

Nitrogen Metabolism in Soybean Tissue Culture

I. ASSIMILATION OF UREA

Received for publication October 30, 1975 and in revised form March 30, 1976

JOSEPH CARMINE POLACCO

Department of Genetics, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504

ABSTRACT

Cultured soybean (*Glycine max*, Kanrich variety) cells grow with 25 mM urea as the sole nitrogen source but at a slower rate than with the Murashige and Skoog (MS) (Physiol. Plant. 15: 473-497, 1962) nitrogen source of 18.8 mM KNO₃ and 20.6 mM NH₄NO₃. Growth with urea is restricted by 18.8 mM NO₃⁻, 50 mM methylammonia, 10 mM citrate or 100 μM hydroxyurea, substances which are much less restrictive or nonrestrictive in the presence of ammonia nitrogen source. The restrictive conditions of urea assimilation were examined as possible bases for selection schemes to recover urease-overproducing mutants. Since urease has higher methionine levels than the soybean seed proteins among which it is found, such selections may be a model for improving seed protein quality by plant cell culture techniques.

Callus will not grow with 1 mM urea plus 18.8 mM KNO₃. Urease levels decrease 80% within two divisions after transfer from MS nitrogen source to 1 mM urea plus 18.8 mM KNO₃. Hydroxyurea is a potent inhibitor of soybean urease and this appears to be the basis for its inhibition of urea utilization by callus cells.

Stationary phase suspension cultures grown with MS nitrogen source exhibit trace or zero urease levels. Soon after transfer to fresh medium (24 hours after escape from lag), urease levels increase in the presence of both MS or urea nitrogen source. However, the increase is 10 to 20 times greater in the presence of urea. NH₄Cl (50 mM) lowers urease induction by 50% whereas 50 mM methylammonium chloride results in more drastic reductions in urea-stimulated urease levels. Citrate (10 mM) completely blocks urease synthesis in the presence of urea.

Ammonia and methylammonia do not inhibit soybean urease nor do they appreciably inhibit urea uptake by suspension cultures. It appears likely that methylammonia inhibits urea utilization in cultured soybean cells primarily due to its "repressive" effect on urease synthesis.

Citrate does not inhibit urease activity *in vitro* and exhibits only a partial inhibition (0-50% in several experiments) of urea uptake. It appears likely that the citrate elimination of urease production by cultured soybean cells is due to its chelation of trace Ni²⁺ in the growth medium. Dixon *et al.* (J. Am. Chem. Soc. 97: 4131-4133, 1975) have reported that jack bean (*Canavalia ensiformis*) urease contains nickel at the active site.

Several investigators have demonstrated that cultured plant cells can use urea as a nitrogen source (3, 11). In the work presented here, the assimilation of urea by cultured soybean cells was studied in some detail. Conditions have also been devised whereby the assimilation of urea, but not that of ammonia, is inhibited. Such conditions may serve as selections for cell lines which overproduce urease. The high urease trait could be agronomically valuable in the intact plant for two reasons: (a) it could permit more efficient assimilation of urea fertilizer; and (b) it may be reflected in higher seed urease levels which would

result in improved nutritional quality since urease is richer in methionine (20) than soybean seed protein (19).

MATERIALS AND METHODS

Culture Media and Conditions. Edible soybeans (*Glycine max*, Kanrich variety, Burpee Seed Co., Warminster, Pa.) were surface-sterilized by shaking for 30 min in 20% Clorox, followed by a 95% ethanol wash and repeated washings with sterile distilled H₂O. They were then germinated on sterile (autoclaved) moist vermiculite in large Petri plates at 25 C. Callus was obtained from shoot tip or hypocotyl sections of 5- to 10-day-old etiolated seedlings.

Soybean cultures were induced and maintained on Murashige and Skoog (16) salts supplemented with 1 mg/l thiamine · HCl, 0.5 mg/l pyridoxine · HCl, 0.5 mg/l nicotinic acid, 100 mg/l myo-inositol, 30 g/l sucrose, 5 mg/l IAA, 0.5 mg/l 2,4-D, 0.3 mg/l 2-IP¹, and 8 g/l Difco agar (12). Agar was omitted from the shake culture medium. The pH was adjusted to 6 before autoclaving; sucrose was autoclaved separately. This is essentially the rice medium described by Chaleff and Carlson (5). To alter the nitrogen source, KNO₃ and NH₄NO₃ were replaced by filter-sterilized additions of urea, NH₄Cl, arginine, or ammonium citrate (NH₄OH adjusted to pH 6 with citric acid). Methylamine · HCl and urease inhibitors were filter-sterilized and added to autoclaved medium cooled to 45 C or below. Cultures were maintained at 25 C in the dark. Calluses were subcultured every 3 weeks, shake cultures every 7 to 14 days.

When utilization of a nitrogen source by callus was determined, they were maintained on that source for 6 weeks (two 3-week passes); this was necessary to deplete endogenous nitrogen reserves. Growth was determined by increase in fresh weight from the 6th to 8th week. Since the calluses were "preconditioned" to the respective media, changes in fresh weight to dry weight ratios brought about by changes in medium constitution do not introduce an error in comparing relative increases in callus weight.

A well dispersed suspension of shoot-tip-derived soybean cells was produced after filtering through a layer of cheesecloth every 3 or 4 days for a month. Growth was measured by turbidity using a Klett-Summerson colorimeter, by dry weight, or by measuring the settled volume of cells. Excellent correlation among these parameters was obtained. To determine dry weight, cells were collected on Miracloth (a nonwoven cellulose fiber mat, Chicopee Mills, Inc., New York) and dried 4 to 5 hr at 75 C to constant weight.

Callus growth rates were measured by weighing pooled calluses aseptically at intervals. As callus growth proceeded, calluses were divided. Between six to 12 calluses (25-50 mg) each

¹ Abbreviations: 2-IP: N⁶-(Δ²-isopentenyl) adenine; MS: Murashige and Skoog (16) salts; DMSO: dimethylsulfoxide.

were maintained per 10-cm plate. Transfers to fresh medium were made at each weighing.

Preparation of Enzyme Extracts. Cultured cells were pooled, washed with distilled H₂O, and collected by suction on Miracloth. One ml of extraction buffer was added for each g fresh weight of tissue. The mixture was ground in a chilled mortar with sand. After addition of another volume of buffer, the extracts were sonicated for 2 min, centrifuged for 15 min at 20,000g, and the supernatant dialyzed 3 hr with hourly buffer changes. To assay urease, plant tissue was extracted with 20 mM sodium phosphate, pH 7, containing 1 mM β -mercaptoethanol and 1 mM EDTA. In later experiments, the extraction buffer was 0.1 M tris maleate, pH 7, containing 1 mM β -mercaptoethanol (added just before use), and 1 mM EDTA. This buffer was used also to extract glutamate dehydrogenase and nitrate reductase activities.

Enzyme Assays. Urease activity was measured using a modification of the Sumner assay (22). EDTA (21) and β -mercaptoethanol (13) were added at 1 mM to both the extraction and assay buffers. Sumner's buffers were later substituted by 0.1 M tris maleate. The reaction medium contained 5 mM urea and was maintained at 30 C. Urea or enzyme was eliminated from blanks. At intervals, 0.5 volumes of 2 N H₂SO₄ were mixed with aliquots of the reaction mixture; urea and/or ammonia were determined after protein was removed by centrifugation. This last step was later eliminated when urea was measured.

Urea levels were measured by modifying the diacetylmonoxime method (17). One-tenth ml of H₂SO₄-treated reaction mixture was added to a mixture prepared just before use consisting of 2 ml 0.015% FeCl₃ in 4 N H₂SO₄ and 3 ml of a solution of 0.6% diacetylmonoxime and 0.03% thiosemicarbazide. After boiling for 10 min and rapidly cooling, absorbance was determined at 535 nm. Ammonia was determined by Nesslerization (4). The analytical techniques employed in these experiments give a ratio close to 2 for the number of mol of NH₄⁺ produced for each mol of urea hydrolyzed by soybean seed urease.

The glutamic dehydrogenase assay was a slight modification of the procedure described by Bayley *et al.* (2). The reaction mixture contained: 160 μ mol Tricine buffer, pH 8; 25 μ mol α -ketoglutaric acid (separately adjusted to pH 8 with KOH); 50 μ mol (NH₄)₂SO₄, and 0.25 μ mol NADH in a total volume of 2.5 ml maintained at 30 C. Blanks did not contain NADH. Change in absorbance at 340 nm was measured on a Gilford recording spectrophotometer. Activity was based on the NADH extinction coefficient of 6.22×10^6 (cm²/mol). All activities were corrected for endogenous α -keto glutarate-independent oxidation of NADH.

For the nitrate reductase assay (10), nitrite production was measured in a reaction mixture which contained: 40 μ mol K phosphate, pH 7.5, 15 μ mol KNO₃, and 1 μ mol of NADH (added as 0.2 ml of a 5 mM solution in 10 mM Tricine, pH 8) in a volume of 0.8 ml. Blanks lacked KNO₃. Enzyme (0.2 ml) was added to start the reaction. Reaction tubes were maintained at 30 C in a water bath. The reaction was stopped by addition of 0.2 ml 1 M zinc acetate, and after the addition of 3.8 ml of 95% ethanol, the precipitate was removed by centrifugation. Then, 0.5 ml each of 1% sulfanilamide in 25% (v/v) HCl and 0.02% naphthylene ethylenediamine in 38% ethanol were added, and absorbance was determined at 540 nm after 10 min.

Protein was determined by the Biuret method (9). Serial dilutions of extracts were precipitated with 4 volumes of 10% trichloroacetic acid. Precipitates were washed once with acetone, and dissolved in 1 ml of 6% NaOH in a boiling water bath. After cooling, 3 ml of a modified Biuret solution consisting of 0.2% CuSO₄, 0.8% NaK tartrate, and 2% NaOH were added and absorbance at 550 nm immediately read. BSA (Sigma Chemical Co., crystallized) served as standard.

Urease Inhibition Studies. Soybean seed urease was extracted

by grinding whole seeds in a Wiley Mill (Filter Mesh No. 20) and adding 6 ml cold extraction buffer to 1 g ground seed. The slurry was stirred in the cold for 1 hr, centrifuged for 10 min at 5,000g, and the supernatant liquid carefully separated from the lipid layer. After overnight dialysis, the extract was diluted 10-fold with extraction buffer. The extracts obtained hydrolyze 265 to 330 nmol urea/min·mg protein.

Seed extract was incubated for 5 min at 30 C in the presence of inhibitor added in 0.5 volumes of assay buffer. Urea (5 mM) and more inhibitor (to avoid dilution) were added, and either urea disappearance or NH₄⁺ production was measured depending on the inhibitor used. Dimethylurea, phenylurea, and hydroxyurea react with diacetylmonoxime reagent. None of these urea analogs was noticeably hydrolyzed under the assay conditions. Controls lacked inhibitor, urea, or both.

Urea Uptake Studies. Logarithmically growing cells were filtered through two layers of cheesecloth and the "fines" collected on Miracloth and washed well with nitrogen-free growth medium. They were suspended in this medium at a concentration of 5 to 10 mg/ml (dry weight) and shaken for 5 hr at 25 C.

To study uptake, 6 ml of cell suspension were stirred with a magnetic stirrer in a water-jacketed beaker maintained at 30 C with a circulating water bath. After 1 min preincubation with or without inhibitors, 6000 nmol urea was added (1190 cpm/nmol) in a 12- μ l volume. Half-ml aliquots of cells were collected at intervals on Millipore filter and washed with 1 ml of growth medium containing 25 mM unlabeled urea. Cells were scraped into scintillation vials to which were added 0.1 ml H₂O and 1 ml Nuclear-Chicago Solubilizer. After overnight incubation at 50 C, scintillation fluid was added and radioactivity determined in a Nuclear-Chicago Isocap/300 liquid scintillation counter.

RESULTS

UTILIZATION OF UREA BY CULTURED SOYBEAN CELLS

Growth Patterns. Increases in callus wet weight during the 6th to 8th week of exposure to urea as the sole nitrogen source were determined for different urea concentrations (Fig. 1). It is apparent that urea can serve as nitrogen source for cultured soybean cells. In all subsequent experiments, urea was provided at 25 mM since this appeared to be the optimum concentration. It is obvious from Figure 1 that 18.8 mM KNO₃ or 50 mM methylammonium chloride depresses growth with urea as nitrogen source, whereas the addition of 10 mM potassium citrate is completely

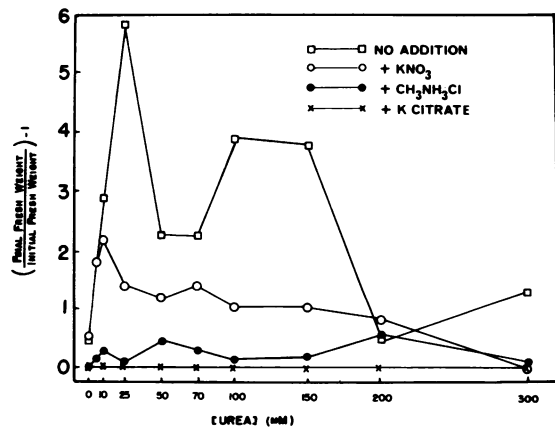


FIG. 1. Utilization of urea by soybean callus. Callus was maintained on different levels of urea for 6 weeks and the relative increases in fresh weight from the 6th to 8th week were determined. □—□, urea alone; ○—○, urea plus 18.8 mM KNO₃; ●—●, urea plus 50 mM methylammonium chloride; ×—×, urea plus 10 mM potassium citrate.

toxic. Soybean callus utilizing any other nitrogen source employed in this work is tolerant of 10 mM potassium citrate.

Hypocotyl-derived callus maintained on urea as nitrogen source for 5 months doubles in fresh weight every 15 days compared with a doubling time of 7.5 days for callus maintained on the MS (16) nitrogen source (Fig. 2). Shoot-tip-derived liquid suspension cultures utilize urea with a doubling time (based on dry weight) of 78 hr compared with 63 hr for cells utilizing MS nitrogen source (Fig. 2), *i.e.* 18.8 mM KNO_3 and 20.6 mM NH_4NO_3 .

The induction of urease was studied in batch cultures (Fig. 3). Within 46 hr after transfer from MS to urea nitrogen source and barely at the start of logarithmic growth, urease levels are maximally induced approximately 100-fold. The levels are half-maximum at 142 hr. Such a reduction could possibly be wholly explained by growth dilution of urease from the 46-hr maximum level.

Urea Tolerance. As shown in Figure 4, soybean callus is tolerant of urea added to MS medium at concentrations approaching 350 mM; indeed, 250 mM seems to be an optimal concentration in urea stimulation of growth. Similar results have been obtained with tobacco callus. These results seem to indicate a lack of urea hydrolysis in the presence of ammonia, since at high urea concentrations, extensive hydrolysis of urea would result in toxic levels (≥ 75 mM) of ammonia (Fig. 4) as well as a sharp inhibitory pH increase.

Inhibition of Urea Utilization by a Nontoxic Urease Inhibitor. Several compounds (*cf.* review by Reithel [18]) were assayed *in vitro* for their inhibitory effect on urease activity from soybean seed. As seen in Table IA, of several compounds which inhibit soybean seed urease *in vitro*, hydroxyurea is the most potent. This was employed at different concentrations to determine if it would selectively inhibit growth on a medium with urea nitrogen source. As seen in Table IB, 100 μM hydroxyurea, which is far below the level tolerated by callus grown on MS nitrogen salts, completely blocks growth with urea nitrogen source. This is strong circumstantial evidence that the urease induced in soybean callus is identical to that found in soybean seed. In results not shown here, the compounds of Table IA, at or near the

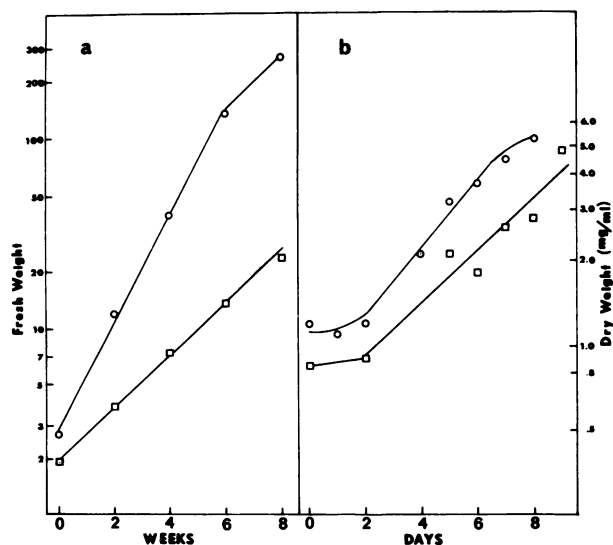


FIG. 2. a: Soybean callus growth (wet weight) with urea as nitrogen source. Hypocotyl-derived callus had been maintained on urea nitrogen source (\square — \square) 5 months before growth determination. \circ — \circ , growth with MS nitrogen salts. Initial combined weight of urea-grown callus was 183 mg, that of MS grown callus, 27 mg. b: Growth of soybean cell suspension with urea (\square — \square) or MS (\circ — \circ) nitrogen salts. Urea-grown suspension had been maintained 1 month before growth determinations.

indicated concentrations, were all toxic to soybean callus growing on MS nitrogen salts.

Methylammonia Inhibition of Urea Utilization. Methylammonia virtually prevents the adaptation of soybean callus cells to urea utilization (Fig. 5). It only partially depresses growth in the presence of MS nitrogen salts (Fig. 5), and has no effect on the growth of soybean callus on a nitrogen source of 20 mM ammonium citrate (Fig. 6). I have observed a similar phenomenon in tobacco callus: methylammonia inhibits growth on urea but not on MS nitrogen source. Methylammonia may block urea utilization for any of the following reasons: (a) it is a nonutilizable nitrogen compound which represses urease synthesis but not the synthesis of the key enzymes involved in ammonia assimilation; (b) it prevents the uptake of urea but not ammonia; (c) it specifically inhibits urease activity but not the enzymes involved in ammonia assimilation; (d) a more trivial reason is that methylammonia uptake, and therefore toxicity, is greater in the presence of urea nitrogen source because there is no ammonia present to inhibit uptake.

In considering the first possibility, soybean callus cannot use 50 mM methylammonia either alone (Fig. 1) or in the presence of 18.8 mM KNO_3 (unpublished data). In the experiments of Table II, soybean suspension cultures were grown for 10 days to stationary phase in a medium containing the MS nitrogen source. There is little or no urease activity in such cells. After the cells are transferred to fresh medium containing urea nitrogen source, there is a large increase in urease production upon escape from lag (1 to 2 days after transfer). The urease level in such cells is 20 times that found in cells transferred to the MS nitrogen source. This urea-stimulated urease production is reduced only 35% by the addition of 50 mM ammonium chloride. These results indicate that in late lag-early log phase, urease is induced approximately 20-fold by urea, and that the induction is partially repressed (about 35%) by ammonia. Matsumoto *et al.* (15) reported that ammonia represses urease in jack bean (*Canavalia ensiformis*) leaves. In cultured soybean cells, methylammonium chloride appears to be a more potent repressor of urease synthesis than ammonium chloride in that urea-induced urease levels are lowered 70% by methylammonia and only 35% by ammonia (Table II). The decreased amount of urease in the presence of methylammonia is not due to reduced "vitality," since cell growth was normal during the induction period. Furthermore, the levels of another nitrogen assimilatory enzyme, *i.e.* glutamic dehydrogenase, actually increased.

To test the second possibility, the effect of methylammonia and ammonia on urea uptake was examined. Cells grown on MS salts exhibit excellent urea uptake which is not inhibited by a 2-fold excess of ammonia or methylammonia (2 mM) (Fig. 7). Unexpectedly, it was found that in cells pregrown 5 days with urea nitrogen source, urea uptake is not increased nor is it more sensitive to inhibition by ammonia or methylammonia. Methylammonia inhibition of urea uptake cannot be responsible for the cessation of growth in a medium containing 50 mM methylammonia in addition to 25 mM urea.

To test the third possibility, the effect of methylammonia on urea degradation of soybean seed urease was examined (Fig. 8A). No inhibition by 10 mM methylammonium chloride was observed in two different buffer systems (0.35 M NaKPO_4 (22) or 0.1 M tris maleate, pH 7). Surprisingly, 10 mM NH_4Cl also failed to inhibit.

It cannot be argued that cells utilizing urea take up methylammonia much more efficiently thereby resulting in selective inhibition of urea-supported growth (the fourth possibility). The data of Figure 3 show that when methylammonia is added after urease is maximally induced, there is no inhibition of growth. Further, callus cells maintained with KNO_3 in the absence of NH_4^+ are not inhibited by methylammonia (unpublished results).

Citrate Inhibition of Urea Utilization. Citrate (10 mM potas-

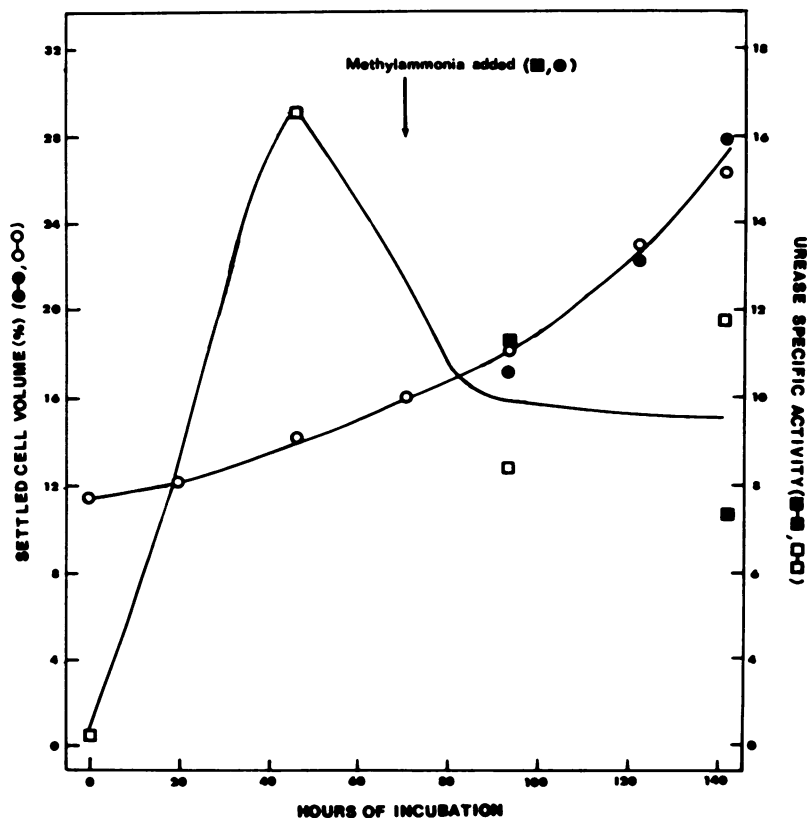


Fig. 3. Production of urease by soybean cells in suspension culture growing with urea as sole nitrogen source. Cells were transferred from MS nitrogen source at zero time and cultured in 300-ml flasks fitted with side arms. The complete contents of a flask (50 ml) were extracted for enzyme determination. Growth is expressed as the settled volume of cells in the side arm as a percent of total medium volume.

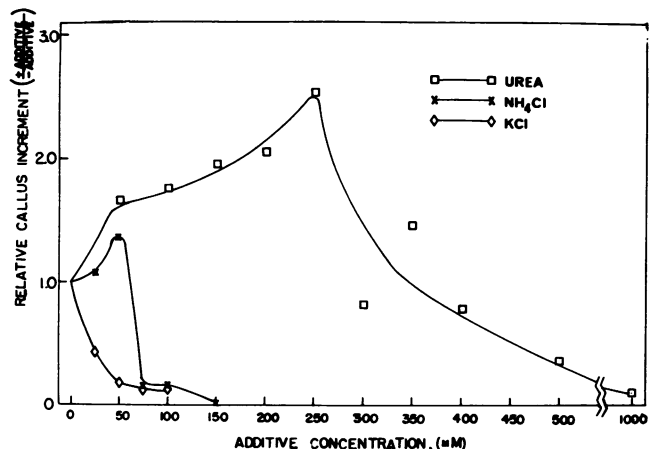


Fig. 4. Urea tolerance of soybean callus. Urea (□—□) at varying concentrations was added to normal (MS) solid growth medium, and growth increments after 2 weeks were divided by increments on nonsupplemented controls and expressed as a unitless ratio. Growth responses to added NH_4Cl (×—×) and KCl (◇—◇) were also determined.

sium salt) exerts a striking toxicity for soybean callus cells utilizing urea over a wide range of concentrations (Fig. 1). This severe inhibition seems due specifically to blocking of urea utilization since the addition of 1 mM NH_4Cl (Fig. 9) results in luxurious growth. The citrate inhibition of urea utilization is especially striking in view of the work of Gamborg and Shyluk (8), who showed that citrate and other Krebs' cycle intermediates enhance utilization of ammonia as sole nitrogen source.

As seen in Table II, cells leaving lag phase in the presence of 25 mM urea and 10 mM potassium citrate exhibit no detectable urease activity. The escape from lag is undoubtedly possible

Table I. Effect of Putative Urease Inhibitors on Soybean Seed Urease and Soybean Callus Growth

A		
Compound	Concentration	Seed Urease % Inhibition
Dimethylurea	5 mM	0
Phenylurea	10% saturated solution	0
Thiourea	5 mM	10
DMSO	5% (v/v)	30
D-Phenylalanine	5 mM	33
Hydroxyurea	5 mM	94

B		
Effect of hydroxyurea on callus growth ¹		
concentration	Urea nitrogen source	Callus Growth Murashige-Skoog salts nitrogen source
μM		
0	++	+++
10	++	+++
20	++	+++
50	++	+++
75	++	+++
100	+	+++
700	-	+++
1000	-	+++

¹ Shoot tip-derived calluses were maintained for 2 weeks on the various media. They were transferred to fresh media and scored visually after 2 more weeks.

because of utilization of nitrogen reserves accumulated in stationary phase. Such a drastic shutoff of urease synthesis cannot be completely explained by a presumptive citrate-inhibition of urea uptake since there is some urease synthesis in the absence of any added urea (in the presence of MS salts, Table II). In several experiments, cells grown with urea nitrogen source take up urea at equal initial rates in the presence or absence of citrate (results not shown here). However, the maximum total uptake is inhibited 50% in the presence of citrate. It appears that citrate does not sufficiently inhibit urea uptake to account for the severe

inhibition of urea-dependent growth. Sodium citrate only partially inhibits soybean seed urease *in vitro* (Fig. 8B). The high concentration (10 mM) of citrate necessary to inhibit urease is undoubtedly many times higher than the concentration to which *in vivo* urease is exposed. The most plausible explanation for the growth inhibitory role of citrate is that it chelates a divalent cation which is an important component in the induction (or structure) of urease or a urea permease. Dixon *et al.* (6) have recently reported that jack bean urease contains Ni²⁺ at the active site. Soybean and jack bean urease have identical amino acid compositions (20).

Nitrate Poisoning of Urea Utilization. Soybean cells in suspension cannot efficiently utilize nitrate unless a proper reduced nitrogen source is also present (1, 2, 7). When ammonia serves as the reduced nitrogen source, it is assimilated preferentially, its disappearance from the growth medium signaling appearance of

nitrate reductase activity to assimilate the quantities of nitrate that support maximal growth.

In agreement with these results reported in the literature, it was found that soybean callus grows poorly, if at all, with a nitrate nitrogen source; under these conditions, nitrate reductase

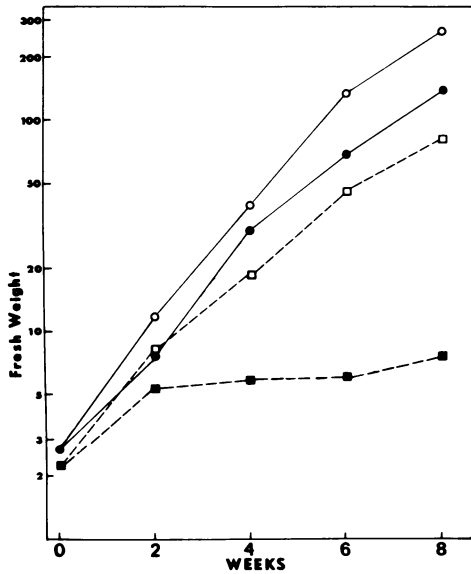


Fig. 5. Methylammonia inhibition of soybean callus adaptation to urea nitrogen source. Soybean callus maintained on MS nitrogen source was transferred to the same medium with (●—●) or without (○—○) 50 mM methylammonia or to medium with urea nitrogen source with (■—■) or without (□—□) supplementation with 50 mM methylammonia. Starting fresh weight of urea-grown callus was 220 mg, while that of MS-grown callus was 27 mg.

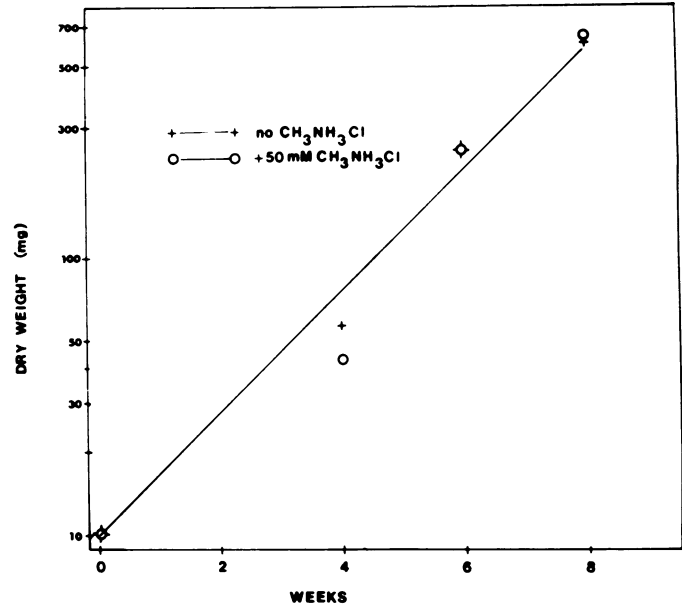


Fig. 6. Lack of inhibition by methylammonia (50 mM) on ammonium citrate (20 mM) utilization. Cells were maintained on 20 mM ammonium citrate nitrogen source for 6 weeks. At the start of the experiment, callus was transferred to medium with (○—○) or without (×—×) 50 mM methylammonium chloride. Dry weights were determined on defined portions of callus at each transfer.

Table II. Nitrogen Assimilation Enzyme Levels in Soybean Suspension Culture Upon Escape from Lag Phase of Growth

Nitrogen Source	% Increase in Cell Volume	Urease	Glutamic Dehydrogenase	Nitrate Reductase
			nmol/min·mg protein	
Murashige-Skoog salts	14	0.32	175	0.23
25 mM Urea	17	6.1	197	0
25 mM Urea + 50 mM methylammonium chloride	24	1.7	149	0
25 mM Urea + 50 mM ammonium chloride	9	4.0	158	trace
25 mM Urea + 10 mM potassium citrate	24	0	153	trace
Murashige-Skoog salts	0(zero time)	0	129	0.1

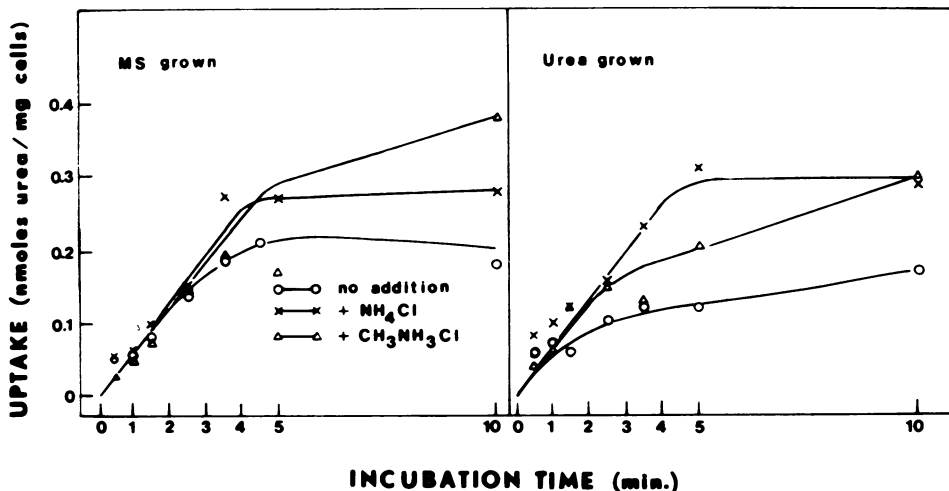


Fig. 7. Effect of ammonium chloride (2 mM) and methylammonium chloride (2 mM) on urea (1 mM) uptake by soybean suspension culture cells. Cells were exposed to ammonia and methylammonia for 1 min prior to addition of 1 mM urea (1190 cpm/nmol).

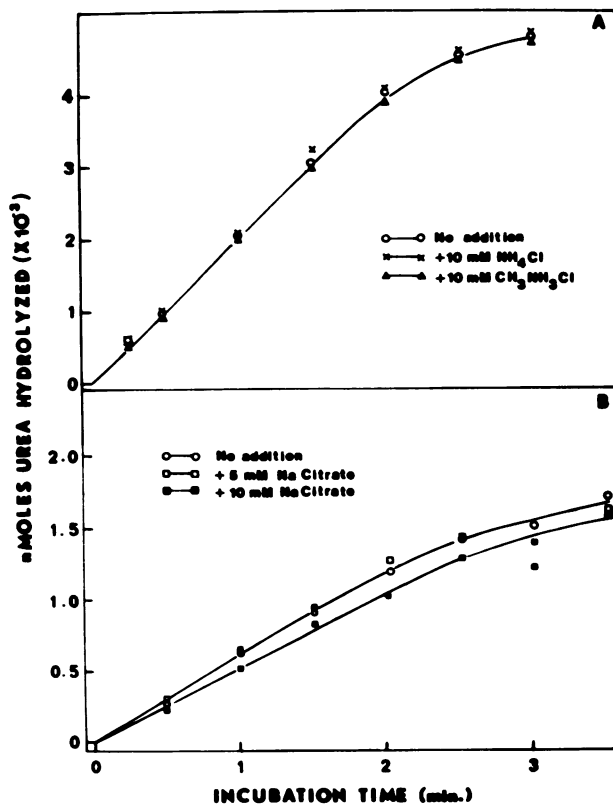


Fig. 8. Effect of inhibitors of urea utilization on soybean seed urease. A: Ammonium chloride and methylammonium chloride were incubated with seed extract for 5 min at 30 C before the addition of 5 mM urea. B: Inhibition by sodium citrate (pH 7) was determined as in A.

levels are basal (Fig. 10). In another experiment (Fig. 11), soybean callus growth was slight after 8 weeks on a medium with 18.8 mM potassium nitrate as sole nitrogen source. The addition of 2 mM ammonium chloride at the 2nd week enhances growth, whereas at 4 mM, the maximal growth rate is restored. The equivalent amounts of reduced nitrogen, added as 1 or 2 mM urea at the 2nd week, do not stimulate growth (Fig. 11). Gamburg (7) showed that 1 to 8 mM urea could not replace NH_4^+ as a reduced nitrogen source in the presence of 25 mM KNO_3 . In fact, as shown in Figure 1, the presence of 18.8 mM KNO_3 inhibits urea utilization at virtually all urea concentrations, hence the term "nitrate poisoning."

Nitrate may "repress" urease since I found that urease levels are reduced about 80% (from 2 units to less than 0.4) when callus is transferred from MS nitrogen source to a medium containing 18.8 mM KNO_3 as sole nitrogen source. Such a severe reduction in urease levels occurs within 2 weeks or 1.4 divisions, after transfer, and well before growth slows appreciably (growth still 80% of control). The addition of 1 mM urea to 18.8 mM KNO_3 causes no elevation in nitrate depressed urease levels.

DISCUSSION AND SUMMARY

Selection schemes for recovering from cultured soybean cells mutants which overproduce urease have been examined. There are several important features of this approach: (a) mutation in cell culture may be used to elevate levels of specific proteins localized in edible plant tissues (seed, tuber, etc.) to improve their nutritional value; (b) enhanced urea assimilation by the intact plant may make the application of urea fertilizers more productive; (c) mutants altered in the regulation of synthesis of urease or any other enzyme would be useful in elucidating genetic regulatory mechanisms in higher plants.

Mutants resistant to hydroxyurea may include the following

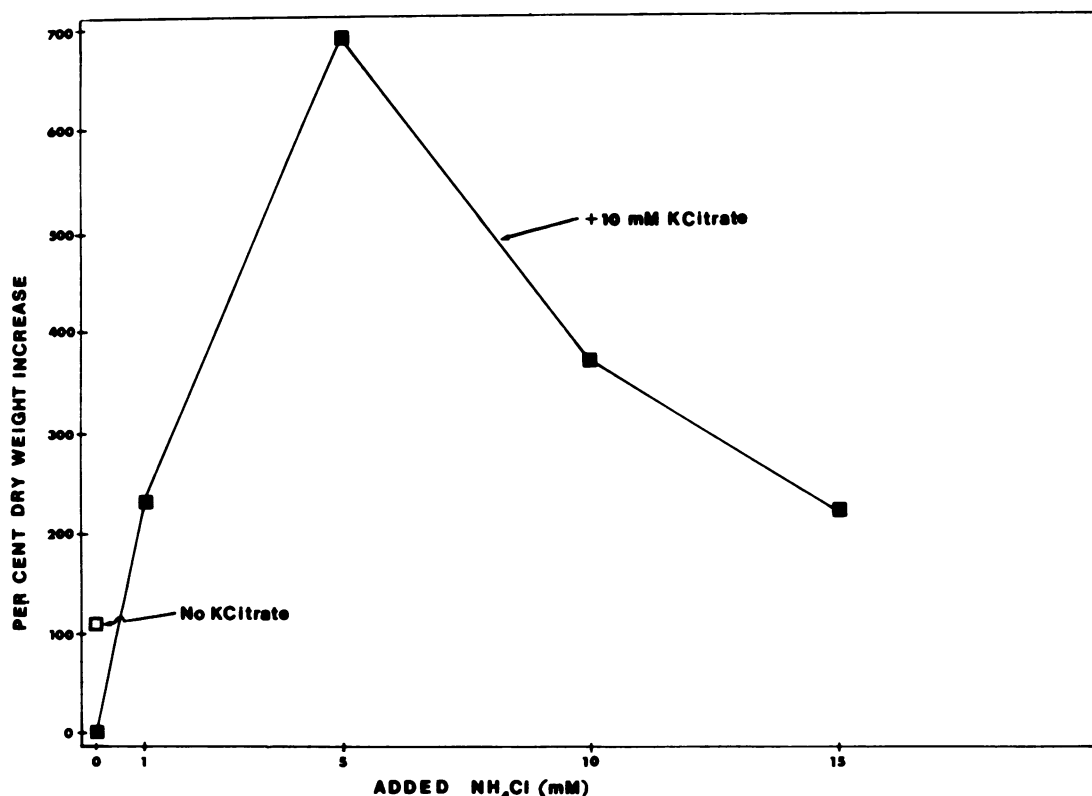


Fig. 9. Ammonia rescue of citrate inhibition of callus cells exposed to urea as sole nitrogen source. Soybean callus which had been maintained many months on urea nitrogen source was grown for 6 weeks on solid media containing 10 mM potassium citrate (pH 6) and varying concentrations of ammonium chloride (■—■) in addition to the urea (25 mM) nitrogen source. The increase in dry weight from the 6th to 8th week was recorded. Cells maintained with 25 mM urea alone (□) underwent one division in the same 2-week interval.

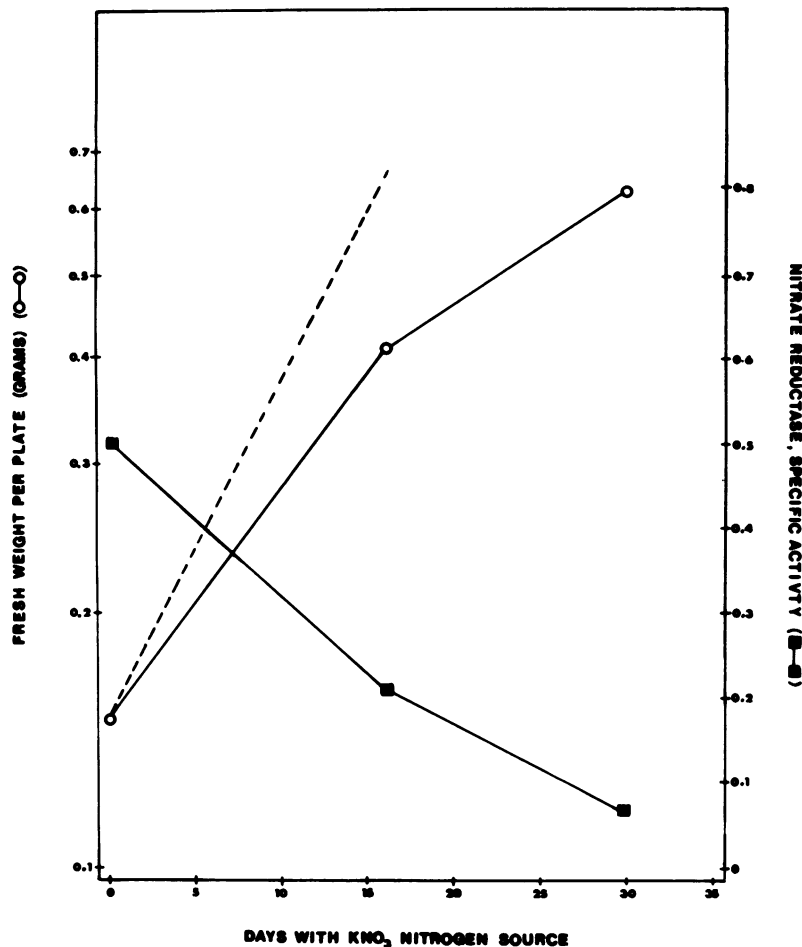


FIG. 10. Nitrate reductase production (■—■) and callus growth (O—O) upon shift in nitrogen source from MS (18.8 mM KNO₃ and 20.6 mM NH₄NO₃) to 18.8 mM KNO₃. Growth rate on MS salts is represented by dotted line. MS nitrate reductase levels (zero time value) generally vary from 0.5 to 1.3 units (nmol nitrite produced from nitrate/min · mg protein in crude extracts).

two classes: (a) those with high urease levels which effectively compete with hydroxyurea; and (b) those which produce an altered urease that retains its catalytic activity for urea hydrolysis while no longer recognizing hydroxyurea. This class could serve as a genetic marker for the urease structural gene.

Mutants which could efficiently assimilate urea in the presence of methylammonia would likely hydrolyze urea at higher rates than wild type. Since methylammonia does not inhibit urease, "urea hydrolysis" mutants would be expected to exhibit higher urease levels rather than an altered urease activity.

Similar arguments may be put forward for mutants able to assimilate urea in the presence of citrate. If citrate somehow represses urease or a urea permease, such mutants would be expected to have high constitutive levels of one of the proteins. Urease is only slightly sensitive *in vitro* to citrate. In the unlikely event that citrate acts primarily by inhibiting urea uptake, mutants would either exhibit a citrate-insensitive urea uptake or extremely active hydrolysis of available intracellular urea, *i.e.* superinducible or constitutive urease.

Citrate is an excellent chelator of divalent cations (14). Since Dixon *et al.* (6) have recently demonstrated that jack bean urease contains nickel at the active site, it appears likely that citrate is chelating or sequestering trace nickel from urease apoenzyme. The MS salts do not contain added nickel (16). Given that sequestering of nickel is the mode of citrate inhibition of urea-supported growth, it is conceivable that citrate-resistant mutants would include those which overproduce urease. Such a

class would produce sufficient urease apoenzyme to compete effectively with citrate for available nickel.

Mutants are now being sought which can grow efficiently with a nitrogen source of 1 mM urea plus 18.8 mM KNO₃. Such a selection could yield two valuable mutant types: (a) nitrate reductase constitutive mutants, *i.e.* those which produce nitrate reductase without the prior metabolism of a reduced nitrogen source, and (b) urease-overproducing mutants whose high levels of urease efficiently convert urea to NH₄⁺ and thereby overcome nitrate poisoning. Mutants have been isolated which appear to fall in the first category. These will be described in a separate report.

The long range aim of this work is to effect changes in seed protein composition by mutant selection in tissue culture. The possibility of eliciting such tissue-specific changes via tissue culture has yet to be demonstrated. The selective techniques described here are based on alteration of metabolic control mechanisms. Advances in the study of plant developmental processes may suggest biochemical selections in tissue culture which recover mutations resulting in beneficial tissue-specific alterations in the intact plant.

Note Added in Proof. Nickel sulfate at 10 μM completely overcomes the citrate toxicity observed on urea nitrogen source. This observation indicates that soybean urease, as well as that of jack bean, contains nickel. It also strongly suggests that cultured soybean cells cannot hydrolyze urea by virtue of an enzyme different from urease.

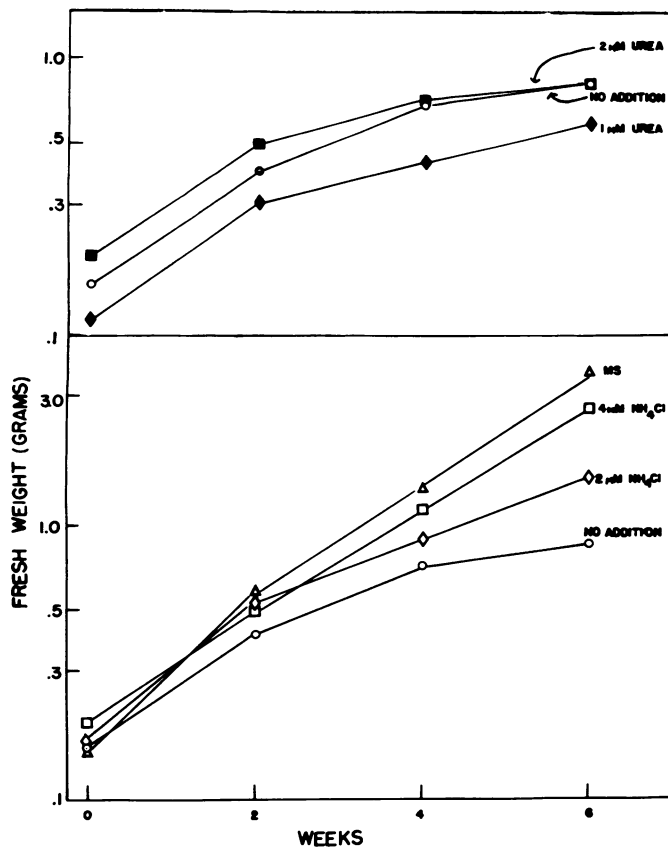


FIG. 11. Urea and NH_4Cl as reduced nitrogen sources for soybean callus. Soybean callus was maintained 2 weeks on a medium containing only 18.8 mM KNO_3 as nitrogen source. At zero time, the additions shown were made. Transfers and weighings were simultaneously made at 2-week intervals. The nonsupplemented controls (containing KNO_3 alone) plotted in each family of curves represent the same experiment.

Acknowledgements—It is a pleasure to acknowledge the expert technical assistance of J. Colacino, C. Forte, and D. Sattelberger-Sligar. The author thanks S. Anagnostakis, P. Day, D. Sands, and I. Zelitch for critical reading of the manuscript.

LITERATURE CITED

- BAYLEY, J. M., J. KING, AND O. L. LAMBORG. 1972. The ability of amino compounds and conditioned medium to alleviate the reduced nitrogen requirement of soybean cells grown in suspension cultures. *Planta* 105: 25-32.
- BAYLEY, J. M., J. KING, AND O. L. LAMBORG. 1972. The effect of the source of inorganic nitrogen on growth and enzymes of nitrogen assimilation in soybean and wheat cells in suspension cultures. *Planta* 105: 15-24.
- BEHREND, J. AND R. I. MATELES. 1975. Nitrogen metabolism in plant cell suspension cultures. I. Effect of amino acids on growth. *Plant Physiol.* 56: 584-589.
- BERNT, E. AND H. U. BERGMAYER. 1963. In: H. U. Bergmeyer ed., *Methods of Enzymatic Analysis*. Academic Press, New York. p. 401.
- CHALEFF, R. S. AND P. S. CARLSON. 1975. Higher plants as experimental microorganisms. In: R. Markham, D. R. Davies, D. A. Hopwood and R. W. Horne eds., *Modification of the Information Content of Plant Cells*. American Elsevier Publishing Company, New York. pp. 197-214.
- DIXON, N. E., C. GAZZOLA, R. L. BLAKELEY, AND B. ZERNER. 1975. Jack bean urease (EC 3.5.1.5). A Metalloenzyme. A simple biological role for nickel? *J. Am. Chem. Soc.* 97: 4131-4133.
- GAMBORG, O. L. 1970. The effects of amino acids and ammonium on the growth of plant cells in suspension culture. *Plant Physiol.* 45: 372-375.
- GAMBORG, O. L. AND J. P. SHYLUK. 1970. The culture of plant cells with ammonium salts as the sole nitrogen source. *Plant Physiol.* 45: 598-600.
- GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the Biuret reaction. *J. Biol. Chem.* 177: 751-766.
- HAGEMAN, R. H. AND D. FLESHER. 1970. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. *Plant Physiol.* 35: 700-708.
- HEIMER, Y. M. AND P. FILNER. 1970. Regulation of the nitrate assimilation pathway of cultured tobacco cells. *Biochim. Biophys. Acta* 215: 152-165.
- LINSMAIER, E. M. AND F. SKOOG. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant* 18: 100-127.
- MAMIYA, G. AND G. GORIN. 1965. Urease. V. Some observations on the procedure for its isolation. *Biochim. Biophys. Acta* 105: 382-385.
- MARTELL, A. E. AND M. CALVIN. 1952. *Chemistry of the Metal Chelate Compounds*. Prentice-Hall, New York.
- MATSUMOTO, H., Y. HASEGAWA, M. KOBAYASHI, AND E. TAKAHASHI. 1968. Inducible formation of urease in *Canavalia ensiformis*. *Physiol. Plant.* 21: 872-881.
- MURASHIGE, T. AND F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- ORMSBY, A. A. 1943. A direct colorimetric method for the determination of urea in blood and urine. *J. Biol. Chem.* 146: 595-604.
- REITHEL, F. J. 1971. Ureases. In: P. D. Boyer ed., *The Enzymes*. Academic Press, New York. pp. 1-21.
- ROBERTS, L. M. 1970. In: *The Food Legumes, Recommendations for Expansion and Acceleration of Research to Increase Production of Certain of These High Protein Crops*. The Rockefeller Foundation, New York. p. 64.
- ROTHERY, W. G. 1962. Investigations on plant ureases. Ph.D. thesis. University of Liverpool.
- SEHGAL, P. O. AND A. W. NAYLOR. 1966. Purification and properties of urease derived from hydrated seeds of jack bean, *Canavalia ensiformis* (L7 DC). *Plant Physiol.* 41: 567-572.
- SUMNER, J. B. 1951. Ureases. In: J. B. Sumner and K. Myrbach, eds., *The Enzymes*, Vol. I, Part 2. Academic Press, New York. pp. 873-892.