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Resistance to Acetohydroxamate Acquired by Slow Adaptive Increases in Urease in Cultured Tobacco Cells[1]

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ABSTRACT

Urease activity of tobacco XD cells (1U cells) had undergone a 4-fold increase (4U cells) during a year of growth on urea (Skokut and Filner 1980 Plant Physiol 65: 995–1003). A clone of 4U cells gave rise to 12U cells during another year of growth on urea. The doubling time of 12U cells on urea is 2.2 days, compared to about 4 days for 1U cells, while 1U and 12U cells double in 2 days on nitrate. Acetohydroxamic acid (AHA), a specific inhibitor/reversible inactivator of jack bean urease, affects tobacco cell urease similarly. Fifty per cent inhibition of growth by AHA occurred at 20 micromolar in 1U cells growing on urea and at 165 micromolar in 12U cells growing on urea, but at 600 micromolar for either 1U or 12U cells growing on nitrate. When 12U cells were grown on urea with 100 micromolar AHA, extractable urease activity decreased 80% within 2.5 hours and remained at this level for 2 weeks; the doubling time increased to 3.7 days, and intracellular urea rose 2-fold, compared to 12U cells grown on urea without AHA. Urease of 12U cells inactivated by AHA *in vivo* could be reactivated to its pre-AHA level by incubation at 30 C after extraction and separation from free AHA. AHA inhibited incorporation of ^{15}N from [^{15}N]urea into Kjeldahl nitrogen in the cells, in spite of the increased intracellular urea. These results indicate that AHA acts primarily by inhibiting urease action, rather than by inhibition of formation of urease protein or of uptake of urea. Because 12U cells are 8 times more tolerant of AHA than 1U cells, it is likely that growth on urea in the presence of AHA should select strongly for cells with high urease.

Higher plants contain urease but attempts to detect in them the allophanate pathway of urea assimilation which occurs in algae and yeast have so far been unsuccessful (see ref. 27). The function of urease in higher plants is not established but most probably it catalyzes the final step in the breakdown of purines via ureides and of arginine after the arginase reaction (20). The ureides are important as transport forms of nitrogen in some species such as apple (4) and in certain legumes such as soybean, especially when they are fixing nitrogen symbiotically (17). Arginine is an important component of the nitrogen stored in some seed proteins (20).

Urease occurs in high concentrations in seed of jack bean, soybean, and cucurbits (4). While jack bean urease was the first enzyme to be crystallized and has been studied extensively (21), ureases from other plant sources have received relatively little attention. Polacco (19) recently purified the urease of soybean seed and found it to be quite similar to that from jack bean seed. In addition to hydrolyzing urea, jack bean urease also hydrolyzes hydroxyurea (3), dihydroxyurea (6), and semicarbazide (8). Hydroxamates are nonhydrolyzable analogs of urea which reversibly inactivate the enzyme, apparently by slowly binding to the catalytic site (3, 11).

Regardless of the normal function of urease in higher plants, both intact plants and cultured plant cells can grow on urea as their sole nitrogen source, probably as a result of the reaction catalyzed by urease (see ref. 27). Polacco provided evidence for this by using citrate to inhibit specifically growth of soybean cells on urea, and nickel ions to overcome the inhibition of this nickel-containing enzyme (18). The enzyme activity appears to be induced by urea in some plants and in cultured soybean cells, but not in the XD strain of cultured tobacco cells, in which it is constitutive (see ref. 27).

Skokut and Filner (27) described slow adaptive increases in urease activity in the XD cells (1U cells). These increases begin to be evident about 40 generations after shifting from nitrate to urea as the sole source of nitrogen, and reach 4 times the basal level after another 20 to 40 generations (4U cells). The doubling time of the 4U cells grown on urea was 3.0 days, compared to 3.6 days for 1U cells. Also, urea accumulation was reduced by a factor of about 3 in 4U cells, compared to 1U cells. These findings are suggestive that growth of the XD cells on urea nitrogen is urease-limited.

Clones of 1U cells went through the same slow increase in urease activity as the uncloned population of XD cells, showing conclusively that 4U cells originate from 1U cells. The time dependence of the rise in urease activity was consistent with 4U cells arising from 1U cells at a frequency of 8 per 10^5 cells per generation, and then multiplying at their characteristic rate. This frequency of origination is much higher than the 10^{-7} to 10^{-8} which has been encountered in selections for other biochemical variants (mutants?) of the XD cells (7, 10, 29).

The paths of nitrate and urea assimilation converge at ammonium. Because the doubling times of XD cells growing on nitrogen supplied as nitrate or ammonium is 2 days in both cases, while that of 4U cells growing on urea is only 3.0 days, it was conceivable that a further increase in urease activity might further reduce the doubling time on urea nitrogen. Therefore, continued growth on urea might select for still higher urease levels. Other selective pressures for increases in urease activity are conceivable. Cells with high urease should be favored if the rate of the urease reaction is slowed, either by growing the cells on a urea analog which is a slowly hydrolyzable substrate of urease or by adding a urea analog such as hydroxyurea (3) or a hydroxamate (3, 11) which inhibits hydrolysis of urea by urease.

We report here observation of a further adaptive increase in urease activity, to about 12 times the basal level, accompanied by a further decrease in doubling time on urea, approaching that on nitrate and ammonium. We also show that acetohydroxamate specifically inhibits growth on urea nitrogen by reversibly inactivating urease, and that its effectiveness as a growth inhibitor decreases as the urease level characteristic of the cells increases.

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MATERIALS AND METHODS

Culture Condition for Tobacco Cells. Cells of tobacco (*Nicotiana tabacum* L. cv. Xanthi, line XD) were grown on nitrate (*i.e.* on MID medium) or urea (*i.e.* nitrogen-less MID plus urea) as a sole nitrogen source under the culture conditions described previously (27). Cultures were maintained by inoculating 200-ml portions of media with 10-ml aliquots of 14-day-old stationary phase cultures of cells grown on nitrate or urea. The fresh weights of inocula were about 0.3 g/200 ml. Urea (ultra pure grade, Schwarz/Mann), AHA[2] (Sigma), and MSO (Sigma) stock solutions were sterilized by filtration through a sterile membrane filter (Type GS, 0.22 μm pore, Millipore Corp).

Preparation of Cell-free Extract. Tobacco cells were harvested by vacuum filtration on Whatman No. 1 filter paper and rinsed with distilled H_2O. After determining the fresh weight, the cells were suspended in ice-cold homogenizing buffer (0.1 M K-phosphate, 5 mM DTE, and 1 mM Na-EDTA, pH 7.5). Three ml of the buffer were used per g fresh weight. The cells were homogenized by 30 strokes of a motor-driven Teflon-glass homogenizer at 4 C and then sedimented at 20,000*g* for 20 min. The supernatant fraction was used as a crude enzyme preparation in some experiments. The crude preparation was then passed through a small gel column which was made by packing a 5-ml plastic syringe with 4 ml of Sephadex G-25. The packed syringe plus 2 ml of the equilibration buffer (10 mM K-phosphate, 0.1 mM DTE, and 0.1 mM Na-EDTA, pH 7.5) was first centrifuged in a clinical centrifuge at 3,400 rpm for 15 s. Then 1 to 2 ml of the crude fraction was loaded onto the tops of the gels and centrifuged for 15 s. The fluid below the syringe was used as the urease sample. Blue dextran and riboflavin could be completely separated by this procedure.

Assay Methods of Urease and GS. Urease activity was determined by measuring the release of ammonium from urea. The complete reaction mixture contained in 1 ml: 100 μmol K-phosphate (pH 7.5), 50 μmol urea, and enzyme preparation containing 100 to 600 μg protein. The reaction was started by the addition of the enzyme. After incubation for 60 min at 30 C, the reaction was terminated by addition of reagents for the ammonium assay and placing tubes in a water bath at 70 C for 5 min. Incubations terminated at zero time were used as control. When the reaction mixture contained AHA, or when crude enzyme which had not been subjected to gel filtration through Sephadex G-25, was used as the enzyme, the reaction was terminated by the addition of 0.1 ml 5 N HCl. GS was assayed by measuring transferase activity according to the method of Rhodes *et al.* (22). GS was assayed in the same fractions as urease. Assays were routinely performed by incubation for 30 min at 30 C. All assays were performed in duplicate.

Determinations of Ammonium, Urea, and Protein Contents. Ammonium was determined by the method of McCullough (14). When the urease reaction was terminated by the addition of HCl, or when ammonium in cell-free extracts was measured, ammonium was distilled at pH 10.1 and trapped in 0.1 N HCl in plastic Conway dishes. Ammonium concentration was determined as described above. Ammonium chloride was used as a standard.

The concentration of urea in cell extracts was determined as ammonium after first hydrolyzing it to NH_4^+ and CO_2 using commercially available urease as described previously (27). Soluble protein content of the cell extract was determined as described previously (27).

Chromosome Counts. Chromosomes were stained with Propiocarmine (0.5 g carmine in propionic acid:H_2O; 55:45, boiled and filtered). Cells were heat-fixed in the presence of the dye, then squashed. The chromosomes in each well spread metaphase figure were counted three times.

Incorporation of ^{15}N into Kjeldahl Nitrogen of 12U Cells Grown on [^{15}N]Urea in the Presence of AHA and/or MSO. 12U cells grown on 3 mM [^{14}N]urea medium for 3 days were harvested, washed with nitrogen-less MID medium and weighed sterilely. Four g fresh weight of the 12U cells were then inoculated into 100 ml of N-less MID medium containing 3 mM [^{15}N]urea (95 A% Merck) either with no addition, with 100 μM AHA, with 1 mM MSO, or with both inhibitors. The cells (about 500 mg fresh weight) were then harvested at 5, 10, and 24 h after the inoculation and duplicate samples of 200 mg fresh weight cells were each digested in 4 ml concentrated H_2SO_4 containing 1.1 g of salt-catalyst mixture (15). NH_4^+ was then steam-distilled and trapped in 2 ml of 0.08 N H_2SO_4 plus 5 ml of H_2O. Distilled NH_4^+ solution was concentrated by heating and then by lyophilization. The [^{15}N]A% in the lyophilized samples of [^{15}N]$(NH_4)_2SO_4$ was then determined by isotope ratio mass spectrometry. Other portions of the cells at 24 h after the inoculation were homogenized in homogenizing buffer as described above, and activities of urease and GS as well as endogenous concentrations of urea and NH_4^+ were measured.

RESULTS

Further Slow Adaptive Channels in Urease Level in Tobacco XD Cells. The specific activity of urease for 1U cells grown on NO_3^-, extracted and assayed by the methods described in this paper, ranged from 0.30 to 0.40 μmol h^{-1} g fresh weight^{-1}, compared to that of about 0.50 in the previous report in which different extraction and assay conditions were used (27). Clones, originally isolated from a population of 4U cells by Skokut and Filner (27), were cultured in liquid urea medium for 5 months, then on agar urea medium for 7 months, after which they were returned to liquid urea medium. Then the urease levels of the clones were determined (Table I). Most clones from 4U cells, which initially had the specific activity of about 2.0 μmol h^{-1} g fresh weight^{-1}, still had urease levels similar to those found a year earlier. However, in three out of 14 clones kept for a year, the urease levels were up to 4.3 to 5.2 μmol h^{-1} g^{-1} fresh weight (12U cells).

Growth rate constants and doubling times of 12U cells and of 1U cells grown on urea or nitrate were determined as described

Table I. *Urease Levels of Isolated Clones Cultured for More than 2 Years on Urea Medium*

The clones isolated from 4U-cells (about 2 μmol NH_4^+ h^{-1} g^{-1} fresh weight) by Skokut and Filner (27) were maintained on agar for more than 2 years. Those clones were transferred to liquid urea medium. After two or three transfers in liquid medium, urease levels were determined for each clone in the exponential phase (4- to 7-day-old cultures).

Clone Number	Urease
	μmol NH_4^+ h^{-1} g^{-1} fresh wt
1	5.24
2	4.32
3	4.28
4	3.09
5	2.70
6	2.63
7	2.40
8	2.38
9	2.25
10	2.21
11	2.17
12	1.95
13	1.85
14	1.15

[2] Abbreviations: AHA, acetohydroxamic acid; MSO, L-methionine-DL-sulfoximine; DTE, dithioerythritol; GS, glutamine synthetase; GDH, glutamate dehydrogenase.

previously (27) and compared. The 12U cells grew rapidly on urea with a growth rate constant of 0.32 day^{-1} and doubling time of 2.2 days. Those values were essentially identical with those for 1U cells grown on nitrate, which had a growth rate constant of 0.33 day^{-1} and doubling time of 2.1 days. The growth rate constant and doubling time for 1U cells newly transferred from nitrate to urea were 0.14 day^{-1} and 5.1 days, respectively (this growth rate is slightly lower than reported previously [27] for XD cells newly transferred to urea). The values for 4U cells on urea were 0.23 day^{-1} and 3.0 days, as reported earlier (27). Thus, accompanying the increase in the urease level during the transition from 4U to 12U cells was a decrease in the doubling time for growth on urea.

Effects of Substituted Ureas and AHA on the Growth and Extractable Urease of 1U and 12U Cells. Several substituted ureas were tested for their effect on the growth of 1U cells on urea and that on nitrate medium. A substituted urea: methylurea, ethyl urea, 1,1-dimethylurea, 1,3-dimethylurea, or 1,1,3,3-tetramethylurea was added to give from 0.01 to 1.0 mM in the medium containing either 3 mM urea or nitrate. None of these substituted ureas affected growth of 1U cells on either medium. Extractable urease activity from the cells was not inhibited by incubation with any of the substituted ureas already mentioned, nor by phenylurea or thiourea, up to 10 mM.

AHA, which is a potent and specific inhibitor and reversible inactivator of jack bean urease (3, 11), inhibited the growth of both 1U and 12U cells grown on urea (Fig. 1). The 1U cells were more sensitive to AHA than the 12U cells: 50% inhibition of growth on urea medium occurred at 20 μM for 1U cells and at 165 μM for 12U cells. However, 600 μM AHA gave 50% inhibition of growth of either cell type on nitrate. Thus, the cells were most sensitive to AHA when grown on urea, and a difference in sensitivity of 1U and 12U cells to AHA was only evident when they were grown on urea.

Urease extracted from both 1U and 12U cells was inactivated by AHA *in vitro*, most effectively in the absence of urea. The inactivation by AHA increased with preincubation time and was temperature-dependent (Fig. 2). No inactivation occurred at ice temperature. Inactivation of urease from both cell types also increased with AHA concentration when the urease fraction was preincubated for 30 min at 30 C; 50% inactivation of urease from both cells occurred at 45 μM AHA (Fig 3A). When the preincubation was omitted, inactivation by low concentrations of AHA was completely prevented by the presence of 50 mM urea. An extremely high concentration of AHA (7 mM) was required to cause 50% inactivation in the presence of 50 mM urea (Fig 3B). The inactivation of 12U cell urease bv AHA was slowly reversed, depending on time and temperature, when excess AHA was removed by gel filtration with Sephadex G-25. Partial reactivation could be observed after urease was inactivated *in vitro* (Fig. 4A). Urease of 12U cells was also inactivated by AHA *in vivo* (see below), and the urease inactivated *in vivo* was largely reactivated to its pre-AHA level by the incubation at 30 C (Fig. 4B). For the reactivation treatment, 0.1 mM chloramphenicol was included in the incubation mixture to prevent bacterial growth. This was found to be necessary during overnight incubations because,

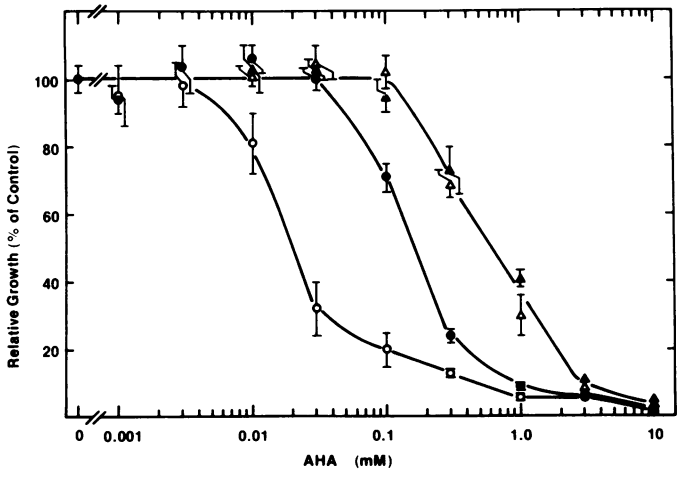

FIG. 1. Effect of AHA on the growth of 1U or 12U cells grown on either urea or NO_3^-. 1U cells (○, △) and 12U cells (●, ▲) were grown on either 3 mM urea (○, ●) or nitrate (△, ▲) medium containing various concentrations of AHA for 18 days. Growth without AHA of: 1U cells on urea; 1U cells on NO_3^-; 12U cells on urea and 12U cells on nitrate was 1.87, 4.15, 3.98, and 3.60 g fresh wt/100 ml, respectively.

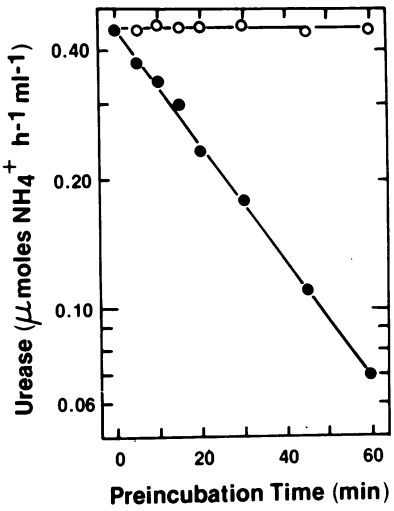

FIG. 2. Inactivation of urease by AHA as a function of preincubation time. Urease fraction extracted from 5-day-old 12U cells (Sephadex G-25 fraction containing 0.084 mg protein/200 μl) was preincubated with 0.1 mM AHA at 30 C (●) or 0 C (○) in 0.1 M K-phosphate (pH 7.5). Urease reaction was then started bv adding 0.1 ml of 0.5 M urea. After 60 min at 30 C, the reaction was terminated by adding 0.1 ml of 0.5 N HCl, then released NH_4^+ was distilled in Conway dishes.

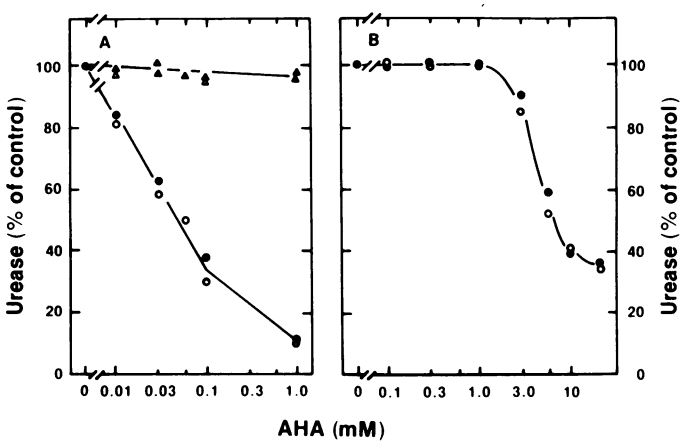

FIG. 3. Inactivation of urease extracted from 1U (○, △) and 12U cells (●, ▲) by AHA as a function of concentrations with (A) or without (B) preincubation. A, crude extracts of urease from 1U cells (0.83 mg protein) and 12U cells (0.18 mg protein) were preincubated with AHA for 30 min at 30 C (○, ●). AHA was also added without preincubation (△, ▲). Urease activity of 1U cells without AHA was 0.061 mol NH_4^+ h^{-1} and that of 12U cells without AHA was 0.13 mol NH_4^+ h^{-1}. B, crude extracts from 1U cells (0.48 mg protein) and 12U cells (0.05 mg protein) were used as the urease. Urease activity of control was 0.03 for 1U cells and 0.06 mol NH_4^+ h^{-1} for 12U cells.

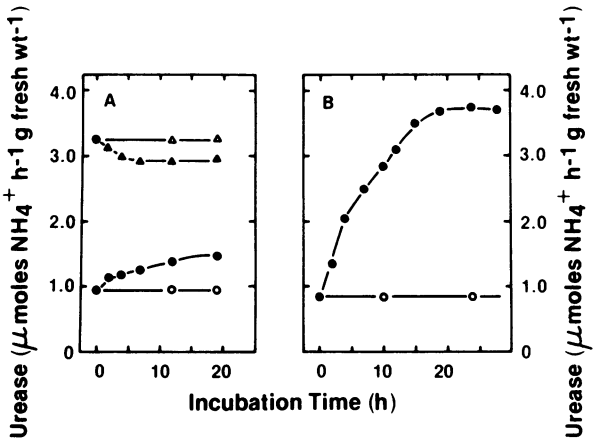

FIG. 4. Reactivation of urease which had first been inactivated by 100 μM AHA *in vitro* (A) and *in vivo* (B). A, crude urease fraction from 7-day-old 12U cells (7 ml containing 6.04 mg protein) was first preincubated with or without 100 μM AHA for 30 min at 30 C. Protein fraction was then separated from excess AHA by Sephadex G-25 gel filtration and 0.3 ml of the fraction was incubated with 0.6 ml 0.1 M K-phosphate (pH 7.5) containing 0.1 mM DTE, 0.1 mM EDTA, and 0.1 mM chloramphenicol at 30 C (●, ▲) or 0 C (○, △). Urease reaction was started at the time indicated by addition of urea. The urease fraction contained 0.14 mg protein for control (△, ▲) and 0.16 mg for AHA treatment sample (○, ●), respectively. B, 12U cells were grown on urea in the presence of 100 μM AHA for 7 days. Urease fraction was then separated from excess AHA by the gel filtration, and 0.2 ml of the fraction was incubated as described above at 30 C (●) or 0 C (○).

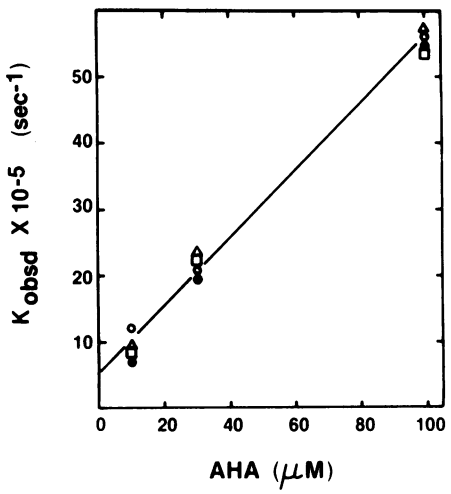

FIG. 5. Dependence of K_{obsd} for inactivation of urease on the concentration of AHA. Crude urease fraction from 7-day-old 12U cells was diluted to give concentrations: 1.87 (○), 0.94 (●), 0.47 (□), and 0.19 (△) mg protein/2 ml. Each concentration of urease was preincubated with 0, 0.01, 0.03, or 0.1 mM AHA for 30 min at 30 C. Then urease activity was assayed after removing excess AHA by gel filtration. K_{obsd} was obtained as described in text.

otherwise, urease activity of proliferating bacterial contaminants began to be detected after 8 h at 30 C. The inactivation of urease by AHA *in vitro* did not cause changes in the K_m for urea, which was estimated to be 0.182 mM before and after inactivation, but it reduced the V_{max}, as expected if AHA acted by titrating a portion of the population of active enzyme molecules. In addition, the concentration of urease had no effect on the rate constant for its inactivation by AHA, when 0.19 to 1.87 mg protein of the 12U cell fraction containing urease was preincubated with various concentrations of AHA (Fig 5). That is, the inactivation reaction was first order with respect to AHA concentration regardless of enzyme concentration.

Those results suggested that the inactivation involved a slowly attained equilibrium as follows:

$$E + I \underset{k_-}{\overset{k_+}{\rightleftarrows}} EI \qquad (1)$$

where E is urease, I is AHA, and EI is inactive urease-AHA complex. The rate constants and the equilibrium constant can be determined by the procedures of Blakeley *et al.* (3). Because the concentration of AHA should remain essentially constant and the time course of inactivation gave first order plots (Fig. 2), this can be treated as a simple first-order approach to an equilibrium for which $\ln E_t/E_o = -K_{obsd} \cdot t$ where E_o = initial enzyme activity; E_t = activity at time t; and $K_{obsd} = k_- + k_+ [I]$. A graph of $-\ln\frac{E_t/E_o}{t} = K_{obsd}$, at constant t, *versus* $[I]$ was linear as required by the equilibrium described by equation 1 (Fig 5). The values of k_+ and k_-, obtained from the slope and the y intercept, respectively, were calculated to be 5.05 s^{-1} M^{-1} and 5.5 × 10^{-5} s^{-1}, respectively. The dissociation constant of the EI complex, calculated from the equation of $K_i = k_-/k_+$, was 1.09 × 10^{-5} M. As a check on this interpretation, k_+ was also calculated from data at constant $[I]$ and varying t (Fig. 2) as 5.12 $s^{-1}M^{-1}$. Rate and equilibrium constants for the inactivation of jack bean urease by AHA were reported to be 1.98 × 10^{-5} M for K_i, 42 s^{-1} M^{-1} for k_+ and 8.3 × 10^{-4} s^{-1} for k_-, respectively, from measurements made at 38 C and pH 7.0 (3). Thus, the tobacco cell urease has a K_i for AHA similar to that reported for jack bean urease but k_+ and k_-, determined at 30 C and pH 7.5, are both an order of magnitude lower than those of jack bean urease at 38 C. The temperature difference could perhaps account for a difference of a factor of two. The origin of the remainder of the difference is unknown, but it may be explained partly by the different pH optima of jack bean urease (6.5) and tobacco cell urease (7.5–8.0). Another factor could be differences in the proportions of the two tautomeric forms of hydroxamic acids—the N-hydroxyamide and the C-hydroxyoxime—at the pH values at which the ureases were assayed.

Inactivation of 12U Cells by AHA *in Vivo*. When 12U cells were incubated with urea medium containing 0.1 mM AHA, 1.0 mM MSO, or 0.1 mM AHA together with 1.0 mM MSO, the extractable urease level decreased about 80% within 2.5 h and remained at this level for 7.5 h in the presence of either AHA alone or AHA with MSO (Fig. 6A). MSO also caused rapid and strong inhibition (about 90%) of GS within the same time (Fig. 6B). AHA alone did not inhibit GS, nor did MSO alone inhibit urease (Fig. 6, A and B). In the presence of AHA alone and AHA with MSO, the endogenous urea pool of the cells rapidly increased about 4 times compared with the control within 2.5 h, and the high level was maintained during the incubation (Fig. 6C). The pool of NH_4^+ increased continuously in the presence of MSO alone (Fig .6D). A slight increase of the NH_4^+ pool was observed in the presence of AHA with MSO, and a slight decrease of that level was detected with AHA alone. Those results are consistent with the hypothesis that urea is assimilated via hydrolysis to NH_4^+ which is then incorporated into glutamine by GS. AHA and MSO seemed to inhibit only the reactions of urease and GS, respectively, but not to inhibit urea uptake (Fig. 6C).

The inactivation of urease by AHA *in vivo* was sustained throughout 2-weeks of growth (Fig. 7B). The urea pool increased (Fig. 7C) and the NH_4^+ pool decreased (Fig. 7D) in the cells during the growth period. Growth rate was reduced by AHA (Fig. 7A) and the growth rate constant and doubling time were 0.19 day^{-1} and 3.7 days, whereas the values without AHA were 0.33 day^{-1} and 2.1 days, respectively. The protein content/g fresh weight was unaffected by 100 μM AHA. Thus, AHA did not create

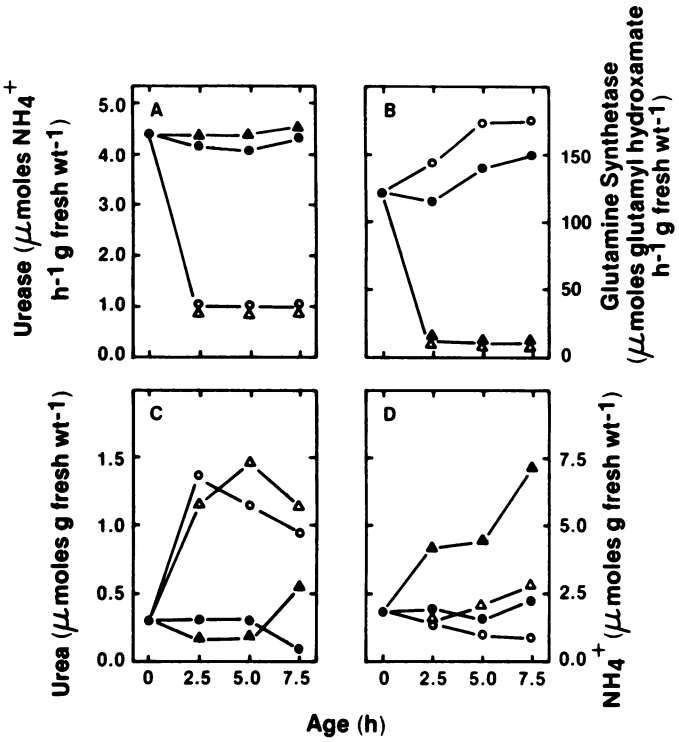

FIG. 6. Inactivation of urease by AHA and its effect on endogenous concentrations of urea and NH_4^+. 12U cells (3-day-old, about 3 g fresh wt) were inoculated into urea medium containing 100 μM AHA (○), 1.0 mM MSO (▲), AHA plus MSO (△), or none of those as a control (●). The levels of urease (A), GS (B), urea (C), and NH_4^+ (D) were determined every 2.5 h.

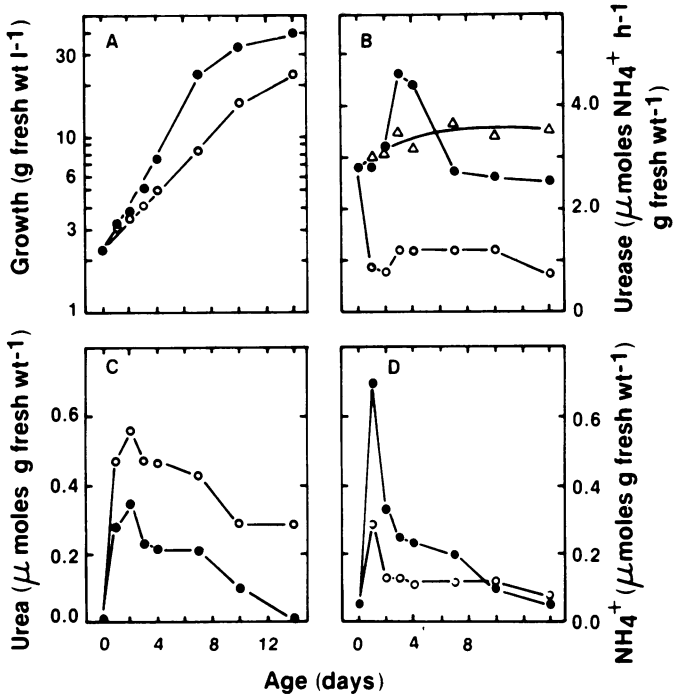

FIG. 7. Effect of AHA on the growth (A), overt and latent urease (B), internal concentration of urea (C), and internal concentration of NH_4^+ (D) of 12U cells grown on urea. 12U cells were grown on urea with (○) or without (●) 100 μM AHA for 2 weeks. The urease inactivated by AHA was reactivated by incubating at 30 C for 16 h (△) as described in legend of Figure 4.

a lag; it truly reduced the exponential growth rate constant.

The urease inactivated by AHA could be reactivated slowly if the inactive urease-AHA complex was separated from excess free AHA and incubated at 30 C (Fig. 4B). The inactive form of urease prepared at various times from the cells grown with AHA could be reactivated to about 3.5 μmol NH_4^+ h^{-1} g fresh $weight^{-1}$ (Fig. 7B). This is close to the activity in untreated controls, 2.9 to 4.6 μmol h^{-1} g fresh $weight^{-1}$, suggesting that AHA had little or no effect on formation of urease, at least over a period of a few days. Urease reactivated bv incubating at 30 C, and the residual urease after partial inactivation by AHA, had the same K_m for urea as urease not subjected to AHA inactivation (182 μM). No activation of urease was observed after passage through Sephadex G-25 and incubation, at 30 C, of extract prepared from 1U and 12U cells grown without AHA. This indicates that there is no naturally occurring latent form of urease comparable to the AHA-inactivated form of the enzyme in those cells.

Inhibition of [^{15}N]Urea Assimilation by AHA and MSO. In the experiments described above, we found that extracts of 12U cells exposed to 100 μM AHA contained reversibly inactivated urease. We also showed that GS in extracts of 12U cells exposed to 1 mM MSO is strongly inhibited. If the nitrogen of urea is assimilated via the action of urease to generate NH_4^+ and then incorporation of NH_4^+ into glutamine via the action of GS, then it should be possible to inhibit assimilation of [^{15}N]urea with AHA and MSO.

To test these predictions, the effects of 100 μM AHA and/or 1 mM MSO on incorporation of ^{15}N from [^{15}]urea into Kjeldahl nitrogen of 12U cells was determined at various times (Fig. 8). The time dependence of ^{15}N incorporation by the control cells was very close to that expected, based on the exponential rate constant of 12U cells, 0.332 day^{-1}, reaching 27.4 A% excess ^{15}N after 24 h, compared to 29.6 A% excess expected. Both AHA and MSO began to inhibit within the first 5 h, but inhibition became more pronounced after 5 h. The inhibition by AHA up to 10 h was fairly close to that expected from the exponential rate constant of 12U cells in the presence of 100 μM AHA, 0.186 day^{-1}. Beyond 5 h, the fraction of control incorporation obtained in the presence of the

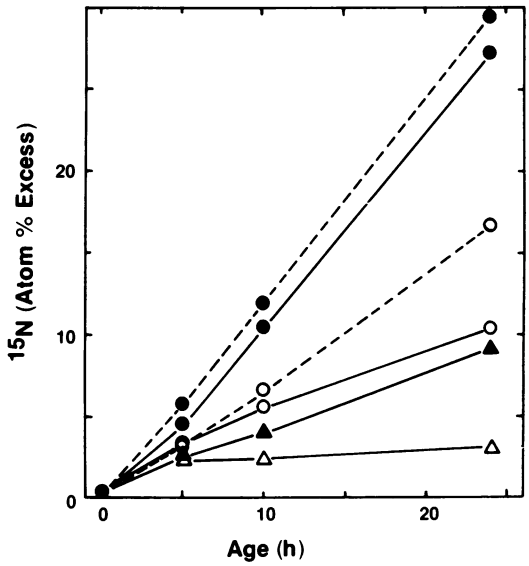

FIG. 8. Incorporation of ^{15}N into Kjeldahl nitrogen of 12U cells incubated with [^{15}N]urea in the presence of AHA and/or MSO. Four g fresh wt of 12U cells (3-day-old culture in early exponential phase) were inoculated into 100 ml of N-less MID plus 3 mM [^{15}N]urea (95 A%) either with no addition (●), with 100 μM AHA (○), with 1 mM MSO (▲), or with both inhibitors (△). Expected incorporation of ^{15}N into Kjeldahl nitrogen (-----) was estimated from the exponential growth rate constant as described in text.

combination of inhibitors was about one-third to one-fourth of the product of the fractions obtained when the inhibitors were used separately (Table IIA), which indicates that there was a synergism between the two inhibitors. That is, the observed inhibition from 5 to 24 h by the combination was substantially greater than expected if the two inhibitors acted entirely independently on two reactions in a sequence.

It is noteworthy that ^{15}N incorporation continued in the presence of sufficient MSO to inhibit GS more than 99% (Table IIB). This indicates that there is very likely a path of urea assimilation which does not go through the GS step. MSO alone caused NH_4^+ to rise 10-fold, which perhaps would enable a GDH with a high K_m for NH_4^+ to function. The combination of AHA and MSO did not cause such a large accumulation of NH_4^+, which could account for the synergistic inhibition by AHA plus MSO of ^{15}N incorporation, if poor affinity of GDH for NH_4^+ resulted in a much slower assimilation rate. Nitrogen/g fresh weight remained quite constant regardless of treatment, as expected if the inhibitors merely lowered the exponential growth rate.

Chromosome Counts. One conceivable way for a 12-fold increase in urease activity to occur is for the cells to acquire 12 times the normal number of copies of the chromosome bearing the urease gene, either as a consequence of a 12-fold rise in ploidy, or merely a 12-fold rise in somy of the particular chromosome. To evaluate these explanations, chromosome counts were done on metaphase figures of 1U and 12U cells of 3-day-old cultures (early exponential phase). Both 1U and 12U cells had very close to 48 chromosomes (49.0 ± 1.8 for 1U cells; 48.4 ± 1.3 for 12U cells), the diploid number for *Nicotiana tabacum* L. Although it is difficult to count tobacco chromosomes because they are small, an increase of 3 to 4 chromosomes could have been detected, had it occurred. It is clear from these results that the increase in urease activity is not associated with a 12-fold increase in ploidy or somy.

DISCUSSION

The finding of a 4-fold rise in urease level in XD cells during prolonged growth on urea (27) had raised the question: could the enzyme level rise still higher? The doubling time had dropped from 3.6 days to 3.0 days in conjunction with the 4-fold rise, apparently because the higher enzyme level gave 4U cells a selective advantage over 1U cells. However, because the XD cells can double in 2 davs on NO_3^- or NH_4^+, it seemed possible that a further increase in urease might further reduce the doubling time closer to the limit of 2 days and thereby convey a selective advantage over 4U cells. In the descendants of a 4U clone which had grown for an additional year on urea after cloning, we found a population of 12U cells, with a doubling time of 2.2 days on urea. Thus, the prediction was correct. These increases are equally evident on a per g fresh weight, or per mg protein basis.

Because 12U cells have a doubling time close to 2 days, further increases in urease would not be expected to convey a selective advantage over 12U cells for growth on urea, as long as the urease is functional. We have shown that AHA, known as a reversible inactivator of jack bean urease *in vitro* (3), affects tobacco cell urease similarly, both *in vitro* and *in vivo*. AHA inactivated more than 80% of urease within 2.5 h in both cases.

The evidence supporting the proposed action of AHA on tobacco cell urease, *in vivo* is: (a) it inactivates extracted urease during preincubation without urea; (b) urease inactivated by AHA *in vivo* or *in vitro* is slowly reactivated when extracted, separated from free AHA, and incubated at 30 C; (c) reactivated urease which had been inactivated *in vivo* has the same K_m for urea as the enzyme not subjected to inactivation; (d) the association constant, *i.e.* the ratio of rate constants determined for formation of urease-AHA complex and its dissociation, is in good agreement with the value reported for jack bean urease (3); (e) AHA caused urea to accumulate *in vivo*, and prevented NH_4^+ accumulation caused by MSO, which is what is expected if AHA inhibits the urease reaction and MSO inhibits the GS reaction; and (f) AHA inhibits incorporation of ^{15}N from [^{15}N]urea into Kjeldahl nitrogen of the cells, while urea accumulates to a higher level in cells treated with AHA, than in untreated controls. The inactive form of urease could be reactivated to pre-AHA levels and a high level of urea accumulated within the cells throughout the growth period on urea with AHA. These observations strongly suggest that AHA does not inhibit formation of urease protein or uptake of urea.

AHA had no inhibitory effect on GS activity from the tobacco cells. While there are many enzymes which are not inhibited by AHA at 1 mM (11), it should be kept in mind that hydroxamates form complexes with other metalloenzymes (25), and inhibit cyanide-resistant respiration (26). Also, tobacco cells have been se-

Table II. *Inhibition of [^{15}N]Urea Assimilation by AHA and/or MSO*

Part A shows their effects on urea assimilation and Part B on total-N content, urease and GS activities, and the endogenous concentrations of urea and NH_4^+. Experimental conditions were the same as in Figure 8.

A

Time Interval	Control ΔA%(a)[a]	AHA		MSO		AHA + MSO		AHA + MSO
		ΔA%(b)	$\frac{b}{a}$	ΔA%(c)	$\frac{c}{a}$	ΔA%(d)	$\frac{d}{a}$	$\frac{b}{a} \times \frac{c^b}{a}$
h								
0–5	4.716	3.375	0.716	2.592	0.55	2.387	0.506	0.39
5–10	5.700	2.168	0.38	1.376	0.241	0.114	0.02	0.092
10–24	16.950	4.822	0.284	5.317	0.314	0.628	0.037	0.089

B

Total-N	3.06	3.03	3.13	2.91 (mg-N/g fresh wt)
Urease	4.02	0.81	3.60	0.86 (μmol/h·g fresh wt)
GS	2.52	2.02	0.4	0.4 (μmol/h·g fresh wt)
Urea	0.33	0.76	0.43	0.95 (μmol/g fresh wt)
NH_4^+	1.93	1.50	14.00	3.84 (μmol/g fresh wt)

[a] ΔA% is the change in atom% ^{15}N in the time interval.

[b] The calculation is based on the assumption that AHA and MSO independently slow down two reactions in series, so that their combined effect on the flux through the path would be the product of their separate effects.

lected by Lawyer *et al.* (13) for resistance to glycine hydroxamate, which inhibits glycine decarboxylation (12). Urease and cyanide-resistant respiration share a lack of specificity for the R group to which the hydroxamate moiety is attached. Also, the urease reaction releases CO_2. If O_2 consumption were directly coupled to assimilation of the other product of the urease reaction, NH_4^+, the urease reaction would resemble cyanide-resistant respiration.

The urease activities of 1U and 12U cells have very similar properties. Urease from both 1U and 12U cells behaved in the same fashion when subjected to DEAE-cellulose column chromatography or gel filtration chromatography on Sepharose 4B (data not presented). The K_m for urea of 1U, 4U (27), and 12U cells is 182 μM. Fifty per cent inactivation by AHA of urease from either 1U or 12U cells occurred at 45 μM AHA, when the enzyme was exposed to AHA for 30 min at 30 C. We have shown further that although the sensitivities of urease from 1U and 12U cells to AHA are identical, growth of 1U cells is more easily inhibited by AHA than is that of 12U cells, which is consistent with the hypothesis that urease level is rate-limiting for growth of XD cells on urea nitrogen. Judging from the concentration dependence of inhibition of growth of 12U cells by AHA on urea *versus* nitrate, AHA is relatively specific for inhibition of urease up to 100 μM. In the presence of 100 μM AHA, the extractable overt urease activity is about 2.4U, and the doubling time is 3.2 days, which is close to that of 1U cells growing in the absence of AHA. Therefore, if 12U cells growing on urea in the presence of 100 μM AHA behave as a phenocopy of 1 to 2.4U cells, a further rise in urease to 115 to 144U is anticipated. The experiment to test this prediction is in progress.

The most intriguing question arising from the work reported here and previously on slow adaptive increase of urease is: What is the mechanism? Anderson and Roth (2) have reviewed cases of increases in enzyme activity in bacteria which are attributable to gene amplifications. Selections done in a chemostat on *Escherichia coli* for hypersynthesis of β-galactosidase yielded strains with up to 4 random copies of the lac operon, which occurred at a frequency of 10^{-3} to 10^{-4} per generation. Similar selections done on *Klebsiella aerogenes* for high ribitol dehydrogenase activity yielded strains with multiple copies of the ribitol dehydrogenase gene. Duplications of the glycinyl-tRNA synthetase gene in *E. coli* occur at a frequency of 7×10^{-5}, and at 8×10^{-5} in *Salmonella typhimurium*. It may be significant that these phenomena occur at frequencies similar to the 8×10^{-5} per generation estimated for the appearance of 4U cells in a 1U population of tobacco XD cells (27).

Two cases of large increases in enzyme activity of cultured animal cells have been found to be attributable to selection for gene amplification. These are dihydrofolate reductase gene amplification, selected on the basis of methotrexate resistance (1) and aspartate transcarbamylase (CAD protein) gene amplification selected on the basis of *N*-[phosphonoacetyl]aspartate resistance (28). The *N*-[phosphonoacetyl]aspartate resistant cells arise at frequencies up to 5×10^{-5}, and the amplified genes are localized at a specific enlarged locus in a particular chromosome (28). Methotrexate-resistant cells arise at high frequency (9) and the amplified genes occur in some lines, which are unstable, on extrachromosomal pieces of DNA (double minutes) (24), and in other more stable lines within enlarged chromosomes (16). The amount of DNA amplified in methotrexate-resistant cells is far greater than can be accounted for as dihydrofolate reductase genes. These cases of gene amplification, while somewhat selective and specific, have nevertheless produced cytologically detectable chromosomal alterations. Similar gross duplications and alterations also occur in bacteria selected for gene amplification (2). Insertion elements and transposons are probably widely involved in gene duplication/amplification. Fink and co-workers (5, 23) have recently shown in *Saccharomyces cerevisiae* that insertion elements are involved in high-frequency reversions of histidine auxotrophs via duplications and transpositions.

There is a growing list of cases in which increases in enzyme levels which occur at frequencies around 10^{-4} are the result of gene amplifications, probably mediated by insertion elements. We suspect that this is the most likely explanation of the heritable increases in urease which we have observed in tobacco XD cells.

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