Hydrogenase in Nitrogenase-Deficient Azotobacter Mutants.* (20117)

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Hydrogenase appears to have a unique significance in biological nitrogen fixation because of its occurrence in most organisms capable of fixing molecular nitrogen and its relationship to the nitrogen metabolism of the organism. Lee and Wilson(1) showed that cultures of *Azotobacter* fixing atmospheric N_2 have a greater hydrogenase activity than cultures grown on sources of fixed nitrogen. More recently, Jensen(2) demonstrated that *Azotobacter indicum* grown on ammonium lactate or glutamic acid has no hydrogenase, whereas cultures grown on N_2 possess the enzyme. If hydrogenase is primarily concerned with nitrogen fixation in the azotobacter, strains lacking nitrogenase‡ should also lack hydrogenase or have a markedly decreased level. This report describes the occurrence of hydrogenase in nitrogenase-deficient mutants and reverted strains of the azotobacter.

Methods. The organisms used were *Azotobacter vinelandii* strain O from the culture collection of the University of Wisconsin, *Azotobacter agile* 4.4 obtained from Dr. H. A. Barker, and nitrogenase-deficient strains of these species isolated by penicillin screening (3,4). Isolation of such mutants is achieved by exposing cultures of the wild type growing in a nitrogen-free medium to 500 units of penicillin per ml. The antibiotic has a bactericidal effect on cells which are growing in this medium, *i.e.*, assimilating molecular N_2, but not on spontaneous mutants which are unable to utilize this source of nitrogen. Cultures were grown in Burk's-sucrose medium(5) with or without 300 ppm $(NH_4)_2HPO_4$-nitrogen in 500 ml Erlenmeyer flasks on a rotary shaker (360 rpm) at 30°C. Growth rates (k values) were determined turbidimetrically. Cultures were harvested, washed with M/15 phosphate buffer, pH 8.0, and suspended in 0.2% KCl. Hydrogenase was assayed in a Warburg microrespirometer at 36°C in an atmosphere of hydrogen purified of oxygen by passage through alkaline pyrogallol. The main compartment of the Warburg flask contained 25 μmoles of methylene blue chloride and 300 μmoles of phosphate buffer, pH 8.0. The center well contained 0.2 ml 20% KOH to absorb CO_2, and the sidearm, the cells. Water was added to the main compartment to give a total volume of 3.2 ml. Specific activity of hydrogenase is expressed as $Q_{H_2}(N)$ value, defined as the microliters of hydrogen taken up per hour per mg nitrogen. Nitrogen was determined by the Kjeldahl method.

Results. The effect of age on the level of hydrogenase in *A. agile* and *A. vinelandii* wild types is shown in Fig. 1. Up to 12 hours, the $Q_{H_2}(N)$ of both species is continually lower in ammonia-grown cells than in cells fixing N_2, indicating that ammonium-nitrogen is inhibiting the formation of hydrogenase. The sharp drop in the hydrogenase activity of ammonia-grown cultures after 12 hours probably arises from the increased hydrogen ion concentration of the medium resulting from the selective utilization of the ammonium ion. Cultures of *A. agile* grown on 400 ppm NH_3-N for 24 hours in which the pH was maintained between 6.7 and 7.5 by addition of sterile base showed no such drop in activity after 12 hours, having a specific activity of 9,100 in 24 hours —a 36% inhibition by ammonia since the comparable N_2-grown culture at this age had a $Q_{H_2}(N)$ of 14,300. These results cannot be accounted for by a resumption of nitrogen fixation since the supernatant still contained 164 micrograms NH_3-N per ml.

Mutants of both species of the azotobacter

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‡ For convenience, we shall term cells unable to use molecular N_2 "nitrogenase-deficient," although obviously the block need not occur in the initial step mediated by the enzyme, nitrogenase.

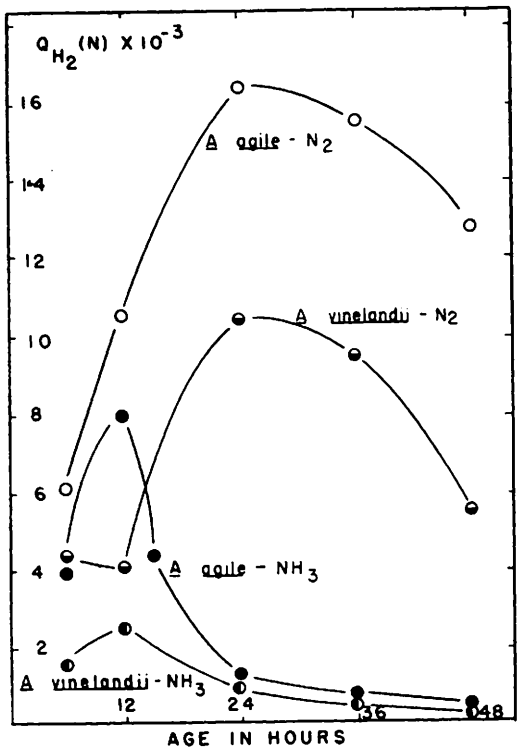

FIG. 1. The time course for production of hydrogenase by the azotobacter. One ml aliquots of 24 hr cultures of the 2 wild types grown in N-free media were inoculated into media either N-free or containing 300 ppm ammonium-N. Cultures were incubated on a rotary shaker (360 rpm) at 30°C and harvested at regular intervals.

unable to use N_2 but capable of utilizing ammonium-nitrogen at varying rates were compared with the wild types with respect to content of hydrogenase. When back mutants which fixed nitrogen arose from the nitrogenase-deficient strains, the hydrogenase activity of these organisms was also assayed to determine the effect of the resumption of fixation on the enzyme content. All cultures were harvested before the pH became sufficiently low to affect the hydrogenase level. One nitrogenase-less mutant of *A. agile* (No. 373) used ammonium-N at a rate approximately equal to that of the wild type, reverting after periods of 3-21 days to nitrogen fixation. Two other types of mutants were isolated and assayed: (a) *A. agile* mutant No. 65 which fixed N_2 at a reduced rate but used ammonium-N at the normal rate; and (b) mutants of *A. agile* and *A. vinelandii* unable to fix and also unable to use ammonium-N at the rate of the wild type. The results of the hydrogenase assays for typical strains are summarized in Table I. All mutants contained hydrogenase, but the specific activity in ammonia-grown cultures was lower than for the same cultures, after back mutation, grown in nitrogen-free media. Repeated transfers of the reverted cultures in nitrogen-free media did not increase the $Q_{H_2}(N)$ value.

Discussion. All non-fixing mutants of the azotobacter had a reduced hydrogenase activity when grown in fixed nitrogen; moreover, those mutants which back-mutated to utilization of N_2 also possessed hydrogenase activity typical of the fixing wild type. In the one mutant (No. 373) which, after reversion to fixation, grew at the same rate as the wild type on N_2, the hydrogenase activity was equal to that of the parent, but it was lower if the growth rate of the mutants (*e.g.*, No. 65) on N-free medium was less than the wild type. These results confirm and extend the previously noted relationship between hydrogenase and the non-symbiotic nitrogen fixing system.

The presence of hydrogenase in the absence of nitrogenase in strains of the azotobacter suggests that hydrogenase functions in other systems of the organism in addition to participation in nitrogen fixation. Since hydrogenase has the lowest reduction potential of any biological system(6), cells of azotobacter may use it in various O/R reactions involving one or more enzymes, coenzymes, or intermediates in the carbon or nitrogen metabolism. For example, the decrease in activity of the serine deaminase of *Escherichia coli* could be prevented by storing in H_2, presumably through the activity of hydrogenase(7).

Study of an azotobacter mutant which does not possess hydrogenase might provide a solution to the nitrogenase-hydrogenase interrelationship. A hydrogenase-deficient strain of *Desulfovibrio desulfuricans* has been isolated by Adams *et al.*(8); although those workers did not determine the ability of their strain to utilize atmospheric nitrogen, Sisler and ZoBell(9) have classified this species among the nitrogen-fixers.

TABLE I. Hydrogenase of Nitrogenase-Deficient Strains of *Azotobacter*.

Organism	Strain	N-free medium		NH_3-N medium	
		k value	$Q_{H_2}(N)$, × 100	*k* value	$Q_{H_2}(N)$, × 100
A. agile	WT*	.36–.39	13†	.38–.41	8†
	373	.00	—	.34–.36	8.6†
	373‡	.32–.36	16†	—	8.5†
	WT	.39	163	.40	46
	373	.00	—	.34–.36	47
	65	.17	62	.36	51
	54	—	53	.14	17
	55	—	41	.15	19
	59	.00	—	.23	56
A. vinelandii	WT	.33	98	.39	21
	1	.06	38	.25	18
	3	.07	41	.27	19
	19	.07	27	.27	16
	22	.00	—	.26	15

* WT = Wild type.
† Hydrogen acceptor was 150 μmoles of $K_3Fe(CN)_6$ rather than methylene blue.
‡ After reversion to nitrogen fixation.

Summary. Non-fixing mutants of both *Azotobacter agile* and *Azotobacter vinelandii* grown on media containing ammonium-N had a reduced hydrogenase activity similar to that of the wild type. Those mutants which reverted to nitrogen fixation had a level of hydrogenase in N-free media at least double that of the cells grown on fixed nitrogen. The specific activity was equal to that of the wild type on N_2 if the growth rate was normal, but was lower if the growth rate was less than the wild type. The results confirm the close relationship between hydrogenase and nitrogen fixation in the azotobacter but also suggest that hydrogenase in these organisms may have same secondary function as it is present, although to a lesser extent, in mutants unable to use N_2.

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