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Title: UREASE INDUCTION IN BARLEY LEAVES BY FOLIAR APPLICATION OF UREA

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Foliar application of urea on 2-3 month-old plants increased in vivo and in vitro urease activity significantly for 5 hours and reached a peak of 20-fold at 2 hours. The increase may be attributed to the induction of urease by the absorbed urea in leaves. To discern the mode of the enzyme induction by urea, biochemical studies were conducted on urea sprayed leaves and leaf sections incubated in urea using polyacrylamide gel electrophoresis (PAGE), ^{35}S-methionine incorporation, fluorography and specific inhibitors for RNA and protein synthesis.

Five isozymes, U_a, U_b, U_1, U_2 and U_3 , of barley leaf urease were found by PAGE. U_a and U_b might be precursors, polymers or less anionic complexes since they occurred only at the peak of induced urease activity. U_1 and U_2 appeared 0.5 hour after urea induction, peaked at 2 hours and existed only in urea treated leaves indicating their induced origin. U_3 was the constitutive preexisting urease

since control leaves had this one only and it also existed in treated leaves.

Protein synthetic ability as shown by ^{35}S-methionine incorporation in urea treated leaves was higher than that of control leaves for 5 hours indicating the effect of urea enhanced leaf growth. The U_1 was increased in quantity with the induction time by fluorography suggesting that U_1 might be the truly induced isozyme.

The inhibitors of RNA (cordycepin) or protein (cycloheximide) synthesis not only reduced both total and specific activity of urease to the control leaves but also nullified the existence of isozymes, U_a, U_b, U_1 and U_2 indicating the mechanism of urease induction appeared to be at transcriptional as well as translational level. An small activation of preexisting urease by urea was also observed in urea sprayed leaves.

UREASE INDUCTION IN BARLEY LEAVES BY FOLIAR APPLICATION OF UREA

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INTRODUCTION

Foliar application of fertilizer, during later stage of crop growth and development become more popular in recent years (13). It is because that the supply of nutrients through older roots in hot and dry weather is generally not adequate to support the rapid growth of crops. This type of supplementary application of N, P and K through foliages has been known to be successful in increasing crop yield in farming practices (19, 27). It is reported that the seed protein of 'Scio' barley (Hordeum, vulgare L.) was increased by 40% through foliar application of urea-ammonium-nitrate(UAN) after anthesis (45). Foliar spray of urea alone also elevated seed protein content in wheat (15). In crop production, urea is an ideal nitrogen fertilizer for foliar application because it contains a high percentage of nitrogen and can be applied at a higher concentration without phytotoxicity (22, 45). In addition, urea uptake and metabolism are rapid and they are quickly assimilated to increase leaf growth and seed protein following several applications (1, 22, 45). The mechanism of these increases is attributed mainly to the stimulation of nitrogen assimilating enzymes by the applied substrates. Nitrate reductase, urease, glutamine synthetase

and glutamate synthetase are all stimulated within hours after UAN spray (45). The induction of nitrate reductase in plants by nitrate has been well documented (2, 36). The induction of urease by urea was studied in jack bean (29) and lichens(47-50). It is known in lichens that the urease induction is at transcriptional as well as at translational levels. The induction of urease in barley leaves has been indicated after foliar application of UAN, but not verified (45). It is, therefore, important to discern the mode of the enzyme induction by urea so that the mechanism of enzyme induction by its substrates can be further explored at each step of its molecular events of synthesis. Thus this study was instigated.

REVIEW OF THE LITERATURE

I. HISTORY AND OCCURRENCE OF UREASE

The enzyme urease (E.C.3.5.1.5., urea amidohydrolase) was first detected by Musculus in 1876 and the enzyme which catalyzes the hydrolysis of urea to ammonia and carbon dioxide was named urease in 1889 by Kuhne (1). Marshall(1913) first used urease in urea determination of human urine that stimulated considerable research interest concerning urea and urease. Urease was first crystallized from jack bean by Sumner in 1926. It is interest to note that urease, the first enzyme crystallized, has its substrate urea being the first organic compounds isolated by Rouelle in 1773 and also the first organic compounds to be synthesized (4). Almost fifty years after Summer's report, urease was identified to be the first known nickel metalloenzyme (1).

Urease occurs in bacteria, yeast, fungi, and in a large number of higher plants both in dicotyledonous and monocotyledonous (4). The first plant which has been identified to contain urease in 1909 was the soybean (Takeuchi) (44), but the richest and now conventional source of the enzyme is the jack bean(Canavalia ensiformis L.). Since both urea and urease occur widely in the plant kingdom,there have been numerous studies of this enzyme,

mostly in legume plants including the molecular characteristics, the physical and chemical properties, the functions, the structure, the isozymes, the kinetics properties of the enzyme and the regulation of enzyme synthesis and activity such as enzyme induction by its substrate (1, 4). But little research of urease was conducted on cereal plants.

II. THE FUNCTION OF UREASE

Urease is an unique hydrolase acting on nonpeptide C-N bonds in linear amides:

$$NH_2CONH_2 + H_2O \text{ ------> } CO_2 + 2\ NH^+_4$$

The existence of urease generally indicates the presence of its substrate, urea, in the plants. Three additional synthetic substrates have been identified: hydroxyurea (Fishbein, et al.., 1965) (1), dihydroxyurea(Fishbein, 1969) (17) and semicarbozide (Gazzota, et al., 1973) (1), although all are far less efficient than the natural substrate, urea. Urea occurs in various plants as metabolic products from canavanine or arginine via the action of arginase, from the degradation of purines via uric acid ,allantoin and allantoic acid(4), and it also could be from exogenous sources such as absorbed from soil fertilizer or from foliar application of urea. Canavanine, $H_2NC(NH)NHOCH_2CH_2CH(NH_2)COOH$, a complex metabolic amino

acid, has been found only in legumes(38), and arginine, a protein amino acid is ubiquitous in plants. Most of the ureido-N in plant is in labile ureides and recently two of these, allantoin and allantoic acid have been shown to be translocated from N_2-fixing nodules to leaves. These ureides may arise through the oxidation of xanthanine, a central product of purine degradation. A series of reactions may then occur through uric acid, allantoin and allantoic acid, subsequently producing urea which could be broken down by urease to ammonia and be re-incorporated into amino acid and then proteins. Therefore, urease plays a significant role in assimilating and recycling nitrogen compounds for the growth and development of plants. Carbon dioxide, the other product of urease activity, could be assimilated to carbohydrates via the process of photosynthesis and then be converted to other C-compounds .

As urease composes 1% of the total protein in jack bean, it is rich in methione, and it is broken down during germination, it has been proposed that it may be a storage protein(4). But the function of high concentration of urease in jack bean seed is still not clear. It may play a role in the catabolism of protein reserves during seedling growth, or that it may function in the assimilation of urea derived from ureides transported from the root nodules to the developing seed (14). If the methionine content of soybean urease is similar to that of jack bean, breeding for high seed urease level may lead to increased seed

methionine levels, as legume seed proteins are deficient in methionine for human and monogastric animal nutrition (42).

III. THE CHARACTERISTICS OF UREASE

Urease is an enzymatic protein which contains nickle strongly bound to the active site, and nickle is needed to synthesize the enzyme molecule. The molecular weight of native urease from jack bean is 483,000 (43). Compared to most enzymes, this is a very high value and indeed the molecule is sufficiently large to be seen in the electron microscope. The units of half this molecular weight still retain enzymatic activity and these units apparently consist of three inactive subunits of about 76,000 (3). The oxidation-reduction conditions have significant effects on the enzyme behavior. Polymeric forms exist depending on the state of the disulfate bonds since jack bean urease is reversibly inhibited by sulphydryl group reagents and SH groups are therefore presumed to be involved in the active center (39). Apart from polymerization, urease isozymes may differ in conformation and amino acid sequence since different sources of jack bean urease exhibited varied electrophoretic mobility (16). The amino acid composition of the purified jack bean enzyme has been established by

Taylor(1969). The N-terminus of the chain is methionine and C-terminal sequence is -Tyr-Leu-Phe-COOH.

Urease from soybean seeds and leaves, other plants and some lower plants have been well described (3, 17, 33, 34, 35, 49). However ,little is known about the characteristics of urease in leaves especially in cereal plant leaves. Kerr et al (21) reported that urease is the primary urea metabolizing enzyme present in soybean leaves as soybean nodules form ureides for transporting nitrogen form to shoot. The most direct route for ureide utilization include the degradation of ureide-derived urea to ammonia and carbon dioxide. Ureolytic activity was found in leaf disks of soybean and exhibited optimal activity at pH7 in the presence of a high concentration of urea(250mM) (21). Soybean leaf urease differs from that of seed in pH optimal, apparent Km value, inhibition by hydroxyurea, electrophoretic mobility and cross-reactivity with soybean seed urease antibodies. The in vitro pH optimum of soybean seed urease was very broad and ranged from 5.0 to 9.7. However, soybean leaf urease activity had two sharp pH optima at pH 5.25 and 8.75. Soybean seed and leaf urease exhibited different responses when assayed in the presence of hydroxyurea and phenylphosphordiamidate. Both enzyme were inhibited 100% by phenylphosphordiamidate, but only the seed urease was inhibited by hydroxyurea indicating that seed urease was more specific to its substrate and hydroxyurea could be a competitive inhibitor as its similar

structure to urea. The in vitro soybean leaf urease had a substantially lower Km for urea(0.85mM) than soybean seed urease (200 to 150mM). The leaf urease was antigenically distinct since leaf extracts were not precipitated by antibodies to soybean seed urease. The electrophoretic mobility in 7.5% polyacrylamide gels indicated that the leaf urease migrates at a faster rate than soybean seed urease, suggesting a difference in charge and/or size between the leaf and seed urease. Davies et al (1984) studied the urease from the leaves of soybean and corn (12). They found that the principal activities of leaf urease at pH 5.5 and 8.8-9 catalysts the same reaction stoichiometery as did urease purified from jack bean seed. Polacco et al (33, 34, 35) did extensive studies on urease in leaves and developing seed of soybean. They found that urease synthesis occurs throughout the normal protein biosynthetic phase of the developing seed. The differential synthesis of urease (the synthesis of urease versus the synthesis of the bulk storage proteins) was greatest during the early stage of development. The development of urease activity, however , lagged behind the synthesis of urease protein during this stage, or the urease peptide synthesized was not the active urease. During the later stage of development, urease specific antigen levels declined whereas urease specific activity increased, or active urease was processed at the later stage. Urease specific activity was at its highest during the late

development and in the mature bean, whereas urease specific antigen or synthesis of inactive urease peptide was at its highest during mid development. The activity:antigen ratios of urease increase throughout the development indicating that the enzyme might have a role in late seed development such as amidation of storage protein. Alternatively, its maximal total activity at maturation could signify a nitrogen assimilatory role in seed germination. In addition to synthesis and possible physiological roles, urease appears to be in a more highly polymerized state in mature beans verus beans in early development. Furthermore, they studied the structure and possible ureide degrading function of the urease of soybean and proposed two forms of urease, seed specific urease and ubiquitous urease. The later urease form has been found in leaves and in suspension cultures as well as in developing seed of a variety(Itachi) which lacks the seed specific urease. The ubiquitous soybean urease, as opposed to the seed specific urease, designates the seemingly identical ureolytic activities of suspension cultures and leaves. The fact that the urease of leaf and cell culture are more closely related to each other than they are to the abundant seed urease suggests that these are identical or closely related tissue-specific isozymes. This second form of urease may have an assimilatory role since it is found in both leaf and seed tissues and is required for urea assimilation or nitrogen assimilation in cell culture and leaves. The

characteristics of urease in barley leaves after foliar application of urea was not yet studied though the importance of urease for nitrogen assimilation in leaves and protein synthesis in seeds has been demonstrated (45).

VI. INDUCTION OF UREASE

It is known that urease may be induced in higher plants including Spirodela oligorrhiza (5), jack bean (29), rice (Oryza sativa) (28), and potato (Solanum tuberosum) (30). Normally urea is the inducer and in the case of Spirodela oligorrhiza, allantoin is also an inducer. In jack bean plants where addition of urea induced urease synthesis in the leaves, serological and tracer evidence showed that the enzyme was synthesized de novo and that ammonia controlled urease activity both by end product inhibition of the activity and by repression of its synthesis. In Spirodela ammonia appeared to exert no control on its activity or synthesis (29).

The urease induction in lower plant lichen has also been extensively studied by Vicente et al (47-50) and by Legar and Brown (25). It is well established that urease activity of lichens was induced when they were incubated in urea. Urease may also be induced in response to endogenous urea production. The induction of enzyme activity was repressed by including 40 uM cyclohexmide in the medium.

They found that when thalli were incubated in urea , the enzyme activity first increased as a function of time and later decreased, after reaching a maximum about 5 hours of incubation. The presence of actinomycin D(Act. D) in the culture media nullified the urease activity for all the times. This result indicated that the induction of urease is at the transcriptional level and could be stopped by the inhibitor of total RNA synthesis (Act. D). Further they found that the presence of actinomycin D (inhibitor for RNA synthesis), cyclohexmide or chloramphenicol (inhibitors for protein synthesis) in the culture medium nullified the enzyme activity for all the experimental time of 5 hours. The induction of this enzyme requires, therefore, newly transcribed RNAs and newly translated proteins. In lichens, urease is either a constitutive, preexistent enzyme in the symbiotic system containing a blue-green alga as phycobiont or an inducible protein which was synthesized in response to exogenous urea.

In higher plants especially in non legume plants, leaf urease activity is important when foliar application of urea as a nitrogen fertilizer is under consideration. Little work has been done on the regulation of cereal leaf urease activity in presence of urea. Matsumoto et al.(28) found that urease activity in green parts (leaf and stem) of urea cultured rice was always higher than that of ammonia cultured rice. The activity often showed maximum level at two hour induction and then decreased. This

increase in activity was inhibited by various protein biosynthesis inhibitors (8-Azaguanine and Ethionine) indicating that the induction of urease in response to substrate requires de novo synthesis rather than activation of any existing proteins or proenzymes(28). Turley and Ching (1986) reported that in barley leaves the endogenous in vivo urease activity increased 18-fold after foliar application of urea-ammonium-nitrate(UNA) within 4 hours (45). The increased activity of urease after UAN spray was apparently attributed to the elevated in situ substrate concentration. The increased activity of the urease by substrate could be a result of enhanced transcription of the enzyme's mRNA, stimulated translation of the enzyme protein, and a protection of the enzyme from inactivation by the presence of added substrate as in the case of nitrate reductase (20), or a combination of these possibilities. Therefore this study was instigated to discern the mode of the increase of urease activity in barley leaves by foliar application of urea.

MATERIAL AND METHODS

I. MATERIAL

`Scio' barley (Hordeum vulgare L) growing in greenhouse was used throughout this experiment. Barley seeds were planted in the greenhouse at $26^{o}/20^{o}C$ day/night temperature condition and with 450 uE M^{-2} S^{-1} supplemental lights in plastic pots containing a soil mixture of clay loam, peat, sand and pumice (1:1:1:2 by volume). Four to five seeds were planted in each pot, and 50 ml Hoagland's solution was applied weekly to each pot to provide the needed nutrients for plant growth. The plants used for the experiments were about two to three months old and prior to anthesis.

All the chemicals were reagent grade and purchased from Sigma, Bio-Rad, Kodak, and New England Nuclear.

II. METHODS

Experiment I: The effect of foliar urea spray of plants on leaf urease

A. Foliar spay of urea

Eight to twelve weeks old plants were divided randomly into control and treated groups. Using a hand atomizer the

treatment group was sprayed 5% urea in 0.1% Triton X-100 on the leaves at 13 mg N/gFW of leaves (approximately 8 ml solution per 15 g fresh leaves from plants in two pots) . Spray was conducted at 8:00 AM on each date of the experiment. The solution was sprayed to both sides of leaves. The control group was sprayed with the 0.1% Triton X-100 using the same method and at the same time.

B. In vivo assay of urease activity

In vivo urease activity refers to the assay of enzyme activity in leaf sections. Approximately 2.5 g of leaf samples from comparable position of the control and treated plants were taken at 0, 1, 2, 3, 4, and 5 hours after spray for assay in the laboratory. Leaves were kept on ice for transporting to the laboratory, then were washed thoroughly and surface dried. Four replicated samples of 0.2 gm leaf sections(3-5 mm in length) were incubated in 5 ml phosphate buffer consisted of 0.1 M K_2HPO_4 and 5% (V/V) n-propanol, pH 7.5, with or without 0.2M urea. Incubation time was 0 and 30 minutes at 30^{o}C in a water bath with rapid shaking, after which a 0.5 ml incubation medium was assayed for ammonia produced using Hogan's method (18). One ml color reagent containing 0.125 M phenol,and 0.25 mM $Na_2Fe(CN)_5NO$ was added , and then one ml of color developer containing 0.5 M NaOH and 0.8%(v/v) chlorox was added to the incubated mixture and mixed well. The color was developed at 37^{o}C bath for 20 min and the absorbance at 560 nm was read

against a reagent blank. In the meantime, a urea standard solution(107 nmol/0.5 ml) was incubated with 0.5 unit of jack bean urease for 10 min and the product was determined accordingly. The ammonia produced as urease activity was calculated as following:

NH^+_4produced in nmol/reaction mixture(RM)

=[A(unknown)/A(standard)] x 107 nmol

Total urease activity(nmol NH^+_4 produced min^{-1} g^{-1}FW)

= NH^+_4 produced/RM x(5/0.5)x(1/0.2)x(1/30)

C. In vitro assay of urease activity

In vitro enzyme activity usually is conducted on cell free extract. Cell free extract was prepared by grinding 1.0 gm of above leaf samples in 12ml 50mM tris-HCl buffer containing 1 mM EDTA and 10 mM mecaptoethenol, pH 7.5 with a polytron for 40 seconds at a setting of 4. The slurry was centrifuged at 20kg for 10 minutes at 4^oC. The clear supernatant(13 ml) was used as cell free extract for in vitro assay of urease activity in the leaves. Four replication of 0.25 ml extract was incubated with 0.25 ml of 0.1 M K_2HPO_4 buffer(pH 7.5) containing 0.2 M urea as substrate for 0, 10 and 20 minutes at 30^oC. After each time the ammonia produced was measured using Hogan's method(section B).

In vitro activity(nmol NH^+_4 produced min^{-1} g^{-1}FW)
=[NH^+_4 produced/RM-endogenous NH^+_4(0 time)]
x(13/0.25)x(1/10 or 20)

D. Determination of ammonia and urea content in leaves

The 0 time results of in vitro urease assay is considered as the leaf ammonia content. For measuring total urea content in the leaves, two replication of 0.2 ml above cell free extract was incubated with 0.5 unit of jack bean seed urease (in 0.1% EDTA,pH6.5) for 15 minutes at 37°C. After incubation the ammonia content of each sample was measured using Hogan's method (section B). The in situ total urea content was calculated from the resulting ammonia quantity minus leaf ammonia content.

Urea content(nmol NH^+_4 produced g^{-1}FW)
=[NH^+_4 produced/RM-endogenous NH^+_4 content]x(13/0.2)x(1/2)

E. Induction of urease by urea with or without inhibitors

a. ^{35}S-methionine incorporation for quantifying newly synthesized proteins

Foliar spray of urea with or without inhibitor of RNA and protein synthesis was conducted in the greenhouse as mentioned above. The specific protein or RNA synthesis

inhibitor, 500nmol/ml cordycepin or 20ug/ml cycloheximide was added to urea (5% in 0.1%Triton X-100) solution for foliar spray. After treatment for 0, 0.5, 1, 2, 3, and 4 hours the leaf samples were taken from greenhouse on ice. Two replications of 0.5 gm leaf sections were incubated in 2 ml of tracer solution containing 20uCi/ml 35S-methionine ,10uM ATP, 10uM ADP and 10mM DTT in K2HPO4 buffer, pH7.4. After incubation for 2 hours at 24^{o}C with 200 uE M^{-2} S^{-1} light the leaf sections were extracted in Tris buffer and partially purified (detail in following section E, b of experiment I). An aliquot of the partially purified urease extract was counted in a liquid scintillation counter(Backman LS7000) and the total radioisotope incorporation was calculated as dpm/gFW(gram,fresh wight). The in vitro urease activity was assayed in partially purified extract.

b. Partial purification method for urease extraction

Because urease is a very small component of the soluble protein of barley leaves, a direct detection of its activity bends on PAGE gel of cell free extract was difficult especially in control leaves. Thus a partial purification procedure was developed. All procedures were conducted at 0 to 5^{o}C except as noted. The partially purified enzyme extract was used for PAGE of protein bends from which urease isozymes were identified by specific activity stain. The stained gel was further processed to

produce a permanent fluorograph for the quantification of radioactive protein bends. Two replications of 0.5 gm leaf section were ground in 5 ml grinding buffer containing 0.1 M Tris-HCl,1 mM EDTA, 10 mM mecaptoethanol, 1.5mM phenolmethyl-sulfonyl fluoride and 0.25 TIU/ml(trypsin inhibitor unit) aprotinin, pH7, with 0.25 g each of grinding buffer saturated PVPP(Polyclar) and XAD-4(Amberlite, nonionic polymeric adsorbent) in mortar with pestle. The slurry was transferred into a 50 ml polyethylene tube and was further ground by a polytron (Brinkman 210) for 2*10''. The mortar and pestle was rinsed with 10 ml grinding buffer and the rinse was used to wash the polytron. The wash was then combined with slurry and centrifuged at 20kg for 10 minutes. The supernatant was collected. Another 7 ml grinding buffer was used to wash the precipitate and the mixture was centrifuged again and two supernatants were combined. Solid ammonium sulfate was added to the supernatant to 70% saturation, and the mixture was stirred for 30 minutes in the cold room. The ammonium sulfate precipitated proteins were centrifuged at 10kg for 10 minutes, dissolved in 1 ml grinding buffer and dialysed against 2*1000ml grinding buffer overnight. The dialysate was centrifuged at 10kg for 10 min to remove the insolubles, and the supernatent was collected as the partially purified enzyme extract. The protein content and urease activity of partially purified extract were measured using coomassie blue method and Hogan's method respectively

(18). Protein content and urease activity in other fractions was also assayed. The distribution of soluble protein was 42-50% in the partially purified extract, 40-56% in the ammonia sulfate soluble fraction, and 4-10% in the insoluble fractions of the dialysate. The distribution of urease activity was 72-75% in the partially purified extract, 15-20% in the ammonia sulfate soluble fraction, and 5-13% in the insoluble fraction of the dialysate. The urease activity was purified by about 13-fold from the cell free crude extract.

c. Electrophoretic separation of urease isozymes

Native polyacrylamide gel electrophoresis(PAGE) of the urease extract was conducted on 5.8% (w/v) acrylamide slab (Bio Rad model 220) according to the Laemmli but without SDS (23) using a discontinuous Tris-HCl and glycine buffer system(Tris: 0.005M, Glycine:0.038M), pH8.3. About 200ul of partially purified extract was loaded to each gel lane. A current of 5 mA per gel was applied for about 4 hours then the current was turned up to 10 mA per gel for 8 hours. After the electrophoretic separation, coomassie blue R250 was used for staining the protein bends.

The method for the detection of urease activity in polyacrylamide gel was used as described by Shaik-M at el (40) based on the following reaction:

$$NH_2CONH_2 + H_2O \xrightarrow{\text{urease}} 2\ NH_4OH + CO_2$$

$$PboAC + NH_4OH \longrightarrow NH_4oAC + Pb(OH)_2 \downarrow$$

white ppt

Lead acetate stop the urease reaction and reacts with ammonia hydroxide produced to form water insoluble lead hydroxide(white precipitate). After PAGE run, the gel was preincubated in 50mM, then in 20mM sodium acetate buffer (pH5.0) each containing 1mM EDTA for 30 min with gentle shaking. The gel was then incubated in a solution containing 1 mM EDTA and 1.5% (W/V) urea. After 5 to 20 min of incubation, the gel was transferred into a solution of 0.1 M lead acetate. The regions on the gel containing urease activity were visible within minutes as white opaque bands. The gel was stored in distilled water and the white opaque bends can be preserved indefinitely. If 10 mM acetohydroxamic acid was added to the urea solution, no white urease bands were observed on the gel from both control and treated materials. This observation verified that the white bands are truly from the urease activity as acetohydroxamic acid is the inhibitor of urease(18).

Experiment II. The effects of urea incubation of leaf sections on urease

A.Leaf sections and induction method

In order to eliminate the possibility of differential absorption of the inhibitors of RNA and protein synthesis under *in situ* condition on urease induction by urea spray in barley leaves, the *in vitro* induction procedure was employed. Whole leaves was cut in greenhouse and the cut ends were immediately soaked in 50mM phosphate buffer, pH 7.0 and transported to the laboratory. Leaves were cut into the long sections(1 inch). Twenty of such leaf sections(about 0.8--0.9g) from comparable position of whole leaves were pre-incubated in 1 ml of 0.05% Triton X-100 in 50 mM phosphate buffer(pH 7) with and without 500uM cordycepin or 20ug/ml cycloheximide for 15min at 24^{o}C under 220 uE M^{-2} S^{-1} light. Special care was taken that the basal end of each section were incubated in the solution to allow a natural absorption transpiration of leaves. Then 0.11 ml of 2.5M urea was added into the incubation solution to a final concentration of 0.25M urea for urease induction. No urea was added in the control treatment.

B. *In vitro* assay of urease activity

After incubated with different solutions, the leaf sections were ground in phosphate buffer and then partially purified(detail in section E,b). The partially purified extract was used to determine the *in vitro* urease activity and the time course of urease induction in leaf sections.

The detail procedure of the enzyme assay is in section C of experiment I.

C. Electrophoretic separation of urease isozymes on gel

Native PAGE of the urease extract was conducted on 5.8% acrylamide slab gel. An aliquot of 220ul of partially purified extract was loaded on each gel lane. The detail procedure is the same as described in the experiment I section E,c.

D. ^{35}S-methionine incorporation and quantification of newly synthesized protein

a. Total incorporation

A protein synthesis tracer of 60uci/ml 35S-methionine(specific activity: 1140 ci/mmol) was added to incubating samples at 0, 15, 45 and 75 minutes after the addition of urea. After one half to 2 hours incubation in 35S-methionine, the leaf sections were washed, weighed, and ground in 10ml phosphate buffer(50mM, pH 7.0) with 0.4 g each of buffer saturated PVPP(polyclar) and Amberlite XAD-4 to remove polyphenols. The partial purification procedure (I.B.e.) was followed. The radioactivity incorporated in leaf proteins of different treatments was determined by counting an aliquot of the partially purified extract in a liquid scintillation counter(Backman LS7000). The total

radioisotope incorporation was calculated as dpm/gram of leaf fresh wight.

b.Fluorographic detection and quantification of newly synthesized proteins and synthesized urease

After proteins were separated on PAGE, a specific method for staining the urease activity on gel(40) was used to localize the urease isozymes on the gel(Detail in section E,c of experiment I) and the picture was taken by a Polaroid camera using Polaroid film type 47 for a permanent documentation. The gel was then processed according to the method of Bonner et al (7) for the preparation of fluorograph. Directly after enzyme staining the gel was soaked in about 20 times its volume of dimethylsulphoxide(DMSO) for 30 min, followed by a second 30min immersion in fresh DMSO. The gel was then immersed in 20% (w/w) 2,5-diphenyloxazole(PPO) in DMSO (22.2%,w/v) for 3 hours followed by immersing the gel in water for 1 hour to enhance the radioactive isotope. Enhanced gel was dried in a slab gel dryer (Bio Rad model 443) for 4 hours at 60^{o}C or 2 hours at 80^{o}C under vacuum condition. Dried gel was mounted tightly on a `X-Omat' film(Kodak) and covered well for exposure at -70^{o}C for 16 hours to 10 days depending upon radioactivity. The exposed X-ray film was developed using Kodak GBX developer(4 min at 30^{o}C without agitate) and fixer(3 min at 30^{o}C with agitate) to yield a fluorograph (38, 44). The relative peak percents of urease

isozymes were estimated from a Bio-Rad densitometer(model 1650)for tracing of the fluorograph. Based on the incorporation counts in the partial purified extract used for PAGE, the newly synthesized urease in terms of ^{35}S-methionine incorporation can be calculated by:

dpm in isozyme^{1}
=dpm loaded on gel x peak percent of isozyme^{1}

Only urease isozyme U_1 formed a distinctive band (Fig. 13). Thus the quantification was conducted.

RESULTS AND DISCUSSION

Experiment I.

The effects of foliar urea spray plants on leaves urease

A. In vivo urease activity

The activity of leaf urease was increased two-fold after 1 hour of urea spray on leaves and maintained at least for four hours (and in Fig. 1). This increased activity could be attributed to the urea content in leaves that was elevated very quickly after foliar urea sprayed (Fig.2). Urea content in the leaves reached the highest level at 1 hour after the spray that almost coincided with the urease activity peak (Fig.1). The urea content in treated leaves was significantly higher than that in control leaves and the elevated level was maintained for four hours then approached to the normal level indicating that barley leaves absorbed urea very quickly and urea was catabolized to carbon dioxide and ammonia gradually by a substrate stimulated enzyme activity. The leaf ammonia content was low in control material, but increased to 27-fold at 2 hour after urea spray and then declined for the subsequent 3 hours (Fig. 3). The ammonia was apparently used for the synthesis of amino acids that enhanced leaf growth and development as reported previously (45).

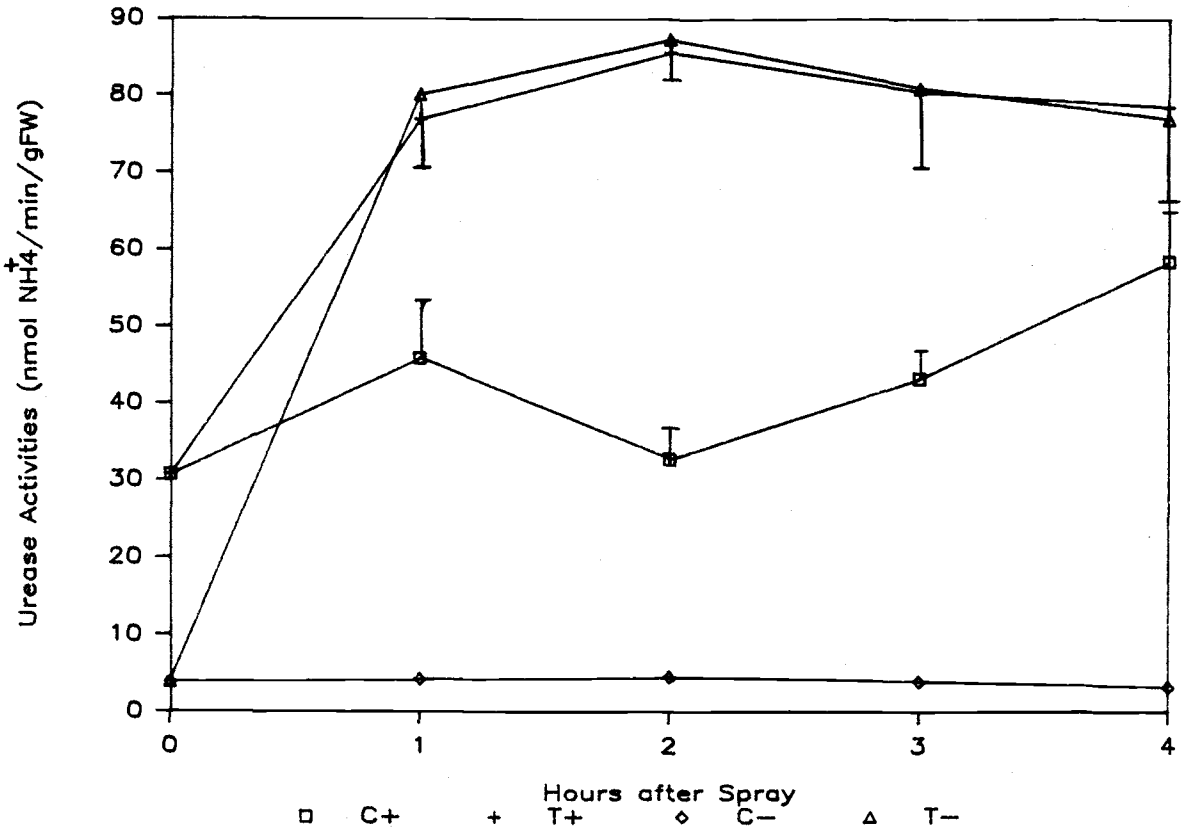

Figure 1. IN VIVO UREASE ACTIVITY IN `SCIO' BARLEY LEAVES AFTER FOLIAR APPLICATION OF UREA(5% in 0.1% Triton X-100) ON GREENHOUSE GROWN BARLEY PLANTS

C: Control
T: Urea Sprayed
+: In vivo assay with exogenous substrate (0.2M urea)
-: In vivo assay without exogenous substrate

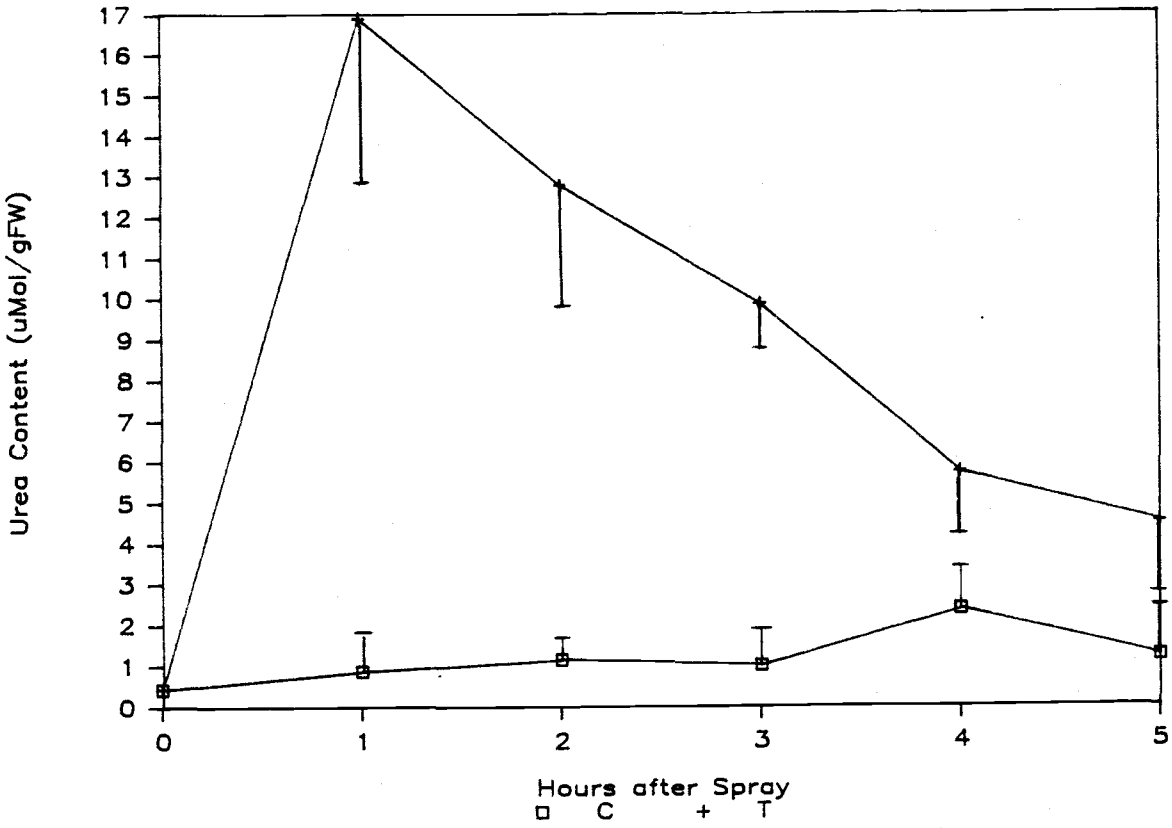

Figure 2. UREA CONTENT IN `SCIO' BARLEY LEAVES SPRAYED WITH OR WITHOUT UREA (5%) ON PLANTS

C: Control
T: Sprayed

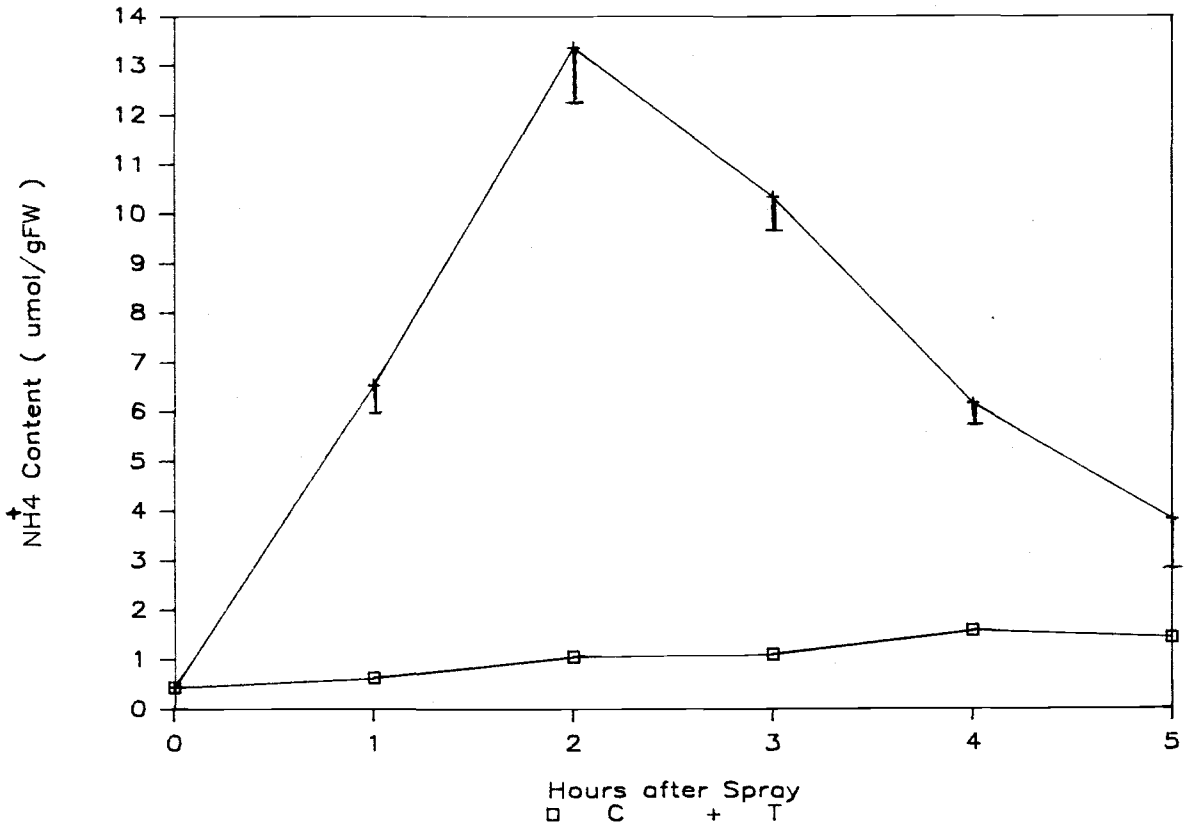

FIGURE 3. AMMONIA CONTENT IN BARLEY LEAVES SPRAYED WITH OR WITHOUT UREA(5%) ON PLANTS

C: Control
T: Treated

In general, the in vivo enzyme assay with exogenous substrate at excess level is for estimating the maximum enzyme activity (and + in Fig. 1). In treated leaves, the assay results would include original and possibly induced enzyme's activity. In control leaves, the assay results would indicate the activity of its pre-existed original enzyme only. An assay without exogenous substrate(and in Fig. 1) was used to estimate the enzyme activity based on available endogenous substrate, thus the activity represents the in situ rates (45). The assay with and without exogenous substrate for urea sprayed samples resulted in an identical rate of urease activity indicating an excess level of in situ substrate was present in leaves after the urea spray as shown in Fig. 2 and more exogenous substrate would not enhance further activity. The endogenous urease activity was 19- to 24-fold higher in treated leaves than that of control(Table 1). With added substrate, treated leaves had 1.3- to 2.6-fold more urease activity indicating either more urease was produced or pre-existing urease become more efficient due to substrate availability and/or protection. Comparing the in vivo urease activities between control plus exogenous substrate and treated material without exogenous substrate , the urea treated leaves have a maximum 2.66-fold more urease activity than that of untreated leaves (Table 1, last column). In this case both samples of treated and control leaves have excess substrate provided. The control leaves

TABLE 1 IN VIVO UREASE ACTIVITY IN BARLEY LEAVES
AFTER FOLIAR APPLICATION OF UREA (5% UREA) ON PLANTS

Hours After Spray	Urease Activities(nmol NH4/min/gFW) Total C+	Total T+	Endogenous C-	Endogenous T-	T- %of C-	T- %OfC+
0	30.8		3.89			
1	45.9 ±7.0 o	77.0 ±7.2	4.18 ±0.6	80.2 ±1.6 **	1919	175
2	32.8 ±1.7	85.7 ±1.0	4.50 ±1.2	87.4 ±2.8 **	1942	266
3	43.3 ±1.5	80.5 ±7.9	3.91 ±0.7	81.0 ±5.8 *	2072	187
4	58.6 ±4.3	78.5 ±9.2	3.19 ±0.7	77.0 ±8.3	2413	131

C---Control
T---Treated
Total activity---Assay with exogenous substrate(0.2 M Urea),C+ & T+
Endogenous activity---Assay without endogenous substrate, C- & T-
o---Data are the mean ± standard error of the mean
* & ** ---LSD analyse for comparing T- and C+, * & ** are significant difference at 0.05 and 0.01 level respectively
LSD(0.01)=28.84
LSD(0.05)=21.63

provided 200mM urea while the sprayed leaves had a maximum endogenous urea of 17 umol/gFW. With an average leaf water content of 70% the maximum concentration of urea in sprayed leaves would be 24 mM. The difference of urease activity, therefore, must attributed to an additional quantity of urease or to a more efficient urease. All these experiment data observed were comparable to what reported previously(45) except the sampling intervals were more close and peaks were more precisely defined.

B. In vitro urease activity

During the five hours after urea spray , in vitro urease activity increased 3-fold in sprayed leaves reaching a peak to 4-fold of control activity after two hours then gradually declined but still significantly higher than that of control material (Fig.4). These changes paralleled that of urea content in leaves(Fig. 2) after the spray except the peak of urease activity being one hour later than that of urea content indicating a causal relationship.

Further nullifying the urease activity with spray of urea plus specific inhibitor of RNA polymerase II, cordycepin, and of protein synthesis, cycloheximide, were not successful. The concentrations used were high enough for complete inhibition of urease induction in lichen (47-50) and jack bean leaves(29). The in vitro urease activity in

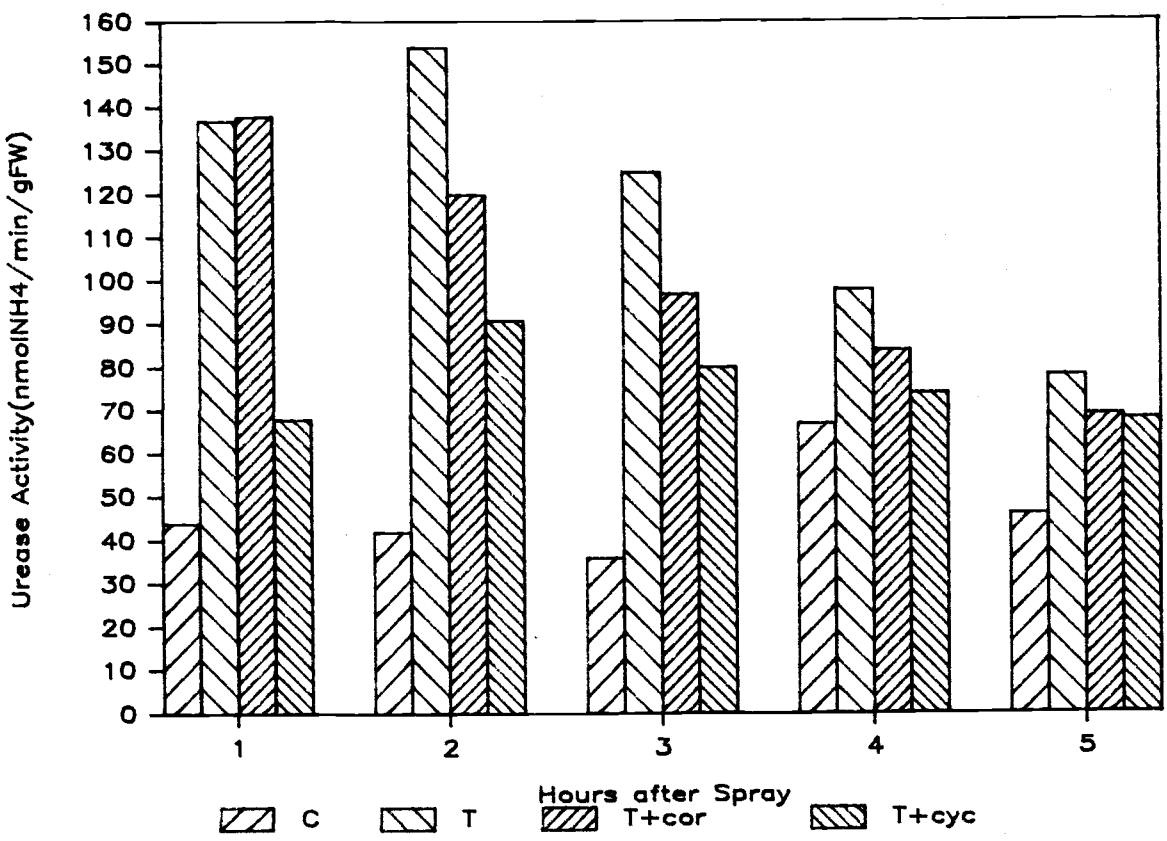

FIGURE 4. IN VITRO UREASE ACTIVITY WITH EXOGENOUS SUBSTRATE IN BARLEY LEAVES AFTER FOLIAR APPLICATION OF UREA +INHIBITORS OF TRANSCRIPTION (cordycepin) AND OF TRANSLATION (cycloheximide)

C: Control
T: Treated

TABLE 2 EFFECT OF RNA AND PROTEIN SYNTHESIS INHIBITORS ON IN VITRO UREASE ACTIVITY IN BARLEY LEAVES AFTER FOLIAR APPLICATION OF UREA WITH OR WITHOUT INHIBITORS OF RNA AND PROTEIN SYNTHESIS

Hours After Spray	In vitro Urease Activities(umol NH4/min/gFW) C	T	T+cordycipin (500uM)	T+cyclohexmide (20ug/ml)
0	29 ±2.5 o			**
1	44 ±6.1	137 ±4.2	138 ±11.1 **	68 ±6.7 **
2	42 ±1.5	154 ±14.5	120 ±4.2 **	91 ±2.7 **
3	36 ±2.3	125 ±14.5	97 ±11.6 **	80 ±3.4 **
4	67 ±4.6	98 ±2.7	84 ±4.8	74 ±1.7 **
5	46 ±2.3	78 ±7.8	69 ±1.1	68 ±1.9

C---Control
T---Treated
o---Data are the mean ± standard error of the mean
* & ** ---LSD analysis for comparing T-cordycipin with T and T-cyclohexmide with T, * & ** are significantly different at 0.05 and 0.01 level, respectively
LSD(0.01)=5.02
LSD(0.05)=3.78

urea + cordycepin sprayed leaves was 101, 77, 77, 86, and 96% of that of urea sprayed alone at 1, 2, 3, 4, and 5 hours after spray, respectively. For urea + cycloheximide sprayed leaves, the urease activity was 46, 58, 64, 75, and 88% of that of urea sprayed alone at respective times(Fig. 4). Even though the data in Table 2 showed that the inhibitors resulted in most cases significantly less urease stimulation than urea spray alone but all were higher than the control. Either the inhibitors didn't reach the site of their action, or a portion of the urea stimulated activity was originated from an inactive precursor. The difficulties in these inhibitors absorption and translocation in intact leaves are plausible and easy to resolve. Consequently , leaf sections were used for the urease induction study in Experiment II.

C. ^{35}S-methionine incorporation for quantifying newly synthesis proteins

The protein synthetic activity as shown by ^{35}S-methionine incorporation in sprayed leaves was higher than that in control leaves within 5 hours after urea spray, and reached a peak at 2 hours and then declined slightly (Fig. 5, C and T). The urea sprayed leaves had higher protein synthetic ability than the control by 45, 22, 23, 21, 18, and 8% at 1, 2, 3, 4, and 5 hours after the spray. The twelve or so

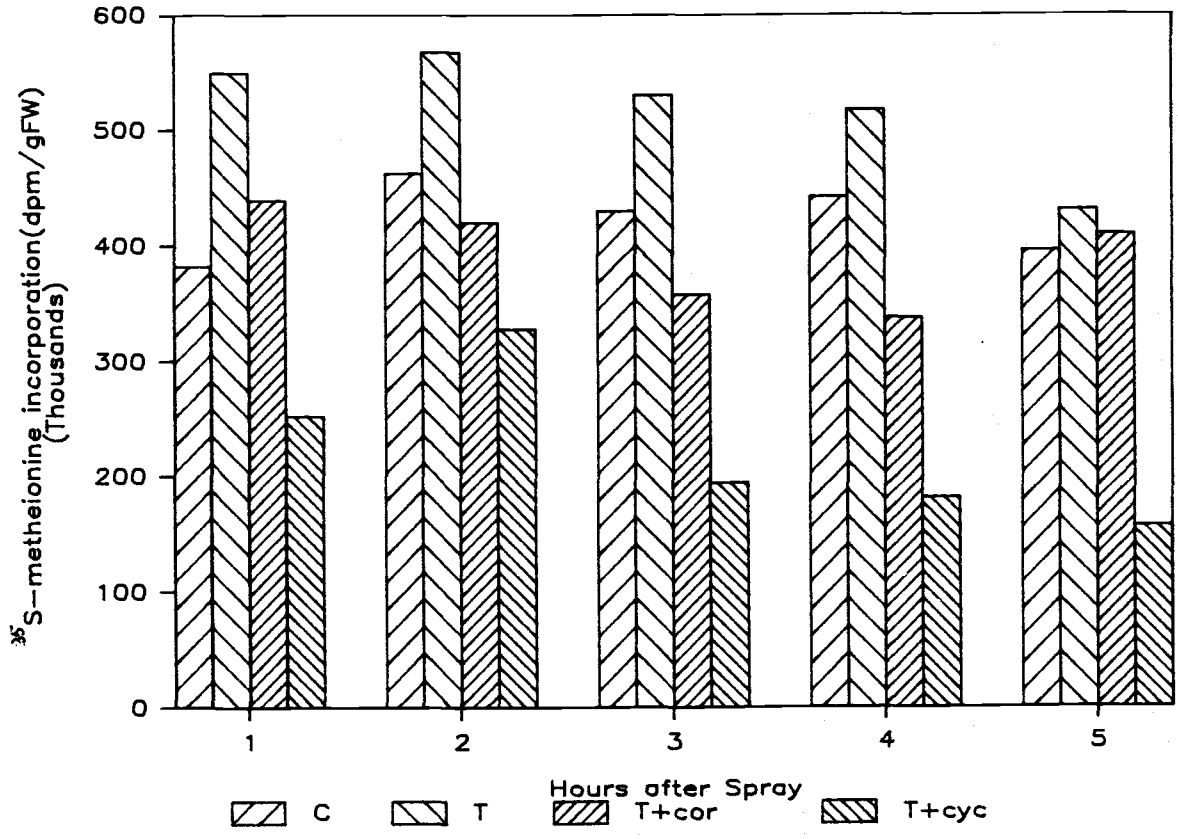

FIGURE 5. INCORPORATION OF ^{35}S-METHIONINE FOR 2 HOURS BY BARLEY LEAVES AFTER SPRAYED WITH UREA ± INHIBITORS OF mRNA (cordycepin) OR PROTEIN (cycloheximide) SYNTHESIS

C: Control
T: Sprayed, 5% urea
T+cor: 500uM cordycepin in 5% urea
T+cyc: 20ug/ml cycloheximide in 5% urea

newly synthesized protein bands were common to both control and treated leaves(Fig.14); urease isozymes were minor proteins and the increased protein synthesis after urea spray must also involve other proteins and enzymes due to the stimulated growth and N-assimilation, such as chlorophyll binding protein, glutamine synthetase, glutamate synthetase, glutamate dehydrogenase etc (44). Therefor the urea stimulated protein synthesis was not proportional to the increased urease activity (Fig.4) which exhibited 3.2-, 3.8-, 3.5-, 1.5- and 1.7-fold higher activity in urea sprayed leaves than the control. Again the inhibitors did not completely stop the protein synthesis indicating the possible problem of their absorption and translocation.

D. Urease isozymes induced by urea spray in barley leaves

After various time of foliar application of urea, the partially purified enzyme extracts of the leaves were separated by PAGE. The leaf urease not only increased its activity with time to 5 hours but also had more isozymes appeared (Fig.6). Five isozymes of urease were observed in barley leaves, namely U_a, U_b, U_1, U_2, and U_3 (Fig.6). U_a and U_b might be polymers or less anionic complexes occurred only at peak urease activity(2 hours, lane 2, Fig.6) in urea sprayed tissue and they might be the precursor of U_1. U_1 and U_2 existed only in urea induced leaves(lane 2, 4),

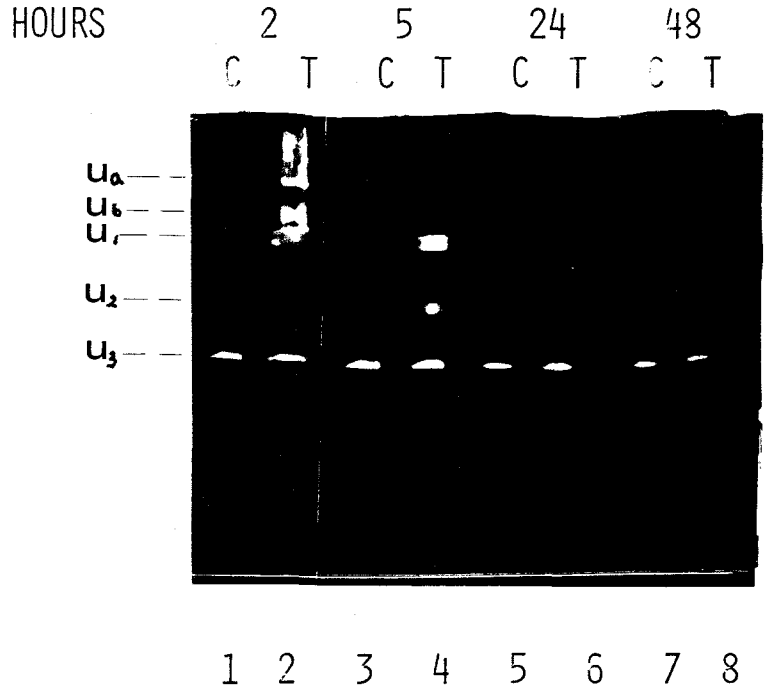

FIGURE 6. UREASE ISOZYME PATTERN IN BARLEY LEAVES AFTER DIFFERENT TIME OF FOLIAR SPRAY OF UREA (5%) ON PLANTS

C: Control
T: Treated

and U_3 was the constitutive preexisting urease isozyme since control leaves had this one only(lane 1, 3, 5, 6, 7, 8). The induced isozymes, U_a, U_b, U_1 and U_2, appeared as transient forms because 24 and 48 hours after the urea spray when absorbed urea was exhausted and urease activity declined to that of control (Fig. 1 and 2), they also disappeared (Fig. 6).

This is the first time that induced urease isozymes were ever been identified on PAGE, their physical and kinetic properties should be characterized for the knowledge of molecular biological events of urease induction.

Experiment II:

The effects of urea induction of leaves sections on leaf urease

A. In vitro assay of urease activity

The urease activity in partially purified enzyme extracts of various treatments at different times of incubation is presented in Fig. 7 as the total activity, umol NH^+_4 produced min^{-1} g^{-1} FW of leaves, and in Fig.8 as the specific activity, umol NH^+_4 produced min^{-1} mg^{-1} protein. It is evident that the constitutive urease activity in the control material was low, 120 nmol total activity, at 0.75 hour's incubation,maintained low for 2.25 hours and then reduced slightly at 3.25 hours. Incubation with 0.2 M urea, the urease in leaf sections increased the total activity to 5-, 11-, 22-, and 24-fold at 0.75, 1.25, 1.75 and 2.25 hours, respectively, then declined to 14-fold at 2.75 and 3.25 hours. These changes paralleled what been observed in leaves of urea sprayed plants (Fig. 4) except the total activity in control leaves was higher than that of the leaf sections.

During the 3.25 hour's incubation, little changes in protein content was observed in the leaf sections. Consequently the time curve of specific activity of urease (Fig. 8) exhibited a similar pattern as the total activity

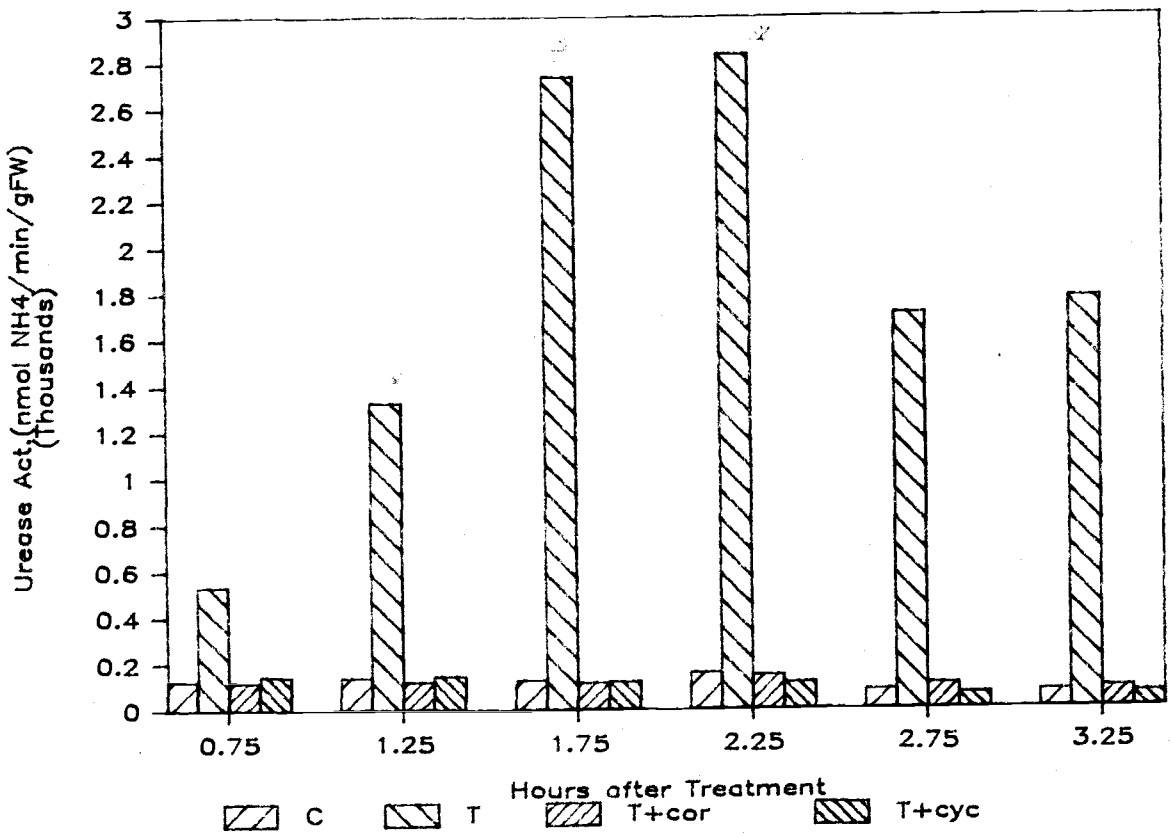

FIGURE 7. In vitro UREASE ACTIVITY IN BARLEY LEAF SECTIONS AFTER VARIOUS TIME OF INCUBATION IN UREA ± INHIBITORS OF TRANSCRIPTION(500uM cordycepin) OR OF TRANSLATION(20ug/ml cycloheximide)

C: Control
T: Treated
*: Data are the average of 2 replications. The variation among replication was less than 2%. Other are one sample.

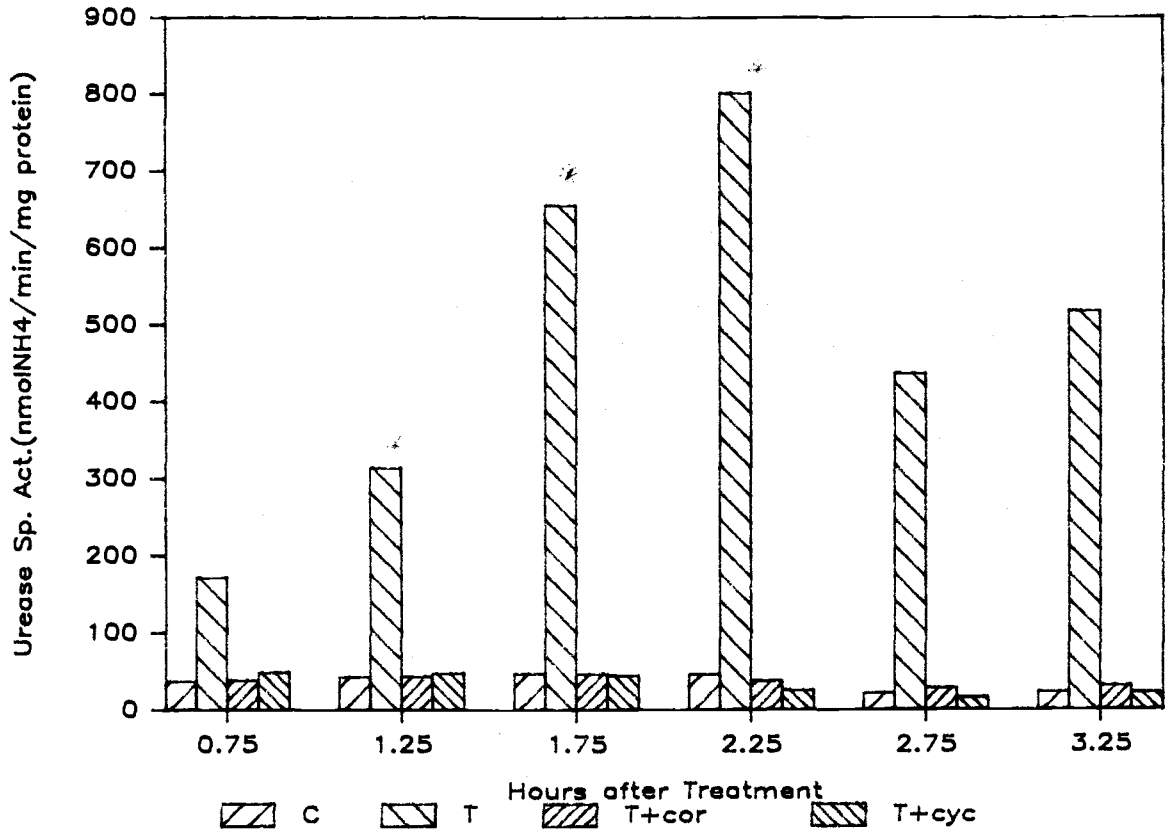

FIGURE 8. SPECIFIC ACTIVITY OF UREASE IN BARLEY LEAF SECTIONS AFTER VARIOUS TIME OF INCUBATION IN UREA(0.2M) ± INHIBITORS OF TRANSCRIPTION (500uM cordycepin) AND OF TRANSLATION (20ug/ml cycloheximide)

C: Control
T: Treated
*: Data are the average of 2 replications. The variation among replications was less than 24%. Others are one sample.

(Fig.7). The 20-fold increase of specific urease activity at 2.25 hours by urea definitely indicates an induction of the enzyme. Further evidences were provided by the nullification of urease stimulation by urea in materials preincubated for 15 min with cordycepin (a RNA polymerase II inhibitor at 500uM), or cycloheximide (a protein synthesis inhibitor at 20 ug/ml) (Fig. 7 and 8). If the mRNA for urease was prevented to be synthesized by cordycepin, no stimulation of urease activity could be observed in leaves even in the presence of urea. If the mRNA for urease was transcribed in response to urea but the translation of the specific mRNA was inhibited by the preincubation of cycloheximide, then again little enhancement of the urease activity would be found. The experimental results complied with these predictions and the stimulated urease activity by urea must be attributed to the newly synthesized urease protein. These results agree with that of urease induction in lichen thelli (47-50), and more direct evidences should be sought to exclusively prove the activation of the urease gene by urea.

B. Polyacrylamide Gel Electrophoretic Separation of Urease Isozymes In Barley Leaf sections incubated with or without urea

In order to discern the mechanism of urease induction in barley leaves after urea incubation, PAGE was employed to detect how many constitutive urease isozymes were in leaves and if any new isozymes were synthesized after the urea treatment. After many trials on gel percentage and separation buffers, the 5.8% native polyacrylamide gel and the specific urease activity stain (40) were used. Fig. 9 illustrates the results: In control material two white bands were observed (Lane 1, 3, 5, 7, and 9). The first one, named as X, was not an urease since it exhibited as an opaque band prior to stain and became whitish after the staining. The second sharp white band was the preexisting urease, U_3, because it existed both in control and treated materials. Half hour after urea incubation,a small U_1 band and a faint U_2 were observed (Lane 2). With increasing urea incubation time, U_1 and U_2 increase in activity up to 3 hours and then declined at 4 hour (Lane 4, 6, 8, 10 respectively). At 2 and 3 hour of incubation, two additional bands, U_a and U_b, appeared at the top of gel as polymers or less anionic complexes. The enzyme activity of U_3 was slightly higher (brighter) in urea treated leaves than that in control leaves indicating that U_3 activity in leaves was also stimulated slightly by the increased substrate. U_1 and U_2 therefore are the induced transient urease by urea treated. U_a and U_b existed only in material with highest induced urease activity, they might be precursors, complexes, or polymers.

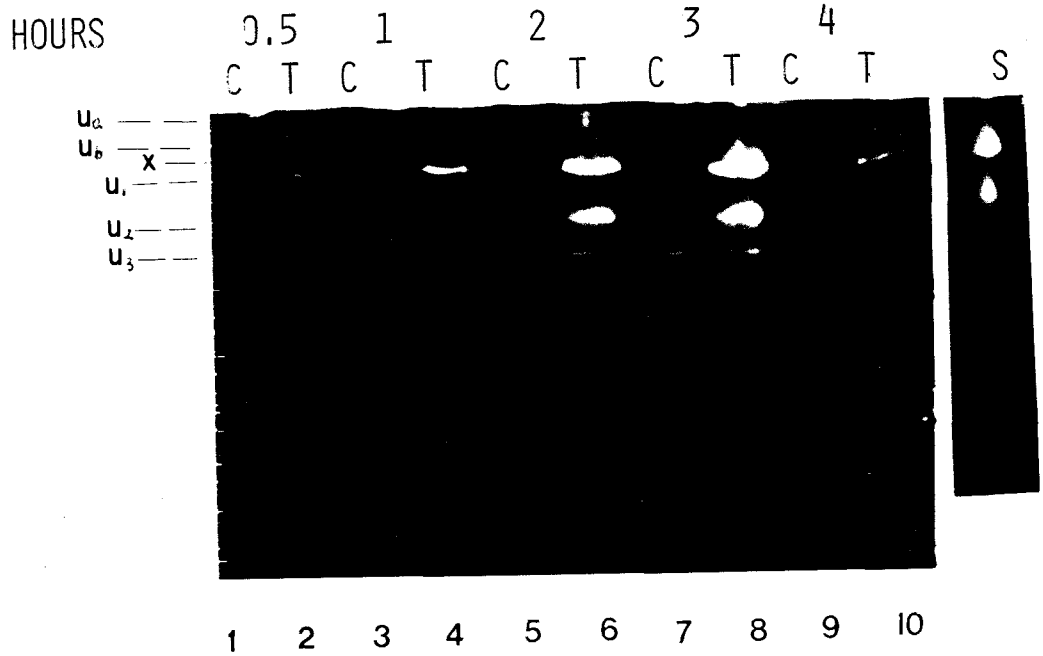

FIGURE 9. POLYACRYLAMIDE GEL (5.8%) ELECTROPHORETICALLY SEPARATED ISOZYMES OF BARLEY LEAF UREASE AFTER INCUBATING LEAF SECTIONS IN 0.2 M UREA FOR 0.5, 1, 2, 3, AND 4 HOURS. EACH LANE WAS LOADED WITH 220 ul OF THE PARTIALLY PURIFIED ENZYME EXTRACT (1.5ml/ 0.8-0.9 g leaf sections)

C : Control
T : Treated
S : Standard jack bean urease

The relative mobility of purified jack bean urease was shown in the last lane of Fig.9. Jack bean urease is composed of two isozymes of lower mobility than that of U_1 and U_2. Some early papers (40, 41) reported that jack bean urease is an oligomer of molecular weight 480,000; Active isozymes may be arisen by different degree of polymerization and changes in conformation, for example the dissociation process such as adding mecaptoethanol and boiling did not alter substantially the enzymatic activity of jack bean urease. This type of dissociation and/or polymerization or other kind of molecular changes in precursor of urease may also occur in barley leaves in response to absorbed urea. The absolute absence of isozymes, U_a, U_b, U_1 and U_2, on PAG gel in leaves incubated with inhibitors of mRNA or protein synthesis and urea (Fig.10, Lane 4, 5, 8, 9, 12, 13, 16, 17, 20, 21, 24, and 25) is the exclusive evidence of urease gene activation by the urea treatment in barley leaves. Apparently there were no precursors of urease or dissociation of polymers involved in the induction process since U_a and U_b were not observed until 1.75 hours into the induction. In addition, the induced isozymes, U_a, U_b, U_1 and U_2, were different from the preexisted isozyme(U_3) in terms of surface charge and possibly molecular weight. More research, however, will be needed to discern their differences. Most importantly the induction process is probably originated at the gene level. What activates the urease gene or how urea derepress

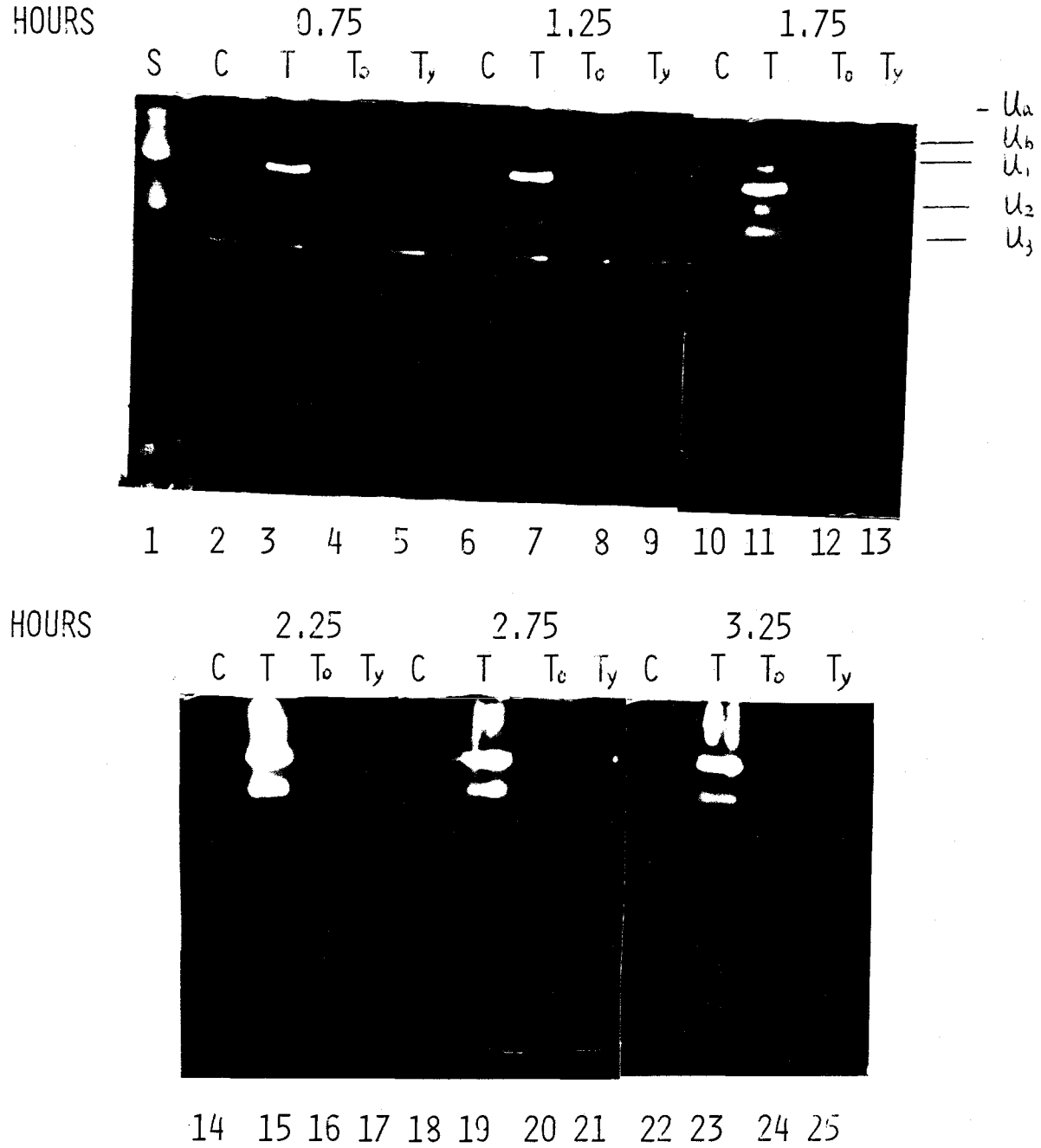

FIGURE.10 ELECTROPHORETICALLY SEPARATED UREASE ISOZYMES IN BARLEY LEAF SECTIONS INCUBATED WITH UREA AND SPECIFIC RNA (500 uM cordycepin) AND PROTEIN (20ug/ml cycloheximide) SYNTHESIS INHIBITORS. EACH LANE WAS LOADED WITH 220ul OF PARTIALLY PURIFIED ENZYME EXTRACT (1.5ml/0.8-0.9g leaf sections)

C: Control
T: 0.2M urea treated
T_o: 500uM cordycepin in 0.2M urea
T_y^o: 20ug/ml cycloheximide in 0.2M urea

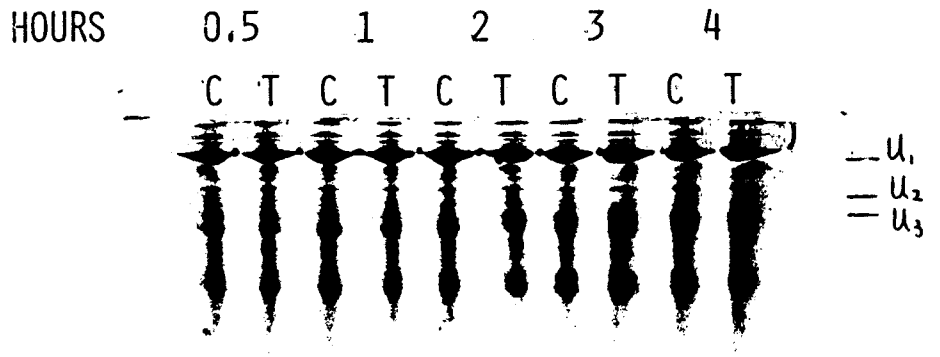

FIGURE 11. PROTEIN PATTERN OF PARTIALLY PURIFIED LEAF EXTRACT FROM LEAF SECTIONS AFTER DIFFERENT TIME OFINCUBATION WITH OR WITHOUT UREA. EACH LANE WAS LOADED WITH 50ul PARTIALLY PURIFIED ENZYME EXTRACT (1.5 ml/0.8-0.9 g of leaf sections)

C: Control
T: 0.2M urea treated

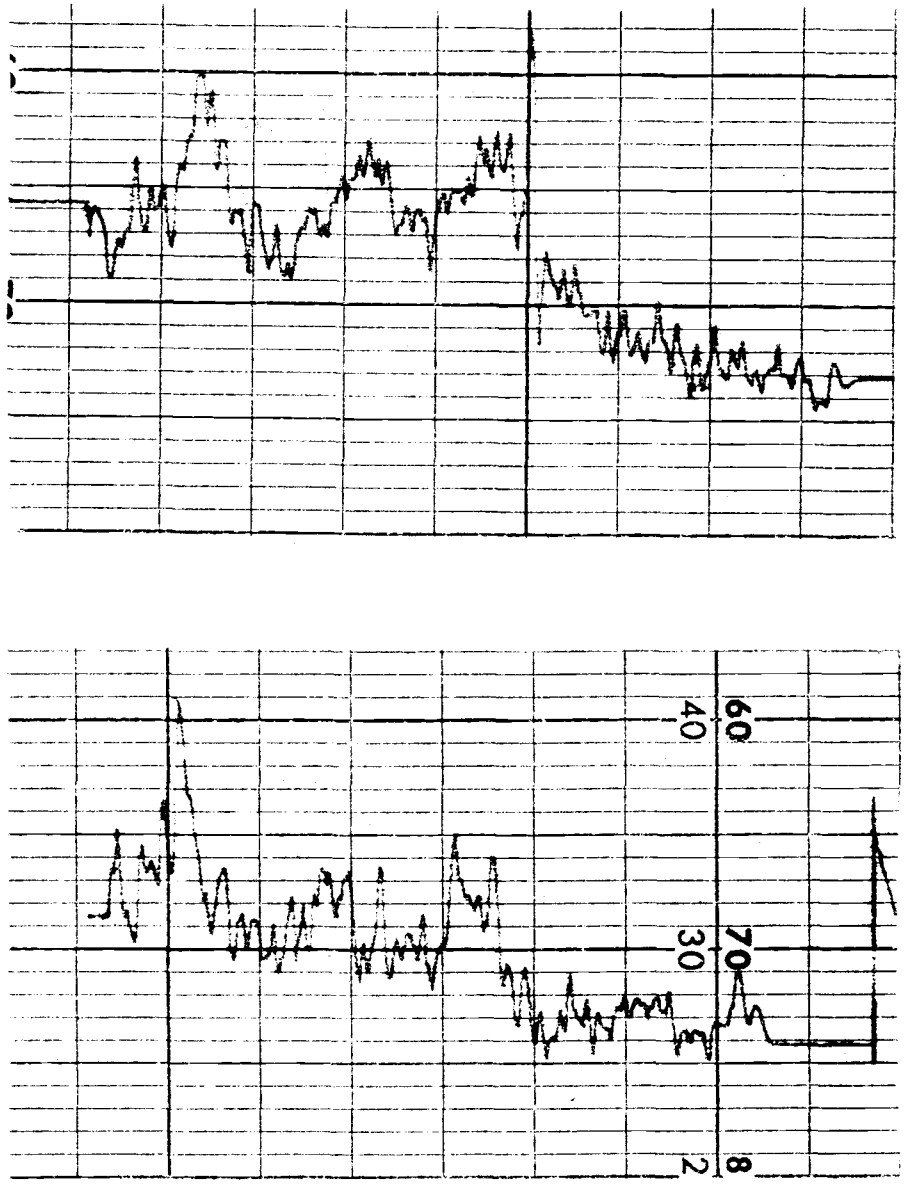

FIGURE 12. SCANNING PATTERN OF PROTEINS ON NATIVE PAGE (5.8%) FOR PARTIALLY PURIFIED LEAF EXTRACT FROM LEAF SECTIONS 2 HOURS AFTER INCUBATION WITH OR WITHOUT UREA.

C: Control
T: Treated

the urease gene become the most interesting subject of future study.

The pattern of protein bands in the partially purified enzyme extract did not show any difference between urea treated and control materials (Fig. 11). The urease isozymes as indicated by arrows were not even distinctive bands, and thus they constituted a very small portion of the extracted leaf proteins. Densitometric profiles of control (Lane 5) and urea treated material (Lane 6) further illustrated the statement (Fig. 12) It appears to be difficult to quantify the induced isozymes on gel. Therefore fluorographic evidence of newly synthesized urease was sought to further verify the induction.

C. Fluorograph of Newly Synthesized Proteins in Barley Leaf Sections Treated with Urea ± Inhibitors

The total ^{35}S-methionine incorporation after different time of urea treatment ± inhibitors in the partially purified enzyme extract are shown in figure 13. The incorporation in each treated sample was higher than that of corresponding control, and all increased with increasing time. The radioisotope incorporation or protein synthesized in the treated leaves was highest at the last stage(3.25 hours) indicating that the synthesis of other nitrogen assimilating enzyme such as glutamine synthetase, glutamate

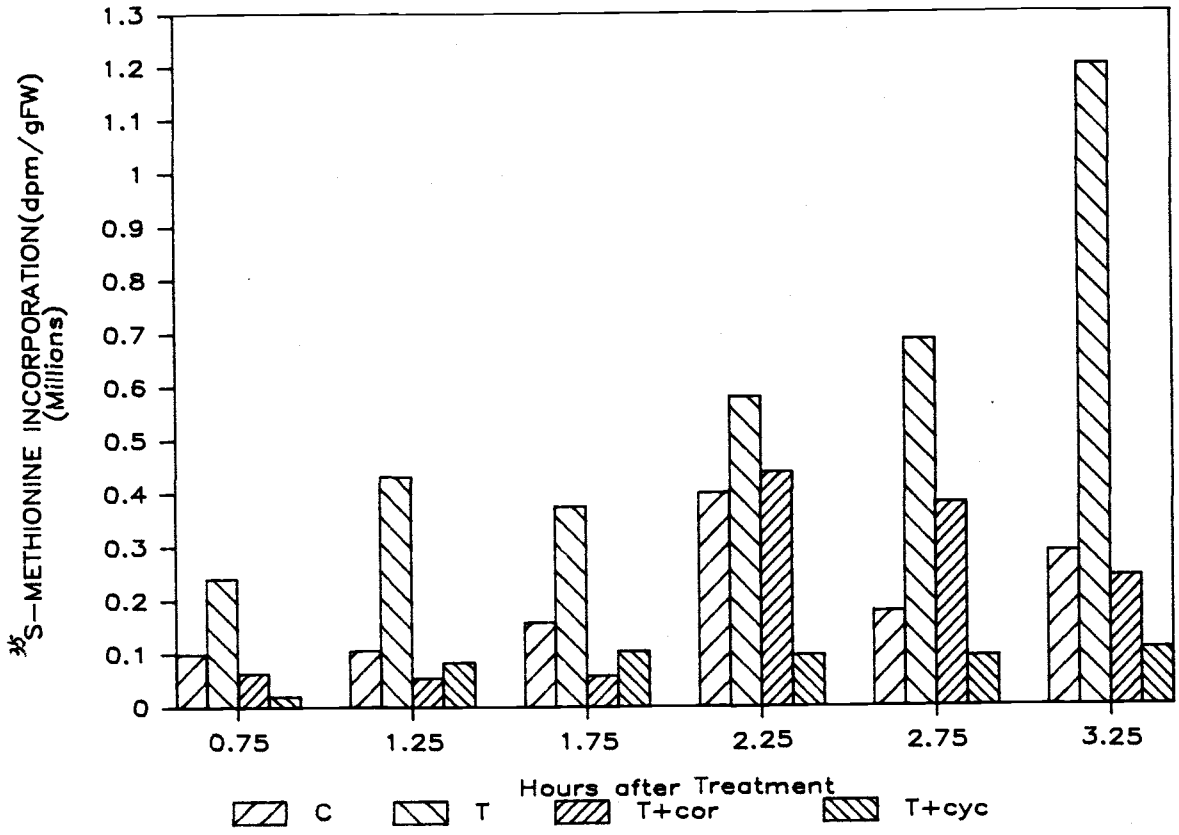

FIGURE 13. INCORPORATION OF ^{35}S-METHIONINE IN NEWLY SYNTHESIZED PROTEINS OF LEAF SECTIONS INCUBATED IN UREA + INHIBITORS OF mRNA(cordycepin) OR OF PROTEIN (cycloheximide) SYNTHESIS. ^{35}S-METHIONINE AT 62 uc/ml MEDIUM WAS PROVIDED FOR THE LAST 30 MIN OF THE INCUBATION TO THE FIRST 3 SAMPLES, AND AT 124uc/2 ml MEDIUM WAS PROVIDED FOR THE LAST 2 HOURS OF THE INCUBATION TO THE SECOND 3 SAMPLES.

C: Control
T: Treated, 0.2M urea
T+cor: 500uM cordycepin in 0.2M urea
T+cyc: 20ug/ml cycloheximide in 0.2M urea

synthetase and glutamate dehydrogenase, etc were stimulated by ammonia which is the product of urea hydrolysis by urease. The inhibitor of RNA or protein synthesis strongly reduced the radioisotope incorporation or protein synthesis in the first three time period, then the inhibiting effects was lessened somewhat due to possibly reduced concentration of the inhibitors. The induced urease activity was absolutely and continuously inhibited by cordycepin or cycloheximide during the whole experimental period either by enzyme assay (Fig. 7 and 8) or by isozyme identification on PAG gel (Fig.9 and 10).

To discern what proteins was synthesized after the urea treatment on leaves, PAGE of ^{35}S-proteins was attempted by fluorography. The specific urease staining method was used to locate the urease position on the gel first and then fluorograph was prepared from the same gel. The fluorograph of the newly synthesized proteins from leaf sections incubated with and without urea is shown in Fig.14. Again distinctive bands of induced urease isozyme was not observable, except one new band comparable to U_1 in R_m was clearly discernible in treated material at two hours incubation(Lane 6).

The fluorograph of the newly synthesized leaf proteins after different time of urea incubation with or without the inhibitors is shown in Fig. 15. At 0.75 hour of urea induction, the newly synthesized proteins in control and treated leaves(Lane 1 and 2) were identical indicating the

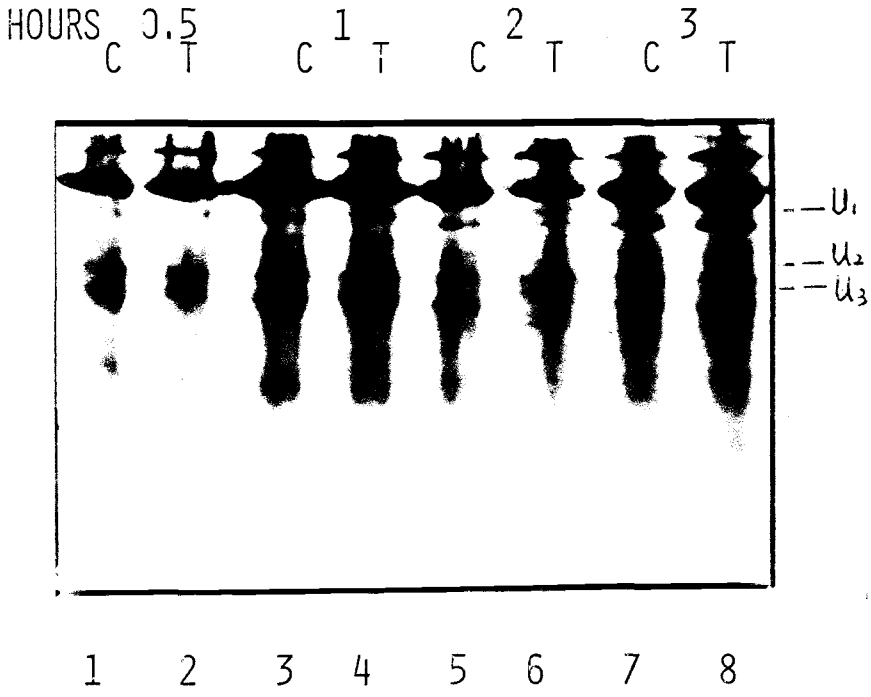

FIGURE 14. FLUOROGRAPH OF PAGE SEPARATED PROTEINS FROM BARLEY LEAF SECTIONS INCUBATED WITH OR WITHOUT UREA FOR 0.5, 1, 2, AND 3 HOURS WITH ^{35}S-METHIONINE INCORPORATION FOR THE LAST 0.5 HOUR

C: Control
T: Treated

synthesized proteins were translated from mRNA's produced mainly prior to the induction, even though the urease activity was already increased to 5-fold (Fig. 7) and induced urease isozymes U_1 was very active and U_2 observable (Fig.10). Perhaps at that time, the U_1 was not synthesized in enough quantity to be shown on fluorograph. At 1.25 hours and 1.75 hours after induction, the induced U_1 is clearly seen in urea treated samples(Lane 6 and 10). Due to some unexplainable reasons, the incorporation in 1.75 hours samples were only 1/2 of that at 0.75 hour and 1.25 hours when the experiments were conducted under identical induction conditions and at the same time. The quantification of U_1 band was attempted by comparing the % of isotope distributing in U_1 with time using densitometric scanning of the fluorograph (Fig. 15, Lane 2, 6, and 10) The result is presented in Table 3. Even though the total ^{35}S-methionine incorporation was different for each treatment, the percentage of incorporation in U_1 or newly synthesized U_1 was increased with the time after urea incubation within 2 hours indicating U_1 was truly induced form of urease by urea. The inhibitory effect of cordycepin or cycloheximide on protein synthesis or radioisotope incorporation was very strong in the first two hours, only two large or least anionic proteins were synthesized as they were located on the top of the gel. The function of these proteins are unknown, thus it is difficult to discern the reason for their presence.

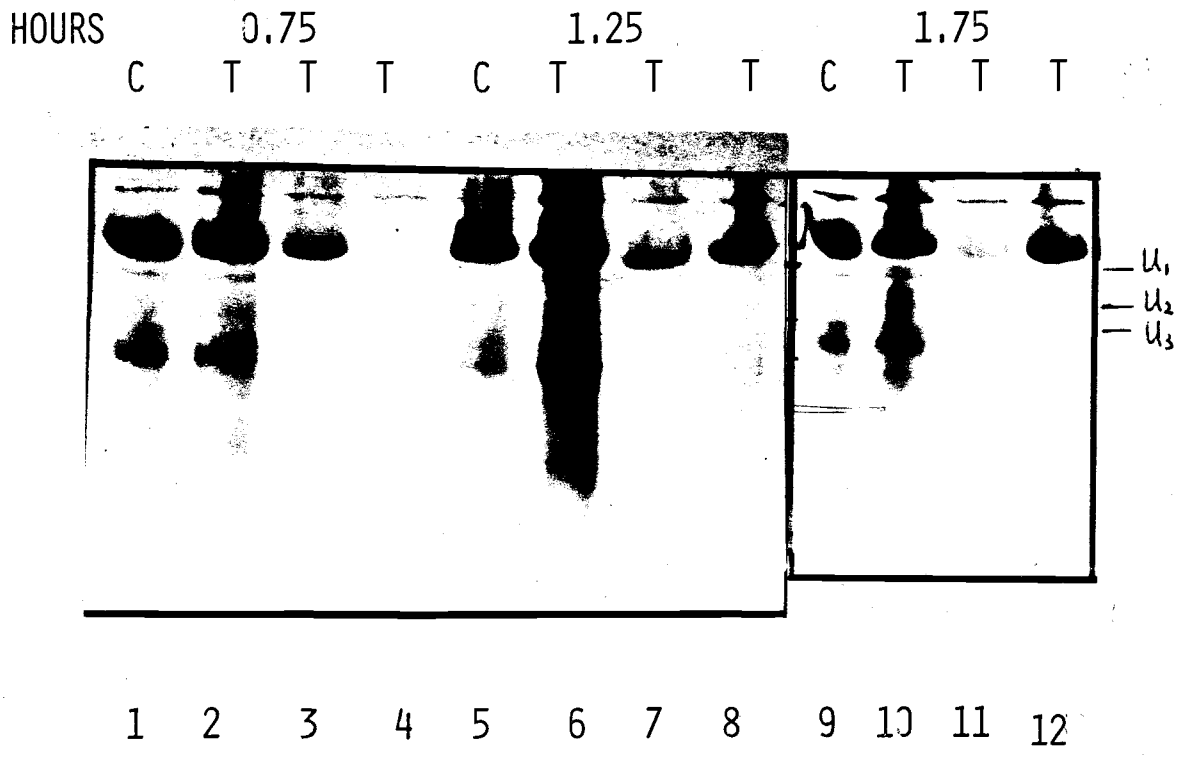

FIGURE 15.THE FLUOROGRAPH OF PAGE SEPARATED PROTEINS FROM BARLEY LEAVES INCUBATED IN UREA PLUS INHIBITORS OF RNA (500uM cordycepin) OR PROTEIN (200ug/ml cycloheximide) SYNTHESIS FOR 0.75, 1.25 AND 1.75 HOURS WITH ^{35}S-METHIONINE INCORPORATION FOR THE LAST 0.5 HOUR. EACH LANE WAS LOADED WITH 220 ul PARTIALLY PURIFIED EXTRACT (1.5ml/0.8-0.9 g of leaf sections)

C: Control
T: Treated
T_o: 500uM cordycepin in 0.2M urea
T_y: 20ug/ml cycloheximide in 0.2 M urea

TABLE 3. QUANTIFICATION OF NEWLY SYNTHESIZED UREASE ISOZYME U_1 ON FLUOROGRAPH (FIG.13) BY DENSITOMETER TRACING

Hours after spray	Total radioactivity incorporation(dpm/gFW)		U_1 synthesized % incorporation	
	C	T	C	T
0.75	100,050	241,976	0	0
1.25	106,750	433,242	0	2.1
1.75	79,827	183,200	0	3.0

C---Control
T---Urease treated

CONCLUSION

The *in vivo* and *in vitro* activity of barley leaf urease was significantly increased within 5 hours after foliar application of urea and reached a peak at two hours after urea spray. The increase of the enzyme activity was attributed to the urea content in leaves that was elevated very quickly and reached a peak at 1 hour after spray indicating a causal relationship between enzyme activity and substrate level. The specific activity of urease increased with the time and also reached a park at 2 hours after urea treatment in leaf sections indicating that urease was induced by urea.

Five isozymes, U_a, U_b, U_1, U_2 and U_3 , of barley leaf urease was found in PAGE separated gel and they were different from jack bean urease in mobility. U_a and U_b might be induced polymers or less anionic complexes since they occurred only at peak urease activity(2-3 hours after treatment) in urea treated tissue. U_1 and U_2 exhibited highest activity at the induced activity peak and disappeared 5 hours later; they appeared to be the final form of induced urease, but were transient in nature. U_3 was the constitutive preexisting urease since control leaves had this one only and it also existed in treated leaves.

The protein synthetic ability as indicated by ^{35}S-methionine incorporation in urea treated leaves was

higher than that in control leaves within 5 hours indicating the effect of urea enhanced leaf growth. The U_1 was increased in quantity with the induction time by fluorography suggesting that U_1 might be the truly induced isozyme.

The inhibitors of RNA (cordycepin) or protein (cycloheximide) synthesis not only reduced both total and specific activity of urease to the control level but also nullified the existence of isozymes, U_a, U_b, U_1 and U_2 indicating the mechanisms of urease stimulation by urea in barley leaves appeared to be a) an activation of urease gene to transcribe mRNA for new urease, b) an enhancement of more efficient protein translation by the newly transcribed urease mRNA, and c) a small activation of preexisting urease in urea sprayed leaves.

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