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Short Communication

The Effect of Tungstate on Nitrate Assimilation in Higher Plant Tissues

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Molybdenum (as molybdate) is an essential mineral nutrient in higher plants and its involvement in nitrate reduction is well documented. Thus, molybdenum deficient plants grow better on ammonia or nitrite as sole nitrogen source than they do on nitrate (2) and nitrate will not induce nitrate reductase in molybdenum deficient plants (1). Furthermore, molybdenum is associated with soybean nitrate reductase through several purification steps (4).

Tungstate is not only a competitive inhibitor of molybdate uptake and utilization in *Azotobacter vinelandii* (8, 9), but also inhibits growth of this organism when nitrate or nitrogen is the sole nitrogen source (13). Tungstate is also a competitive inhibitor of molybdate function in *Aspergillus niger* when nitrate is the sole nitrogen source (7). We have examined the effect of tungstate on the formation of active nitrate reductase in suspension cultures of tobacco XD cells and in intact barley shoots. In both cases tungstate prevented the formation of active enzyme.

Materials and Methods

Tobacco XD cells were grown and harvested as previously described (5, 6). Cells were subcultured into M-1D medium with or without sodium tungstate and were harvested at the times indicated in the figure and table legends. Barley plants, variety Himalaya, were grown from seed in pots in vermiculite at 25° and 1500 ft-c of continuous fluorescent light supplemented with tungsten light. Plants were treated as described in table and figure legends. Induction of nitrate reductase with 3 mM nitrate was carried out when the plants were 5 days old. Shoots were harvested at the times indicated and ground in a porcelain mortar with 0.1 M tris-HCl, 0.001 M cysteine, pH 7.4, and the brei was centrifuged at 12,000*g* for 20 min. The 12,000*g* supernatant was used for both the enzyme assays and for the assay of nitrate. Nitrate reductase was assayed essentially by the method of Paneque, Del Campo, Ramirez and Losada (12) and nitrate by the method of Lowe and Hamilton (10).

Results and Discussion

The effect of tungstate on the formation of active nitrate reductase and the accumulation of nitrate in tobacco XD cells is shown in table I. As the tungstate concentration is increased, the amount of nitrate reductase activity detected after a 24 hr growth period is lowered, no formation of active enzyme occurring above 50 μM tungstate. At the same time, the nitrate level of the cells is increased, presumably because nitrate reduction is much diminished or absent. Even at the higher tungstate levels no inhibition of net nitrate uptake is apparent, suggesting that tungstate does not adversely affect the nitrate uptake system over the time period used here. Molybdate added 24 hr after tungstate, overcomes the tungstate inhibition of development of nitrate reductase activity in the XD cells (Fig. 1). The tungstate inhibition of nitrate reductase activity can be partially overcome by molybdate even if the XD

Table I. *The Effect of Tungstate on the Formation of Active Nitrate Reductase and on Nitrate Uptake in Tobacco XD Cells*

XD cells were subcultured into M-1D medium containing different levels of sodium tungstate and were grown for 24 hr before harvesting and assaying for nitrate reductase and nitrate. Tungstate at the levels used in the experiments reported in this communication did not interfere with either the nitrate reductase assay or the nitrate assay.

Growth period (hr)	Tungstate (μM)	Nitrate reductase mμmoles NO_3^- reduced per hr per g tissue	Nitrate μmoles/g tissue
0	0	0	0.04
24	0	760	18.70
24	5	385	61.40
24	10	187	47.70
24	20	75	46.80
24	50	0	44.20
24	100	0	41.70

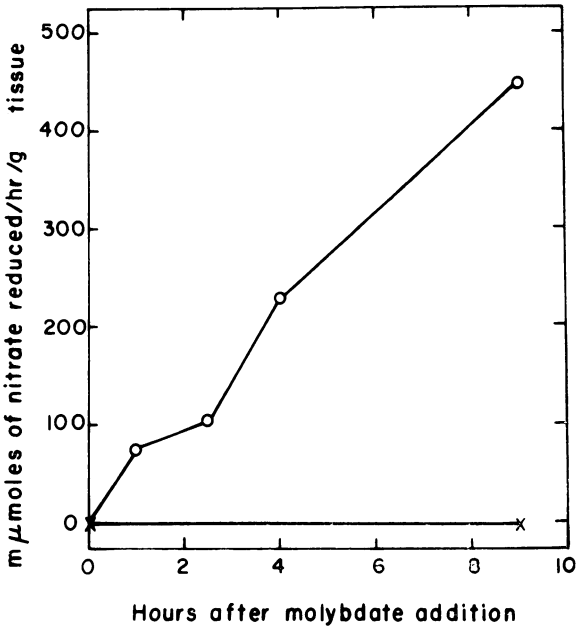

FIG. 1. Reversal of the tungstate inhibition of formation of nitrate reductase by molybdate in tobacco XDR^{thr} cells. XDR^{thr} cells were subcultured into M-1D medium containing 0.1 mM tungstate. After 24 hr molybdate was added to a final concentration of 0.1 mM and nitrate reductase was assayed at the times indicated (o). Molybdate was not added to the control culture (x).

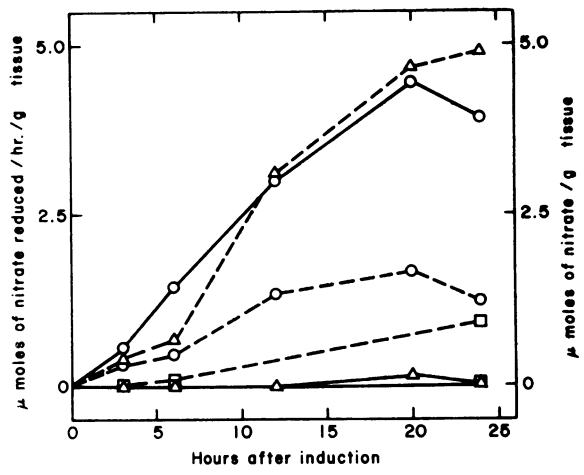

FIG. 2. The effect of tungstate on the development of nitrate reductase activity and the uptake of nitrate in barley shoots. Plants were grown from seed for 5 days before addition of 3 mM nitrate (0 hr) and during this 5 day period were treated with molybdenum-less, nitrate-less half Hoagland's solution containing either no sodium tungstate (o), 0.1 mM sodium tungstate (△), or 0.5 mM sodium tungstate (□). Shoots were harvested at the times indicated and assayed for nitrate reductase (——) and nitrate (----).

cells are pretreated with 4 μg/ml of cycloheximide (which reduces incorporation of ^{14}C-arginine into protein by 98 %) for 2 hr before addition of molybdate (10^{-4} M final concentration). The nitrate induced formation of nitrite reductase in XD cells is not adversely affected by tungstate at levels which cause a 95 % lowering of the detectable nitrate reductase activity (3). The effect of tungstate on the development of nitrate reductase activity is also observed in the intact shoots of barley plants (Fig. 2). Higher levels of tungstate are required to inhibit the formation of active enzyme in this tissue than in tobacco XD cells. Tungstate at a level of 10^{-4} M completely prevents the formation of nitrate reductase activity while at the same time having no adverse effect on plant growth or nitrate uptake. In fact the nitrate content of the shoots is increased (Fig. 2). Higher levels of tungstate inhibit root and shoot development as well as nitrate uptake (Fig. 2). Again, inhibition of the development of nitrate reductase activity is overcome by molybdate. Molybdate also brings about a decrease in the nitrate content of the shoots (table II).

The data indicate that there is no obligatory coupling between nitrate uptake and nitrate utilization in either tobacco XD cells or in barley seedlings. Inhibition of nitrate reduction by tungstate does not inhibit nitrate uptake. There are 3 possible explanations for these observations. They are 1) tungstate has a nonspecific toxic effect, one of the consequences being inhibition of the development of functional nitrate reductase, 2) tungstate does not inhibit the formation of nitrate reductase apoenzyme, but inhibits the incorporation of molybdate into it, thereby rendering the enzyme nonfunctional, or 3) tungstate specifically inhibits the formation of nitrate reductase apoenzyme. A general toxic effect seems unlikely because neither nitrate uptake nor the

Table II. *The Reversal by Molybdate of the Tungstate Inhibition of the Formation of Active Nitrate Reductase in Intact Barley Shoots*

Plants were grown from seed for five days and were treated during this period with molybdenum-less, nitrate-less half Hoagland's mineral solution supplemented with 100 μM sodium tungstate and the levels of sodium molybdenum indicated. At 5 days the plants were induced with 3 mM nitrate and 24 hr later shoots were harvested and assayed. 0.184 μM molybdate represents the concentration of molybdate in half Hoagland's solution. The nitrate content of uninduced plants has never been found to be higher than 0.06 μmoles/g tissue.

Treatment		Nitrate reductase mμmoles NO^-_3 reduced per hr per g tissue	Nitrate μmoles/g tissue
Tungstate	Molybdate		
μM	μM		
100	0	262	4.03
100	0.184	2271	3.56
100	50	3980	1.76
100	100	4280	2.19
100	200	4921	2.43

nitrate induced formation of nitrate reductase is inhibited by tungstate. Also, barley plants and tobacco XD cells appear normal when treated with optimal tungstate levels. The fact that the xanthine dehydrogenase of *Pseudomonas aeroginosa* also exhibits *in vivo* tungstate inhibition which is overcome by molybdate (11) supports the suggestion that the effect of tungstate is specific to molybdoenzymes. Since some nitrate reductase activity develops in tungstate treated cells in response to molybdate despite essentially complete inhibition of protein synthesis by cycloheximide, at least part of the tungstate effect is due to inhibition of the function of the nitrate reductase protein, rather than inhibition of its formation.

The study of nitrate uptake and its regulation in higher plants is complicated by the fact that nitrate is reduced within the cell. Were it possible to isolate nitrate reductase negative mutants from higher plants, this problem could be overcome in the classical way of the microbiologist. Nitrate uptake and accumulation could then be studied in the absence of derivative effects. Such mutants are not yet available in higher plant systems. The data presented in this communication suggest that by using tungstate to selectively prevent nitrate reduction, it will now be possible to examine the regulation of nitrate uptake and of other steps of the nitrate assimilation pathway in higher plants without the complications introduced by the functioning of the pathway.

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