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UREASE ASSAY AND AMMONIA RELEASE FROM LEAF TISSUES

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Key Word Index—*Glycine max*; Fabaceae; soybean; *Triticum aestivum*; Poaceae; wheat; urease assay *in vivo*; distribution; permeability; ammonia; urea.

Abstract—The rate of release of ammonia from leaf tissues of *Glycine max* and *Triticum aestivum* suspended in urea solutions was relatively low for initial periods of up to 40 min, due to retention of ammonia by the tissues. Urease, EC 3.5.1.5, could be assayed by the subsequent rate of ammonia release. The method was applied to leaves of 29 other species.

INTRODUCTION

The release of ammonia from urea, catalysed by urease, provides substrate derived from certain intermediates in the nitrogen metabolism of many plants to the basic enzyme systems which assimilate inorganic nitrogen [1]. Arginine, allantoin and allantoic acid are the main metabolites so involved [2]. Leaf urease activity is also relevant when foliar application of urea as a nitrogen fertilizer is under consideration. Thus while the enzyme may assist efficient nitrogen assimilation, ammonia toxicity [3] could result in cases where activity is high.

Thomas and Schrader, in a recent review of ureide metabolism in higher plants [4], concluded that additional information is required on the assimilation of these compounds, particularly with respect to legume seed production. Although the exact role of the photosynthetic apparatus in ureide metabolism is still obscure and requires further investigation [4], it has been shown [5] that light-dependent assimilation of ammonia readily takes place in isolated chloroplasts.

We have investigated the release of ammonia from leaf tissues *in vivo* and results reported in this paper indicate that suspensions of intact leaf tissues may be used in a convenient assay for urease activity in plants where assimilation of urea nitrogen is of interest.

RESULTS AND DISCUSSION

It has sometimes been found preferable to incubate the intact plant tissue rather than an extract with substrate, in order to assay enzyme activity [6,7]. Nitrate reductase has been assayed frequently by *in vivo* methods, and Jaworski [8] found that the rate of release of nitrite from nitrate by soybean leaf tissue was much greater in the presence of certain organic solvents. He advocated the use of solutions containing *n*-propanol (5%).

Intact tissue suspension (ITS) assay of leaf urease activity

When we incubated leaf tissues from soybean and wheat, respectively, in urea solutions the rate of release of ammonia from the suspension was increased in the presence of certain organic solvents (Fig. 1). The presence of 5% *n*-propanol in the incubation solution led to the most effective enhancement of ammonia release from soybean and wheat leaves. *iso*-Propanol had smaller effects. Siegel and Halpern [9] found that *n*-propanol increased the permeability of beetroot tissue, whereas, *iso*-propanol did not. The possible formation of ammonia from urea by microbial contaminants seemed to be ruled out. No micro-organisms grew on nutrient agar plates inoculated with samples taken after the incubations from preparations which contained either *n*-propanol or *iso*-propanol.

A toluene treatment, which was used recently to induce leakage of low MW solutes from sorghum leaf tissues [10], led to no enhancement of urease activity in wheat leaf tissue (Fig. 1b). The values obtained (μmol ammonia/g fr. wt · hr) were 2.53 and 2.57 for the toluene-treated and the control (no treatment) tissues, respectively. This treatment, however, may have caused a reduction in the initial relatively low rate of ammonia release. Pretreatment of peach leaves with acetone doubled the urease activity [11] but no effects were detected when we applied that treatment to wheat leaves.

There was a marked initial lag phase in the rate of ammonia release in all cases, so that initial readings could not be used for activity estimations. However, more detailed examination of the lag phase together with other investigations, reported below, indicated that a reliable urease assay could be based on the subsequent rate of ammonia release. It was necessary to follow ammonia release for a considerable time and establish the extent of the lag phase for a particular type of sample, then estimate activity from later readings. Details are given in the Experimental. Activities determined from data in Fig. 1 are presented in Table 1.

The rate of release of ammonia (μmol/g fr. wt · hr) in control samples incubated without urea was taken into account in the ITS assays. It was relatively low, varying with the source of leaves, e.g. wheat seedlings, 1.08 ± 0.37; soybean seedlings, 1.10 ± 0.49; mature soybean plants, 0.192 ± 0.078. No ammonia was released from urea during incubation of control samples which had been heated at 100° for 15 min. $V_{max}/2$ occurred at 3.3 and 7.7 mM for wheat and soybean leaf tissues, respectively. However,

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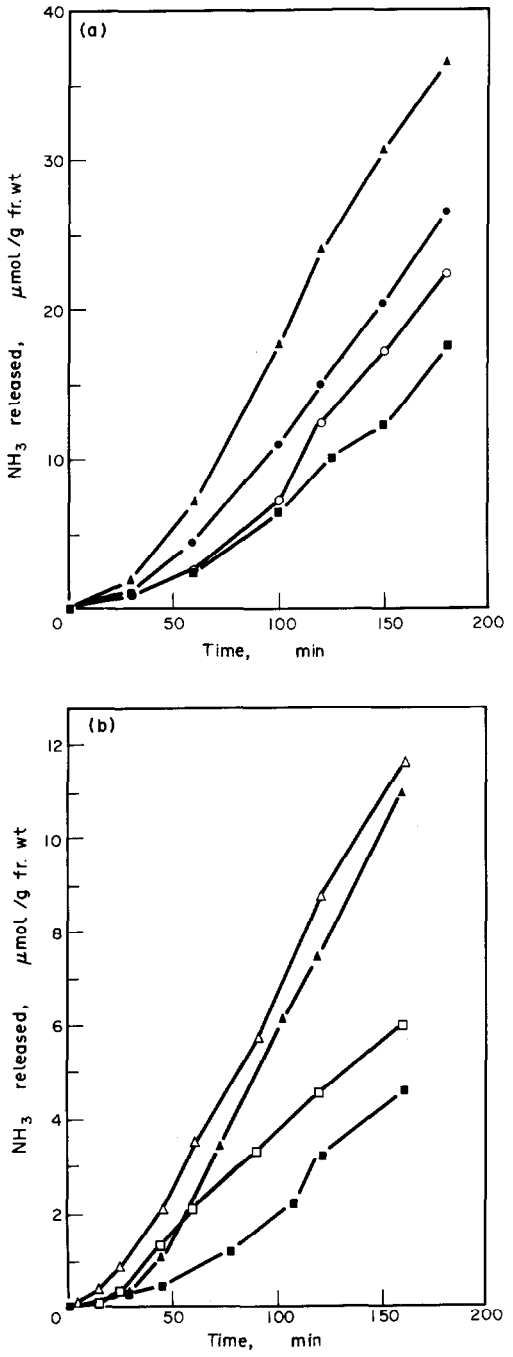

Fig. 1. Ammonia released from leaf tissues of (a) soybean and (b) wheat suspended at 30° in urea solutions (210 mM in KPi buffer, 0.1 M, pH 7.5) which contained no solvent (■); 5% ethanol (○), *iso*-propanol (●), *n*-propanol (▲); 1% toluene (□); 1% toluene plus 5% *n*-propanol (△).

since no inhibition was detected with urea concentrations up to 1 M, a high urea concentration (210 mM) was used in the assay routine in order to expedite diffusion of the substrate into tissues during the prolonged incubations. Acetohydroxamic acid, an inhibitor specific for urease [12] completely inhibited soybean leaf activity at a 10 mM concentration but 1 mM had no detectable effect.

Table 1. Effects of alcohols (5%) or toluene (1%) on urease activity of leaf tissues

Treatment	Activity (μmol NH_3/g fr. wt·hr)	
	Soybean	Wheat
No alcohol	7.40	2.57
Ethanol	10.2	—
iso-Propanol	11.1	—
n-Propanol	14.9	5.29
Toluene	—	2.53

Plots of ammonia released against time were always linear when cell-free extracts of either soybean or wheat leaves were incubated in urea solution. However, the reaction rates found in conventional assays with cell-free extracts (CFE) were 40–70% of those given by ITS assays; e.g. duplicate assays of leaf samples from wheat seedlings gave 7.03 and 2.87 μmol/g fr. wt·hr for the ITS and CFE methods respectively, while duplicate assays of soybean were 5.44 and 3.69 μmol/g fr. wt·hr, respectively, for lower leaves from a mature plant in the field. Other workers have found nitrate reductase activity to be higher in intact leaf tissue than in extracts of wheat [13] and soybean [8].

The initial lag in release of ammonia from intact leaf tissues

The initial lag was apparently species specific in the following cases where samples had been assayed on 15 or more occasions [figures in parentheses give the mean value (min) of the intercept with the abscissa of the extrapolated line of best fit to the later points of the plots of ammonia release/time]: *Pisum sativum* (15.1), *Glycine max* (21.3), *Triticum aestivum* (31.6). The lag time for material from *Casuarina littoralis* was *ca* 110 min. However, in this genus leaf sizes are greatly reduced and the bulk of the samples consisted of the narrow terminal stems wherein practically all photosynthesis must occur.

The observed lag in ammonia release could possibly be related to the heterogeneous nature of the ITS system. Surfactants have been found to eliminate an initial lag in nitrite release during *in vivo* assays of nitrite reductase [14, 16], but we found no such effect on ammonia release with either Triton X-100 (0.5% v/v) or Tween 80 (0.5% v/v). Vacuum infiltration also had no effect. The length of the lag phase did not change when either wheat or soybean leaf tissue was treated with *n*-propanol before addition of urea, so the lag was also not due to a changed permeability which was relatively slow to develop.

When some samples of wheat leaves were removed during the course of a routine ITS assay, washed and again incubated with fresh substrate solution, no lag was detected during the second incubation (Fig. 2). The results of several such experiments could be explained by substrate induction. Substrate induction of urease has been reported in detached leaves of *Canavalia ensiformis* [15] and *Malus pumila* [17]. We found no evidence for this when wheat (Table 2) or soybean leaves (results not shown) were incubated in urea solutions for different times, washed and then assayed by the CFE method.

Two other interesting observations were made during these experiments. (1) Rapid ammonia formation took place when extracts from tissue, which had been incubated

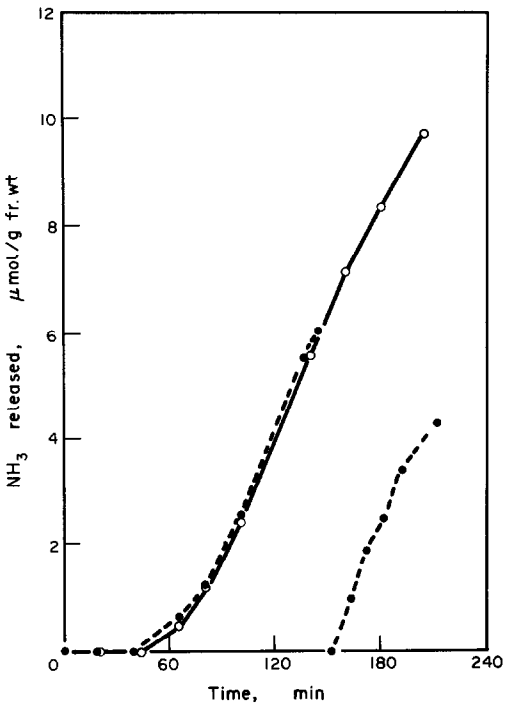

Fig. 2. Ammonia released into urea solutions (210 mM in KPi buffer, 0.1 M, pH 7.5, *n*-propanol 5%) from wheat leaf tissue during incubation (30°), which was either continuous (○), or interrupted at 145 min, after which the tissue was washed and resuspended in fresh solution (●).

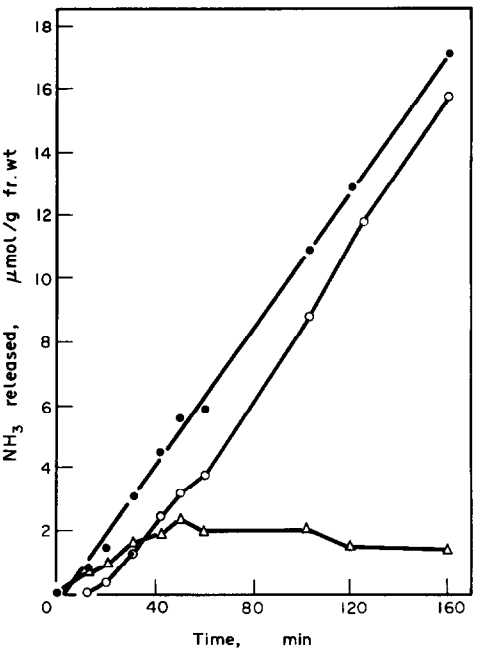

Fig. 3. Partition of ammonia between wheat leaf tissue and the substrate solution. A series of samples were incubated according to the ITS procedure: some were removed at various times, washed and ammonia determined in extracts prepared from them (△); samples were also analysed for ammonia in solution (○). Calculated values of total ammonia (●) are given together with the line of best fit.

Table 2. Urease activity (CFE method) in tissue extracts from first leaves of wheat following prior incubation of intact leaves for various times in buffer (KPi 0.1 M, pH 7.5, *n*-propanol 5%) either with urea (210 mM) or without urea

	Activity (μmol NH_3/g fr. wt·hr)		
Treatment	30 min	60 min	120 min
With urea	2.56	2.38	2.46
Without urea	3.10	2.52	2.63

Activity with no prior incubation = 3.61 μmol NH_3/g fr. wt·hr.

with urea and thoroughly washed before CFE preparation, were incubated in urea-free buffer. Indeed, in several instances the urea apparently retained was adequate for maximum activity. (2) The ammonia content of the tissues, which had been incubated for 60 min in the urea solution and subsequently washed thoroughly, was *ca* four times higher than its initial value. These observations indicated that there had been retention, despite washing, of both urea, which had entered cells during the pre-incubation, and ammonia formed from it. This led us to examine the variation of tissue ammonia during the ITS assay procedure.

The released ammonia and that retained in the tissues were both measured in a series of samples of wheat leaves which had been incubated for different times (Fig. 3). The calculated total yield of ammonia (Fig. 3) was directly proportional to the time of incubation ($r = 0.998$). Thus the early lag in ammonia release was not due to a lower rate of formation at that stage but rather to it being retained in the tissue. The experiment was repeated, with closely similar results. Activity measured from data in Fig. 3 by the ITS procedure was 7.09 μmol/g fr. wt·hr, being calculated from readings taken later than 40 min from the start of incubation. This was reasonably close to the value obtained from the graph of total ammonia formed (6.72 μmol/g fr. wt·hr).

There was no indication that the ammonia formed in the urease assay was subsequently removed by further enzyme action. The glutamine synthetase activity of *T. aestivum* cv Timgalen leaves has been evaluated [18]. There was *ca* 5 μmol γ-glutamylhydroxamate formed/mg leaf protein·min and the K_m with respect to ammonia was 0.04 mM. However, it seems possible that the concentrations of the other substrates for glutamine synthetase were not adequate for detectable amounts of ammonia to be removed by that enzyme. The K_m values for the partially purified enzyme were 5.28 and 0.53 mM for glutamate and ATP, respectively [18].

Urease activity in the leaves of different species

Table 3 summarizes the results of a limited survey. Leaves were selected which were close to the fully-expanded stage. Activity was found in all species examined, except for the two Eucalypts, *E. citriodora* and *E. racemosa*. These were also the only cases where solutions became dark brown during assay so that any urease

Table 3. Urease activity (μmol NH_3/g fr. wt · hr) (ITS assay) in leaves of different species

0.10–0.59	0.60–2.4	2.5–20
Callistemon quinquenervia	*Beta vulgaris* var *cicla*	*Arachis hypogea*
Citrus sinensis	*Brassica oleracea*	*Casuarina littoralis*
Grevillea robusta	*Citrus limon*	*Chloris gayana*
Lactuca sativa	*Fragaria vesca*	*Crinum pedunculatum*
Lycopersicon esculentum	*Grevillea banksiae*	*Cucumis sativa*
Petroselenum crispum	*Melaleuca viminalis*	*Glycine max*
Spinacia oleracea	*Stellaria media*	*Musa sapientum*
Tradescantia albiflora		*Phalaris aquatica*
Tropaeolum majus		*Phaseolus vulgaris*
Vicia faba		*Pisum sativum*
		Triticum aestivum

present may have been inactivated by polyphenol breakdown products.

Bollard [17] and Reinbothe and Mothes [2] discuss urea utilization by urease-free plants but it seems likely that such plants are less common than has been supposed. We found, using the ITS assay, activity in *C. sativa* (8 μmol ammonia/g fr. wt · hr) and *L. esculentum* (0.24 μmol ammonia/g fr. wt · hr), plants which had been reported as urease-free by Bollard [17] but not by Dilley and Walker [11]. Freiberg and Payne [19] demonstrated foliar absorption of urea by banana plants. They detected no urease activity in the leaves and it was suggested that assimilation of the nitrogen was made possible by transport of urea to the vegetative growing points where urease activity had been found. Again, we found urease activity in several samples of banana leaves by the ITS assay (3–6 μmol ammonia/g fr. wt · hr).

Very high urease activity, (*ca* 100 μmol ammonia/g fr. wt · hr), has been reported in *Canavalia ensiformis* leaves [15, 20]. No material from this plant was available, but leaves of *C. rosea* were found to have similar activity (113 ± 15 μmol ammonia/g fr. wt · hr). Samples of three cultivars of *G. max*, grown in the field, had the following activities (μmol/g ammonia fr. wt · hr): Forrest, 18.2 ± 2.5; Lee, 12.7 ± 1.2; Ruse, 20.9 ± 1.0. However, high activities were also found in leaves of non-nodulated plants from the greenhouse, e.g. Lee, 21.6. The relatively high activity in *C. littoralis* samples (Table 3) is of interest since these plants are also able to form an association with nitrogen-fixing symbionts. The five monocot species assayed were also allocated to the group with highest activity.

EXPERIMENTAL

Plant material. Young plants of *A. hypogea*, *C. rosea*, *C. pedunculatum*, *G. max*, *P. aquatica*, *P. sativum* cv Massey Gem, *S. oleracea* Hybrid 102 and *T. aestivum* cv Timgalen were grown in a greenhouse. Other samples were taken from the field.

Assay of urease activity. ITS method. Leaf tissue (usually 0.20 g) was cut into pieces only small enough for complete submersion in 8 ml buffer A (KPi, 0.1 M, pH 7.5; *n*-propanol, 5% v/v) containing urea (210 mM). The mixture was incubated at 30° in a shaking water-bath. 0.2 ml samples of soln, taken at intervals from this suspension and from a second one without urea (buffer A), were used for NH_3 estimation by the indophenol method [21]. For large batches of samples, the Roche Centrifichem spectrophotometer was used. A computer–plotter assembly was programmed to give, initially, a plot of μmol NH_3 formed from urea/g fr. wt against sample time. Vol. changes and NH_3 removal, brought about by sampling, were taken into account. From inspection of the graph, a decision was made on the extent of the lag phase and the reaction rate (μmol NH_3 from urea/g fr. wt · hr) was computed from the line of best fit to the later points by the method of least squares.

CFE method. Leaf tissue of soybean (0.6 g) or wheat (1.0 g) was ground with sand and 4 ml buffer A and the mixture was centrifuged for 10 min at 10000 *g* at 4°. The rate of NH_3 formation was found when 0.5 ml of the supernatant was incubated with 2 ml buffer A containing urea (260 ,mM).

NH_3 content of leaf tissues. Extracts were made in the manner described for the CFE assay method. Interference from pigmentation was corrected for by subtracting the *A* observed when the two reagents were mixed at least 30 min prior to the addition of one portion of extract, from the *A* measured after the indophenol reaction [21] had been completed with a second portion. The indophenol reaction did not occur with the first portion.

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