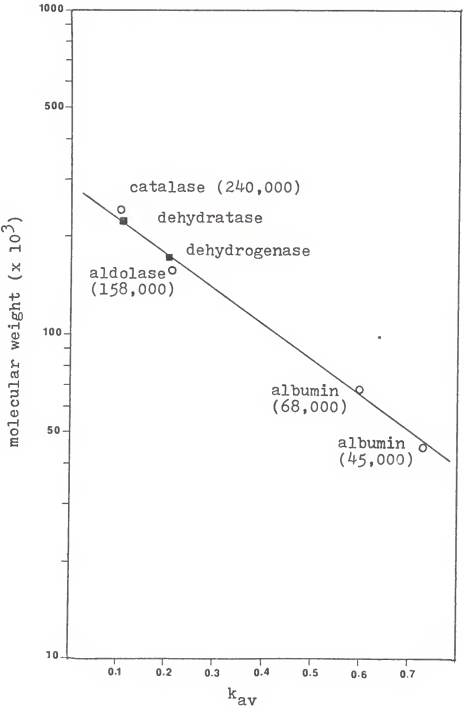

100
80
60
40
20
percent activity
6
7
8
9
pH

Table 2-5. Effect of divalent cations and reducing agents on L-arabonate dehydratase activity.

Reagent	Concentration mM	Relative Activity %
None		100
$ZnCl_2$	1	34
$MgSO_4$	10	135
$(NH_4)_2SO_4$	10	100
$CaCl_2$	10	55
$FeSO_4$	10	55
$MnCl_2$	10	156
$CoCl_2$	10	11
EDTA	2.5	5
2-mercapto-ethanol	1	93
Glutathione (reduced)	1	92
Dithiothreitol	1	41

Figure 12. Molecular weight determination of L-arabinose dehydrogenase and L-arabonate dehydratase by gel filtration technique (see Materials and Methods).


1000
500
100
50
10
molecular weight (x 10^3)
catalase (240,000)
dehydratase
dehydrogenase
aldolase
(158,000)
albumin
(68,000)
albumin
(45,000)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
k_{av}

Polyacrylamide Gel Electrophoresis

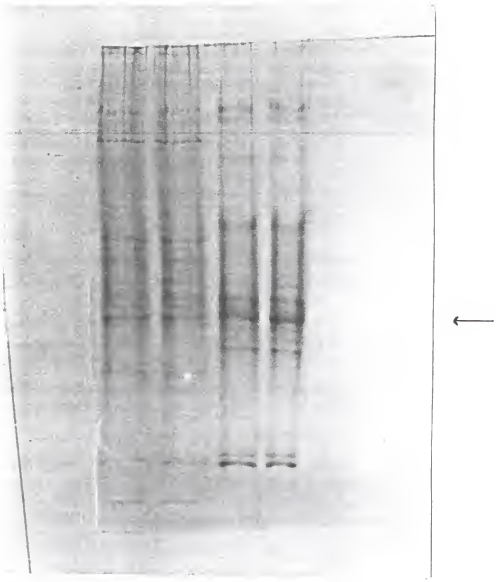
Gels of purified dehydrogenase and dehydratase still showed several bands indicating that the enzymes were not totally pure. Assay of dehydrogenase activity in the gel showed the enzyme activity to be located 60 mm from the top of the gel (Fig. 13). Two dark bands, 41 mm and 53.5 mm from the top of the gel (Fig. 14), appeared in the gels run with partially purified dehydratase. Dehydratase activity could not be located within the gel. It is possible that the dehydratase dissociated into two subunits which could not individually catalyze the dehydration reaction or that the enzyme was inactivated in the gel without disassociation.

Discussion

L-Arabinose dehydrogenase from *Azospirillum brasilense* apparently metabolizes only substrates having the L-arabono configuration. Other aldose dehydrogenases are generally not specific for this configuration (9). According to K_m values the dehydrogenase had a greater affinity for substrate with NADP as cofactor than with NAD, although, V_{max} was twice as great with NAD as cofactor than with NADP. The enzyme had no requirements for any of the divalent cations tested nor was there enhancement of activity in the presence of reducing agent. The enzyme had a high pH optimum of 9.5.

L-Arabinose dehydrogenase from *A. brasilense* shows several similarities to dehydrogenases from other bacteria metabolizing L-Arabinose through similar pathways. L-Arabinose dehydrogenase from *R. japonicum*

Figure 13. Polyacrylamide slab gel of crude and partially purified extracts of L-arabinose dehydrogenase. Lane A and B; protaminesulfate supernatant fraction (50 µg); Lanes C and D; DEAE-G-25 washed fraction (50 µg). Arrow points to area of enzyme activity.



A B C D

Figure 14. Polyacryamide slab gel of partially purified extracts of L-arabonate dehydratase. Lanes A and B contain DEAE-G-25 washed fraction (50 µg).

A B

(31) and D-fucose dehydrogenase from *Ps.* MSU-1 (9) are both capable of reducing NAD and NADP. L-arabinose dehydrogenase from *Ps. saccharophila* is reported only capable of reducing NAD (42). L-arabinose dehydrogenase from *Ps. saccharophila* (42); *R. japonicum* (31), and *Aquaspirillum gracile* (23) are all assayed at high pH, although, optimum pH is not reported. D-Fucose dehydrogenase from *Pseudomonas* MSU-1 is similar to L-arabinose dehydrogenase from *A. brasilense* in its lack of divalent cation requirement, lack of reducing agent requirement, heat stability, and pH optimum as well as substrate specificity (9).

The immediate product of L-arabinose dehydrogenase was L-arabono-γ-lactone, which was cleaved to L-arabonic acid by a lactonase. Even at pH of 6.6, though, there was considerable spontaneous breakdown of the lactone.

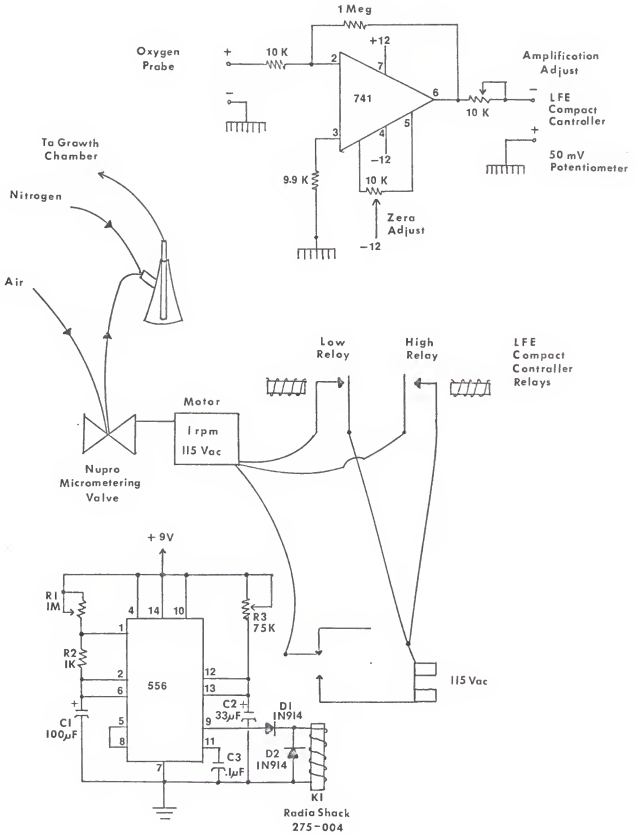
L-arabonate dehydratase showed greatly enhanced activity in the presence of $MgSO_4$ or $MnCl_2$. Its requirement for cations was clearly demonstrated by the near total loss of activity in the presence of the chelating agent EDTA. This was similar to that found for D-fuconate dehydratase (11). Unlike D-fuconate dehydratase this enzyme showed no enhancement of activity in the presence of any of the reducing agents tested. In fact reducing agents had a negative effect on activity. The semicarbazide positive product of the dehydratase reaction has been identified as L-2-keto-3-deoxyarabonate.

APPENDIX
DESIGN OF OXYGEN CONTROLLER

A simple oxygen controller has been built for use in growth and nitrogen fixation studies. The sensing part of the instrument consists of a Borkowski and Johnson (2) oxygen probe. The probe acts as a tiny battery with electrons moving from the pure lead cathode to a pure silver anode covered by a teflon membrane. The amount of current running between cathode and anode is proportional to the rate at which oxygen is reduced at the anode. A resistor placed between the anode and cathode produces a potential drop which drives the sensing system. The whole system is outlined in Figure 1. A 741 operational amplifier allows the probe signal to be amplified up to 100 times. This signal is then fed to a 50 mv potentiometer (as part of the LFE compact controller). The system is calibrated by bubbling pure nitrogen (Matheson) through the sterile medium and setting the mv meter to zero with the 741 zero adjust. Two percent oxygen (Matheson) is then bubbled through the sterile medium and full scale is set with the amplification adjust.

The heart of the controller part of the instrument is an LFE Compact Controller with high and low relays. If the mv indicator drops below the low relay setting, the relay closes; likewise, if it rises above the high relay setting, the high relay closes. The motor (115 Vac, 1 rpm) which drives the micrometering valve has a common, clockwise, and counterclockwise control wire. If the common and clockwise wires are

Figure 15. Schematic of oxygen controller.


1 Meg
+12
Oxygen Probe
10 K
741
Amplification Adjust
10 K
LFE Compact Controller
50 mV Potentiometer
9.9 K
10 K
−12
Zero Adjust
To Growth Chamber
Nitrogen
Air
Low Relay
High Relay
LFE Compact Controller Relays
Motor
1 rpm
115 Vac
Nupro Micrometering Valve
+9V
R1 1M
R2 1K
R3 75K
556
C1 100μF
C2 33μF
C3 .1μF
D1 IN914
D2 IN914
115 Vac
K1
Radio Shack 275-004

connected to 115 Vac the motor turns clockwise, if the common and counterclockwise wires are connected to 115 Vac the motor runs counterclockwise. The common wire runs from the motor through a relay (connected to a 556 timer) to line voltage. The clockwise wire runs through the controller high relay and the counterclockwise wire runs through the compact controller low relay. The high and low relay wires come together to form the other line for 115 Vac connection.

In use the 556 timer is set for 1 second on time and 1 minute delay time (time between on periods). The common line therefore closes for 1 second every minute. The high relay is set at approximately 14.5 and the low relay at 10.5. The mv indicator starts out at 12.5 on the 50 mv scale (0.5% 0_2). Every minute the instrument "checks" to see if either the high relay or low relay is closed. If, for instance, the high relay is closed, the motor will move approximately one-sixtieth of a turn (1 second on a 1 rpm motor) counterclockwise closing the valve a fraction of a turn and decreasing 0_2 concentration in solution. This method manages to keep the 0_2 concentration at 0.5% $\pm$ 0.05%. Typically with *A. brasilense* 0_2 levels being pumped into the growth chamber will rise from 0.5% at the beginning of growth to 5.0% 0_2 by late exponential phase (in order to keep dissolved 0_2 at 0.5%).

LITERATURE CITED

1. Albersheim, P. 1975. The walls of growing plant cells. Sci. Am. 232: 81-94.

2. Borkowski, J. D., and M. J. Johnson. 1967. Long-lived steam-sterilizable membrane probes for dissolved oxygen measurement. Biotech. and Bioeng. 9: 635-639.

3. Buchanan, R. E., and N. E. Gibbons (ed.). 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

4. Burris, R. H., Y. Okon, and S. L. Albrecht. 1978. Properties and reactions of *Spirillum lipoferum*. Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria. Ecol. Bull. (Stockholm) 26: 353-363.

5. Cavallini, D., N. Frontali, and G. Toschi. 1949. Keto acid content of human blood and urine. Nature 164: 792.

6. Child, J. J. and W. G. W. Kurz. 1978. Inducing effect of plant cells on nitrogenase activity of *Spirillum* and *Rhizobium* in vitro. Can. J. Microbiol. 24: 143-148.

7. Dagley, S., and P. W. Trudgill. 1965. The metabolism of galactarate, D-glucarate and various pentoses by species of *Pseudomonas*. Biochem. J. 95: 48-58.

8. Dahms, A. S., and R. L. Anderson. 1969. 2-keto-3-deoxy-L-arabonate aldolase and its role in a new pathway of L-arabinose degradation. Biochem. Biophys. Res. Commun. 36: 809-814.

9. Dahms, A. S., and R. L. Anderson. 1972. D-fucose metabolism in a pseudomonad. II. Oxidation of D-fucose to D-fucono-γ-lacone by an L-arabino-aldose dehydrogenase and the hydrolysis of the lactone by a lactonase. J. Biol. Chem. 247: 2228-2232.

10. Dahms, A. S., and R. L. Anderson. 1972. D-fucose metabolism in a pseudomonad. I. Oxidation of D-fucose to D-fucono-γ-lacone by a D-aldohexose dehydrogenase. J. Biol. Chem. 247: 2222-2227.

11. Dahms, A. S., and R. L. Anderson. 1972. D-fucose metabolism in a pseudomonad. III. Conversion of D-fuconate to 2-keto-3-deoxy-D-fuconate by a dehydratase. J. Biol. Chem. 247: 2233-2237.

12. Dahms, A. S., and R. L. Anderson. 1972. D-fucose metabolism in a pseudomonad. IV. Cleavage of 2-keto-3-deoxy-D-fuconate to pyruvate and lactaldehyde by a 2-keto-3-deoxy-L-arabonate aldolase. J. Biol. Chem. 247: 2238-2241.

13. Day, J. M., and J. Dobereiner. 1976. Physiological aspects of N_2-fixation by a *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. 8: 45-50.

14. Dische, Z., and E. Borenfreund. 1949. A specific color reaction of glycolic aldehyde. J. Biol. Chem. 180: 1297-1300.

15. Duncan, M. J. 1979. L-Arabinose metabolism in rhizobia. J. Gen. Microbiol. 113: 177-179.

16. Duncan, M. J., and D. G. Fraenkel. 1979. Alpha-ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*. J. Bacteriol. 37: 415-419.

17. Eskew, D. L., D. D. Focht, and I. P. Ting. 1977. Nitrogen fixation, denitrification, and pleomorphic growth of a highly pigmented *Spirillum lipoferum*. Appl. Environ. Microbiol. 34: 582-585.

18. Hestrin, S. 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. 180: 249-261.

19. Jones, C. W., and E. R. Redfearn. 1966. Electron transport in *Azotobacter vinelandii*. Biochem. Biophys. Acta. 113: 467-481.

20. Keister, D. L., and W. R. Evans. 1976. Oxygen requirements for acetylene reduction in pure cultures of rhizobia. J. Bacteriol. 129: 149-153.

21. Kersters, K., and J. De Ley. 1971. Tests with cell free extracts. p. 46-51. *In* J. R. Norris and D. W. Ribbons (ed.) Methods in Microbiology. v. 6A. Academic Press, New York.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 222: 680-685.

23. Laughon, B. E., and N. R. Krieg. 1974. Sugar catabolism in *Aquaspirillum gracile*. J. Bacteriol. 119: 691-697.

24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

25. MacGee, J., and M. Doudoroff. 1954. A new phosphorylated intermediate in glucose oxidation. J. Biol. Chem. 210: 617-626.

26. Moore, S., and K. P. Link. 1940. Carbohydrate characterization. I. The oxidation of aldoses by hypoiodite in methanol. J. Biol. Chem. 133: 293-311.

27. Nelson, L. M., and R. Knowles. 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. Can. J. Microbiol. 24: 1395-1403.

28. Okon, Y., S. L. Albrecht, and R. H. Burris. 1976. Factors affecting the growth and nitrogen fixation of *Spirillum lipoferum*. J. Bacteriol. 127: 1248-1254.

29. Okon, Y., S. L. Albrecht, and R. H. Burris. 1976. Carbon and ammonia metabolism in *Spirillum lipoferum*. J. Bacteriol. 128: 592-597.

30. Okon, Y., J. P. Houchlins, S. L. Albrecht, and R. H. Burris. 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen and the properties of nitrogenase in cell free extracts. J. Gen. Microbiol. 98: 87-93.

31. Pedrosa, F. O., and G. T. Zancan. 1974. L-Arabinose metabolism in *Rhizobium japonicum*. J. Bacteriol. 119: 336-338.

32. Reed, L. J. and C. R. Williams. 1966. Purification and resolution of the pyruvate dehydrogenase complex. p. 258-259. *In* W. A. Wood (ed.) Methods in Enzymology. V. 9. Academic Press, New York.

33. Reeves, H. C., R. Rabin, W. S. Wegener, and S. J. Ajl. 1971. Assays of enzymes of the tricarboxylic acid and glyoxylate cycles. p. 425-462. *In* J. R. Norris and D. W. Ribbons (ed.) Methods in Microbiology. V. 6A. Academic Press, New York.

34. Rigaud, J., F. J. Bergersen, G. L. Turner, and R. M. Daniel. 1973. Nitrate dependent anaerobic acetylene reduction and nitrogen fixation by Soybean bacteroids. J. Gen. Microbiol. 77: 137-144.

35. Singer, T. P., and T. Cremona. 1966. D-(-)-Lactate cytochrome c reductase. p. 303-305. *In* W. A. Wood (ed.), Methods in Enzymology V. 9. Academic Press, New York.

36. Smith, I. (Ed.) 1960. Chromatographic and Electrophoretic Techniques. I. Chromatography. Interscience Publishers, Inc., New York.

37. Stoolmiller, A. C., and R. H. Abeles. 1966. Formation of alpha-ketoglutaric semialdehyde from L-2-keto-3-deoxy-arabonic acid and the isolation of L-2-keto-3-deoxy-arabonate dehydratase from *Pseudomonas saccharophila*. J. Biol. Chem. 241: 5764-5771.

38. Tarrand, J. J., and N. R. Krieg. 1978. A taxonomic study of *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. 24: 967-980.

39. Veeger, C., D. V. DerVartanian, and W. P. Zeylemaker. 1969. Succinate dehydrogenase. p. 81-90. *In* J. M. Lowenstein (ed.), Methods in Enzymology. V. 13. Academic Press, New York.

40. Weimberg, R. 1959. L-2-keto-4,5 dihydroxyvaleric acid: An intermediate in the oxidation of L-arabinose by *Pseudomonas saccharophila*. J. Biol. Chem. 234: 5764-5771.

41. Weimberg, R. 1961. Pentose oxidation by *Pseudomonas fragi*. J. Biol. Chem. 236: 629-634.

42. Weimberg, R., and M. Doudoroff. 1955. The oxidation of L-arabinose by *Pseudomonas saccharophila*. J. Biol. Chem. 217: 607-624.

43. Weissbach, A., and J. Hurvitz. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. 234: 705-709.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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