Metabolic Control of Urea Catabolism in *Chlamydomonas reinhardi* and *Chlorella pyrenoidosa*

ROBERT C. HODSON, STUART K. WILLIAMS II, AND WILLIAM R. DAVIDSON, JR.

*Department of Biological Sciences, University of Delaware, Newark, Delaware 19711*

Received for publication 6 December 1974

In the unicellular green alga *Chlamydomonas reinhardi* (strain y-1), synthesis of the enzymes required for urea hydrolysis is under substrate induction control by urea and under end product repression control by ammonia. Hydrolysis of urea is effected by the sequential action of the discrete enzymes urea carboxylase and allophanate lyase, collectively called urea amidolyase. The carboxylase converts urea to allophanate in a reaction requiring biotin, adenosine 5'-triphosphate, and Mg\(^{2+}\). The lyase hydrolyzes allophanate to ammonium ions and bicarbonate. Neither activity is present in pure cultures when cultures are grown with ammonia or urea plus ammonia, or when they are starved for nitrogen for 8 h. Urea in the absence of ammonia induces both activities 10 to 100 times the basal levels. Addition of ammonia to an induced culture causes complete cessation of carboxylase accumulation and an 80% depression of lyase accumulation. Ammonia does not reduce urea uptake by repressed cells, so it does not prevent induction by the mechanism of inducer exclusion. The unicellular green alga *Chlorella pyrenoidosa* (strain 3 Emerson) also has discrete carboxylase and lyase enzymes, but only the carboxylase exhibits metabolic control.

Urea arises in the metabolism of autotrophs and some heterotrophs as an intermediate in the recycling of endogenous nitrogen. The characteristic reaction of urea catabolism in these organisms is hydrolytic and catalyzed by urease (EC 3.5.1.5), a highly specific enzyme with no known activators or cofactors. Recently, it has been shown with some species of yeast and green algae lacking urease that urea amidolysis (urea carboxylase [hydrolyzing], EC 6.3.4.6), which requires adenosine 5'-triphosphate (ATP), bicarbonate, Mg\(^{2+}\), biotin, and a univalent cation that may be K\(^+\), Na\(^+\), or NH\(_4\)\(^+\) (37, 39, 51, 52, 55), can serve as an adaptive system for urea hydrolysis.

The urea amidolyses from the yeasts *Candida utilis* (39) and *Saccharomyces cerevisiae* (56, 57) and the green algae *Chlorella vulgaris* var. *viridis* (51) have been found to consist of two distinct enzymatic activities. These activities are called urea carboxylase and allophanate lyase (51) (or allophanate hydrolase [57]), and they catalyze the hydrolysis of urea to ammonium ion and bicarbonate as follows:

\[
\begin{align*}
O & \quad \text{Urea carboxylase} \\
H_2N-C-NH_2 + ATP + HCO_3^- & \xrightarrow{\text{Mg}^{2+}K^+(Na^+, NH_4^+)} \text{Urea}
\end{align*}
\]

\[
\begin{align*}
O & \quad \text{Allophanate lyase} \\
H_2N-C-N-C-O^- + ADP + P_i & \xrightarrow{\text{Allophanate lyase}} \text{Allophanate}
\end{align*}
\]

\[
\begin{align*}
2NH_4^+ + 2HCO_3^- & \quad (2)
\end{align*}
\]

The yeast urea carboxylase and allophanate lyase activities remain together through several purification steps (39, 56), whereas the same enzyme activities from *Chlorella* are readily separated (51). Several other species of green algae have been shown to possess urea amidolysis (25), but further characterization of these...
enzymes has not been reported.

Initial experiments in our laboratory (17) and by Roon and Levenberg (39) on the biochemistry of urea metabolism in the green alga *Chlamydomonas reinhardtii* indicated that it might possess an inducible catabolic pathway for urea which is also under control by repression. The evidence for this was that the amidolylase could be detected only in cells grown on urea, but not in cells deprived of combined nitrogen for several days or grown on ammonia (17). Additionally, the amidolylase could be obtained in two separate fractions exhibiting the properties of urea carboxylase and allophanate lyase (17). Thus, it appeared to be similar to the *Chlorella* activity but quite unlike that in yeast.

In contrast to bacteria and fungi, less attention has been given to enzymes in algae subject to induction (or derepression) or repression (e.g., 5, 13, 16, 20–23, 27, 28, 31, 34, 45–47, 49, 52) and even less to the combined controls of induction (or derepression) and repression operating on a single pathway or enzyme (16, 27, 31, 45, 49). Furthermore, in many of the existing algal studies, the focus has been on temporal control of structural gene expression during the cell cycle (22, 23, 27, 31, 52), or the species studied does not possess a mechanism (so far as is known) for genetic recombination (5, 13, 23, 28, 31, 34, 49, 50).

*Chlamydomonas* has a history of genetic manipulation and many genetic markers are available (11, 26). Its possession of two urea catabolic enzymes operating in sequence and the potential for genetic recombination affords the opportunity to study the mechanism of dual control over the expression of enzymes in a single algal pathway by both biochemical and genetic approaches.

This paper reports the results of initial studies on the induction and repression of the *Chlamydomonas* urea amidolylase system and some biochemical properties of the enzymes involved. All studies were with a strain wild-type for urea catabolism and with nonsynchronous cultures of vegetative cells. The terms “induction” and “repression” are used in their operational sense (33). A few results from a *Chlorella* system are included for comparison. Preliminary accounts of this work have appeared (17, 60).

**MATERIALS AND METHODS**

**Organisms and growth conditions.** *C. reinhardtii*

Dangeard (strain y-1) and *Chlorella pyrenoidosa*

Chick (strain 3 Emerson) were maintained in Erlenmeyer flasks on a rotating shaker platform under 500 ft-c (ca. 5,000 lx) of cool white fluorescent illumination and at 25 to 30 C. *Chlamydomonas* was grown in a mineral salts medium (35) supplemented with 0.2% sodium acetate as the carbon source and with either 5 mM NH₄NO₃ or 3 mM urea as the nitrogen source. Nitrate is not metabolized by this strain. *Chlorella* was grown in a mineral salts medium (40) with either 10 mM NH₄Cl or 3 mM urea, and with 1% glucose.

**Preparation and partial purification of cell extracts.** Unless stated otherwise, 10 mM sodium tricine [N-tris(hydroxymethyl)methylglycine], pH 8.0, plus 2 mM dithioerythritol was used in all procedures requiring buffer. All manipulations of enzymes were at 0 to 4 C, and glass-distilled water was used throughout.

*Chlamydomonas* was harvested by centrifugation for 10 min at 4,000 × g, washed once by suspension in 10 mM sodium tricine, and finally suspended in 4 volumes of 100 mM sodium tricine containing 2 mM dithioerythritol. Extraction was by either of two essentially equivalent methods. For method A, the cell suspension was passed once through a chilled French pressure cell (American Instrument Company) at 10,000 lb/in² (ca. 7 × 10⁶ kg/m²) and centrifuged for 60 to 90 min at 275,000 × g. The clear, pale-yellow supernatant solution, comprising the crude extract, was subjected to fractional precipitation with solid ammonium sulfate. Those proteins precipitating between 35 and 45% saturation were dissolved in buffer to 1/10 the original volume of crude extract, giving a protein concentration between 10 and 20 mg/ml. For method B, the cell suspension was placed in 50-ml polycarbonate centrifuge tubes, frozen in a dry ice-ethanol bath for 1 h, and thawed slowly in cool water. The resulting suspension was centrifuged for 10 min at 12,000 × g, the pellet was washed once by suspension in 4 volumes of 100 mM buffer, and the combined supernatants were brought to 50% saturation with solid ammonium sulfate. The precipitated protein was dissolved in buffer as in method A. Extracts obtained either way could be stored for several weeks at −20 C with retention of at least 90% of the initial urea amidolylase activity. Numerous thaw-freeze cycles over a period of 4 months decreased the urea carboxylase activity to 1% and the allophanate lyase activity to 50% of the initial values.

Cell extracts of *Chlorella* were prepared as for method A, except that the cells were disrupted in a French pressure cell operating at 20,000 lb/in² (ca. 14 × 10⁶ kg/m²). To reduce the volume, urea amidolylase activity was precipitated with ammonium sulfate between 30 and 60% saturation.

**Partial purification and separation of Chlamydomonas urea amidolylase activities.** The activities were achieved essentially by the method of Thompson and Muenster (51, 53). The protein obtained from precipitation with ammonium sulfate was diluted to 1 mg/ml and placed on a column of brushite previously equilibrated with buffer, using a ratio of 2 mg of protein to 1 or 2 ml of packed brushite. The column was eluted stepwise with 2 volumes of buffer containing 5 mM potassium phosphate, followed by 2 volumes of buffer with 20 mM potassium phosphate.
Enzyme assays. Urea amidolyase activities were assayed with radioactive substrates by procedures similar, in principle, to those published by others (38, 55). All incubations were carried out in 25-ml Erlenmeyer flasks held in a water bath at 25 or 30 C and sealed with a rubber stopper. Each flask had a center well containing 0.5 ml of 10% KOH. The enzymatic reactions were always started by the addition of 1\(^{14}\)C-labeled substrate and stopped with 0.5 ml of 6 N H\(_2\)SO\(_4\). Radioactive carbon dioxide was allowed to diffuse into the KOH for at least 30 min with continuous shaking. A 0.2-ml sample from the center well was combined with 0.8 ml of water and 10 ml of either a standard Triton X-100 and toluene-based scintillation mixture, or a 1:1 (vol/vol) mixture of Omnifluor (New England Nuclear) in toluene and 2-ethoxyethanol, and the radioactivity was determined by liquid scintillation at room temperature. The counting efficiency of about 80% was checked occasionally with an internal standard of 1\(^{14}\)C toluene. One unit of activity is equivalent to that amount of extract liberating 1 \(\mu\)mol of carbon dioxide per min at pH 8.0 and the stated temperature.

1\(^{14}\)C urea uptake and intracellular 1\(^{14}\)C urea. An ammonia-grown Chlamydomonas culture was harvested by centrifugation, washed with sterile 10 mM potassium phosphate (pH 6.5), and suspended in fresh nitrogen-free medium to yield a density of 150 Klett units. Portions of this suspension were placed in 250-ml flasks either with or without 10 mM ammonium nitrate and returned to the growth shaker for 15 min. 1\(^{14}\)C urea was added to a final concentration of 0.3 mM and the flasks were returned to the shaker. At intervals, duplicate 2.5-ml samples were filtered through 25-mm diameter fiber glass disks (Klenow type E) and rinsed with 20 ml of ice-cold phosphate buffer containing 10 mM urea. The filters were placed in 10 ml of a 2-ethoxyethanol and Omnifluor mixture, and the radioactivity was determined by liquid scintillation. Corrections were made for quenching by chlorophyll.

Nonspecific binding of 1\(^{14}\)C urea to cells and filter was estimated with similar suspensions previously killed by freezing to -70 C. Corrections also had to be made for 1\(^{14}\)C urea enzymatically hydrolyzed to 1\(^{14}\)CO\(_2\) and not assimilated by photosynthesis. To do this, immediately after addition of 1\(^{14}\)C urea, 1-ml portions of suspension were sealed in 25-ml flasks possessing 10% KOH in a center well. These flasks were incubated under the above conditions of light and agitation. At various times 1\(^{14}\)CO\(_2\) was liberated with sulfuric acid and trapped in the KOH, and the radioactivity was determined as for the urea amidolyase assay.

Intracellular 1\(^{14}\)C urea was estimated with filtered and washed cells. The filter disks were quickly placed in 4 ml of ice-cold 0.75 M sodium phosphate (pH 8) and frozen with liquid nitrogen. Upon thawing and centrifugation, 1-ml samples of supernatant were placed in 25-ml center well flasks together with 1 ml of jack bean urease (Sigma type IV dissolved in 0.02 M sodium phosphate [pH 8]; 1 U/ml) and incubated for 30 min at 30 C. Radioactive carbon dioxide was estimated by microdiffusion into KOH and liquid scintillation.

Kinetics of induction and repression. We do not have a nonrepressing nitrogen source for Chlamydomonas that will allow rapid growth rates, and so for induction experiments cells were grown with ammonia. In the first experiments, cultures were grown to about 50 Klett units with limiting ammonia (0.5 mM) and then given inducer without changing the medium. To provide more uniform material, in later experiments cells were grown on excess ammonia (5 mM), harvested during exponential growth by low-speed centrifugation at room temperature, washed once by suspension in nitrogen-free medium, and resuspended in fresh nitrogen-free medium to give an absorbance of 100 Klett units or less. Such cells gave rapid induction rates whether or not they were preincubated to deplete endogenous pools. Cells grown with limiting ammonia exhibited a lag in enzyme accumulation during the early minutes of induction.

Cells for repression experiments were grown with excess urea in a single culture, which was divided into separate flasks just before making further additions.

Extracts were prepared from duplicate 15-ml samples of culture. The cells were sedimented in 15-ml Corex tubes at 12,000 \(\times\) g for 10 min at 0 to 4 C, washed once by suspension in 5 ml of 10 mM sodium tricine (pH 8.0) containing freshly added 20 mM mercaptoethanol, and finally resuspended in 5 ml of the same buffer.

Disruption of cells held in an ice-salt bath by sonic oscillation with a Branson microtip (Heat Systems, Inc.) operating for 20 s at the lowest power setting gave at least 99% breakage and maximum recovery of urea amidolyase activity. A few remaining cells and the larger cellular debris were removed by centrifugation for 10 min at 12,000 \(\times\) g. The clear, green supernatant fluid was used within 1 h, although activity was stable for at least 6 h. Usually 1 ml of extract was used for each enzyme assay.

Analytical procedures. Protein concentration was determined by the Lowry procedure (24) or turbidimetrically (24) after precipitation in 5% trichloroacetic acid. Crystalline bovine serum albumin was used as a standard. To remove thiol, which gives a color reaction with the Folin reagent, protein in extracts was precipitated with 8% trichloroacetic acid, sedimented by centrifugation, and dissolved in 1 N NaOH with heating at 60 C for 10 min. This method gave reproducible and quantitative extraction of protein from whole cells as well.

Ammonia in culture media was determined by direct Nesslerization. Growth of algal cultures was measured as apparent absorbance with a Klett-Summerson colorimeter using a green filter whose transmission centered at 540 nm (18). Absorbance was proportional to cellular dry weight in the range of 10 to 200 Klett units.

Ascending paper chromatography employed Whatman no. 1 filter paper and the following solvents: (i) methanol-pyridine-water (85:4:4) and (ii) 95% ethanol-water (7:3). High-voltage paper electrophore-
sis in a water-cooled apparatus (Savant Instruments, Inc.) was carried out with 0.05 M tris-(2-amino-2-hydroxymethyl)-1,3-propanediol acetate buffer (pH 7.9), or 1% sodium tetraborate, and at 30°C in a gradient of 80 V/cm. Ureido compounds were located on the paper with the usual Ehrlich reagent.

**Reagents.** 14C-labeled potassium allophanate was prepared from [14C]urea either enzymatically by the action of *Chlamydomonas* urea carboxylase or chemically. The enzyme reaction mixture contained the usual components of the urea carboxylase assay (see Fig. 3) but without allophanate lyase. The progress of the reaction was determined by passing a sample through a small column of quaternary amine resin in the hydroxide form and counting the amount of unreacted [14C]urea in the effluent. When less than 1% of unreacted urea remained, carrier allophanate was added, the pH was adjusted to approximately 10 with concentrated NaOH, and the mixture was heated at 60°C for 3 min to destroy residual enzyme activity. This preparation of [14C]allophanate was stored at −20°C and used without purification. The chemical preparation of [14C]allophanate followed the procedure of Whitney and Cooper (57), except that more KOH was added to completely dissolve the methyl ester. The product was stored in a desiccator at −20°C, and solutions were made up in 0.1 M sodium carbonate as needed. Unlabeled potassium allophanate was obtained by the action of 1 N KOH in 60% (vol/vol) aqueous ethanol on methyl allophanate and purified by repeated crystallization from aqueous ethanol. Methyl allophanate was prepared by the method of Dains and Wertheim (9).

Brushite was made by the method of Siegelman et al. (43). [14C]urea was purchased from Calbiochem and New England Nuclear. All other reagents were obtained from commercial sources in the highest purity available.

**RESULTS**

**Properties of urea amidolyase from Chlamydomonas.** Extracts from urea-grown cells catalyze the hydrolytic decomposition of [14C]urea to 14CO2. Ammonia, the other product expected, has been detected indirectly by coupling its formation to the oxidation of reduced nicotinamide adenine dinucleotide with bovine liver glutamate dehydrogenase (EC 1.4.1.3). The hydrolytic activity requires ATP and Mg++; it is lost when extracts are treated with heat or avidin prior to incubation with substrate (Table 1). These characteristics of the *Chlamydomonas* activity are similar to the urea amidolyase system obtained from yeast (39, 55) and Chlorella (51, 53). This, together with an earlier report (39), demonstrates that *Chlamydomonas* employs urea amidolyase rather than urease for catabolism of exogenous urea.

The *Chlamydomonas* urea amidolyase system can be separated into two components with distinctly different activities. Crude extract subjected to fractional precipitation with ammonium sulfate followed by adsorption to brushite in the presence of phosphate ions gives two heat-labile components (Table 2), designated for convenience fractions 1 and 2, which must be recombined to obtain 14CO2 from [14C]urea. The resolution of these two components varied from one experiment to the next, and complete separation has not been obtained by this method. Fraction 1 is the component with less affinity for brushite in the presence of 5 mM phosphate. It does not have appreciable activity with [14C]urea (Table 3 and Fig. 1B), but it hydrolyzes [14C]allophanate to 14CO2 (Table 3). Fraction 2, on the other hand, adsorbs to brushite in the presence of 5 mM phosphate and is eluted by 20 mM phosphate. It is unable to hydrolyze either urea or allophanate (Table 3), but it converts [14C]urea to another radioactive substance (Fig. 1C). This substance is identified as [14C]allophanate by its lability to mineral acid and by its co-electrophoresis and co-chromatography with authentic material (Fig. 2). Based on these observations, fraction 1 contains the activity of allophanate lyase (allophanate hydrolyase, 56), whereas fraction 2 contains urea carboxylase activity.

**Table 1. Requirements for urea amidolyase of *Chlamydomonas* in a cell extract**

<table>
<thead>
<tr>
<th>System</th>
<th>Activity (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Complete system</td>
<td>5.9</td>
</tr>
<tr>
<td>−MgSO4</td>
<td>0.037</td>
</tr>
<tr>
<td>−ATP</td>
<td>0.24</td>
</tr>
<tr>
<td>Heated 100°C, 3 min</td>
<td>0.016</td>
</tr>
<tr>
<td>B. Native enzyme</td>
<td>2.1</td>
</tr>
<tr>
<td>+Avidin</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*Experiments A and B were with separate enzyme preparations. The extract was a 35 to 45% ammonium sulfate fraction from a crude homogenate of ureagrown cells. (A) The extract was desalted through a column of Sephadex G-25; (B) the extract was not desalted. The complete system had the following additions to a final volume of 2.5 ml: KCl, 200 μmol; MgSO4, 20 μmol; disodium ATP, 20 μmol; sodium tricine (pH 8.0), 500 μmol; β-mercaptoethanol, 50 μmol; NaHCO3, 20 μmol; [14C]urea, 2.1 μmol (1,610 disintegrations/min per nmol); and extract equivalent to 2.3 ml of culture at 190 Klett units. Incubation was at 25°C for 60 min. One milliunit, 1 nmol of 14CO2 formed per min. 200 μg of avidin was added 30 min before [14C]urea.
Table 2. Separation of Chlamydomonas urea amidolyase activities by chromatography on brushite

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Total activity a (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample for column a</td>
<td>91</td>
</tr>
<tr>
<td>Column fractions:</td>
<td></td>
</tr>
<tr>
<td>1 (not adsorbed)</td>
<td>0.43</td>
</tr>
<tr>
<td>2 (adsorbed)</td>
<td>0.98</td>
</tr>
<tr>
<td>1 + 2</td>
<td>63</td>
</tr>
<tr>
<td>1 + 2 heated</td>
<td>0.47</td>
</tr>
<tr>
<td>1 heated + 2</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Urea amidolyase activity was assayed in the complete system listed in Table 1, except [14C]urea specific activity was 1,524 disintegrations/min per nmol.
* A 35 to 45% ammonium sulfate fraction from crude homogenate of urea-grown cells. Two milligrams of protein (equivalent to 171 ml of culture at 200 Klett units) was applied to 2 ml of brushite. Heating was at 100 C for 3 min.

Discrete enzymes with similar properties have been obtained from the green alga C. vulgaris var. viridis (51). Thus, separate carboxylase and lyase enzymes may be a characteristic of green algae, in contrast to yeast systems in which these activities remain together through several stages of purification (39, 56).

Whether allopahante is a free or bound intermediate in Chlamydomonas is uncertain. However, cell extracts incubated with [14C]urea and then immediately chilled to 0 C and subjected to electrophoresis at an alkaline pH show an appreciable accumulation of [14C]jallophanate (Fig. 1A). In addition, urea amidolyase activity exhibits in vitro a nonlinear relationship to extract concentration (Fig. 3A) which is absent from the individual urea carboxylase and allopahante reactions (Fig. 3B, C). This nonlinearity may reflect the time it takes for free allopahante to accumulate to a steady-state concentration. Both of these observations are consistent with readily reversible binding of allopahante to urea carboxylase or allopahante lyase, and they support the notion that allopahante is a free intermediate in cell extracts and, therefore, in the intact cell. Of course, it is also possible that urea carboxylase and allopahante lyase are weakly associated in the cell as a complex, serving to channel allopahante, which is very unstable at neutral and acidic pH.

The non-linearity in the urea amidolyase assay cited above has raised a technical problem. It has been difficult to quantitate this activity from the usual fixed-timed assay. An improvement is obtained if the rate is calculated from some time interval during the reaction when steady state is approached, for instance from 60 to 90 min, but even this has not been entirely satisfactory. For this reason, and also for the sake of economy, a fixed time, usually 60 min, was employed for this work. The values of urea amidolyase reported are therefore minimum estimates. The urea carboxylase and allopahante lyase activities were proportional to the incubation times and enzyme concentrations employed throughout this work.

Table 3. Activity of Chlamydomonas urea amidolyase fractions incubated with [14C]urea and [14C]jallophanate as substrates

<table>
<thead>
<tr>
<th>Extract fraction</th>
<th>Total enzymatic activity a (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35-45% ammonium sulfate a</td>
<td>731</td>
</tr>
<tr>
<td>Brushite column fractions:</td>
<td></td>
</tr>
<tr>
<td>1 (not adsorbed)</td>
<td>31</td>
</tr>
<tr>
<td>2 (adsorbed)</td>
<td>0</td>
</tr>
<tr>
<td>1 + 2</td>
<td>407</td>
</tr>
</tbody>
</table>

* Urea amidolyase ([14C]urea to 14CO2) was assayed in the complete system listed in Table 1, except KCl was omitted and the incubation was for 30 min. [14C]urea specific activity, 1,470 disintegrations/min per nmol. The allopahante lyase assay ([14C]jallophanate to 14CO2) had the following additions in a total volume of 2.5 ml: sodium tricine (pH 8.0), 500 μmol; β-mercaptoethanol, 50 μmol; NaHCO3, 20 μmol; [14C]jallophanate, 1.1 μmol (460 disintegrations/min per nmol); and extract. Incubation was at 25 C for 30 min. One milliunit: 1 nmol of 14CO2 formed per min.
* Prepared from crude homogenate of urea-grown cells and equivalent to 1,500 ml of culture at 178 Klett units.
UREA CATABOLISM IN CHLAMYDOMONAS AND CHLORELLA

5 10 15
DISTANCE TRAVELLED (CM)

exogenous ammonia, induction was prevented (Table 4).

Induction kinetics with Chlamydomonas.
The results of induction of urea amidolyase in ammonia-grown cells during approximately two

FIG. 1. Electrophoretic pattern of products from Chlamydomonas urea amidolyase components incubated with [14C]urea. The components given in the footnotes to Table 1, together with 5 μCi of [14C]urea (43 μCi/μmol) in a total volume of 0.5 ml, were incubated with enzyme at 25 C for 60 min. Heating was at 100 C for 3 min. The reactions were stopped with 4 volumes of ice-cold 95% ethanol, and 20-μl samples were subjected to electrophoresis for 30 min at pH 7.9. The enzymes were as follows: (A, D) 35 to 45% ammonium sulfate fraction, equivalent to 14 ml of culture at 81 Klett units; (B) nonadsorbed fraction, equivalent to 7 ml of culture; (C) adsorbed fraction, equivalent to 10 ml of culture. The incomplete recovery of radioactivity in (A) is due to 14CO2 production.

the maximum specific activity was also short lived. This latter decline may have been caused by an accumulation of ammonia, which has a repressive action (see below). Indeed, if the culture medium had more than about 1 mM

FIG. 2. Identification of [14C]allophanate synthesized with Chlamydomonas extract by (A) co-electrophoresis and (B) co-chromatography with authentic unlabeled potassium allophanate and unlabeled urea before and after hydrolysis with mineral acid. The radioactive material was the product obtained in the enzymatic synthesis of [14C]allophanate. Hydrolysis was with 1 N HCl for 5 min at ambient temperature. Electrophoresis was for 15 min at pH 7.9, and chromatography was in solvent i. The untreated and acid-hydrolyzed samples contained different amounts of radioactivity.
generations are presented in Fig. 5A. For this experiment, the culture was grown initially with 0.5 mM ammonia, a concentration that does not prevent induction (Table 4). Enzyme activity per milliliter of culture increased during a period of exponential growth (about 16 h) and then decreased rapidly as the culture approached the stationary phase. At about this same time, ammonia first appeared in the culture medium (limit of detection: 0.05 mM) and began to accumulate. The near coincidence between exogenous ammonia accumulation and the decreasing rate of urea amidolyase accumulation suggests that the pathway is under control by repression, with ammonia or a metabolic product acting as corepressor. The subsequent decline of enzyme activity may reflect inactivation of urea amidolyase enzyme without concomitant synthesis, or perhaps senescence and death of some cells not accompanied by a decrease in the culture absorbance. If the same data are normalized to unit culture absorbance (not shown), then the specific activity increased for only the first 12 h as the culture absorbance increased about 2.4-fold. This relationship suggests that the induction potential was realized in about one cell generation. Essentially the same temporal relationship among induction of urea amidolyase, growth, and ammonia excre-

FIG. 3. Relationship between Chlamydomonas extract concentration and activities of (A) urea amidolyase, (B) urea carboxylase, and (C) allophanate lyase. The activities were measured on separate occasions with different extracts. All incubations were for 30 min at 25 C. Other assay conditions were as follows: (A) reaction components given in the footnotes to Table 1, [14C]urea (1,383 disintegrations/min per nmol); (B) as for (A) plus 2 mU of Chlorella allophanate lyase (brushite fraction 1); (C) reaction components given in the legend to Table 3, [14C]allophanate (456 disintegrations/min per nmol).

FIG. 4. Specific activity of Chlamydomonas urea amidolyase from cultures induced with various urea concentrations and for various times. (---) Cultures grown on 0.5 mM NH₄NO₃ and urea added to the concentration given after each curve. (-----) Culture grown on 10 mM urea. Urea amidolyase was assayed in the complete mixture of Table 1. [14C]urea specific activity: 1,798 disintegrations/min per nmol. Incubations were for 60 min at 25 C.
tion was obtained with a culture started in urea medium (Fig. 5B).

Since urea amidolyase embraces two enzymes acting in series, induction could be due to accumulation of one or both enzyme activities. The kinetics of induction during one cell generation in which urea carboxylase and allophanate lyase were assayed separately is shown in Fig. 6. For this experiment, *Chlamydomonas* was grown with 5 mM ammonia, suspended in nitrogen-free medium, and incubated for 2 h to deplete endogenous ammonia pools. Prior to adding inducer, there was a small but significant amount of each enzyme (Fig. 6A), and this was increased only slightly during a further 6 h in nitrogen-free medium. Upon addition of urea, both enzyme activities increased with biphasic rate curves; an initial exponential rate gave way to an approximately linear one. Ammonia did not accumulate in the medium, so it is assumed that the culture was under control only by inducer. These kinetics are similar to the induction rate curve for nitrate reductase in *Aspergillus nidulans* (6) and, as pointed out by Cove (7), they contrast with most *Escherichia coli* systems in which the rate of induction is linear and proportional to cell mass.

Some enzyme systems in fungi “induced” by substrate have turned out to be derepressed by the unintentional removal of corepressor (33). Induction, rather than derepression, of urea carboxylase and allophanate lyase in *Chlamydomonas* is demonstrated by the data in Fig. 6A. Incubation in nitrogen-limited medium for 8 h “derepressed” the carboxylase only 1.6-fold and derepressed the lyase only 1.8-fold. With 3 mM urea in the medium of a companion culture, the carboxylase was induced 79-fold and the lyase was induced 26-fold over the derepressed levels of these enzymes. Growth with urea, therefore, involves induction of both enzymes over the basal (derepressed) levels.

The data in Fig. 6 indicate also that the ratio of carboxylase to lyase during induction does not remain constant (Fig. 6B), and that carbox-

---

**Table 4. Ability of urea to induce amidolyase activity in *Chlamydomonas* cultures grown with various concentrations of ammonia**

<table>
<thead>
<tr>
<th>No.</th>
<th>Nitrogen source for growth (initial concn, mM)</th>
<th>Density of culture at time of assay (A)</th>
<th>Urea amidolyase sp act (µU/mg of culture. A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urea (10)</td>
<td>70</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>NH₄NO₃ (0.25)</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>NH₄NO₃ (0.50)</td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>NH₄NO₃ (1.0)</td>
<td>53</td>
<td>0.018</td>
</tr>
<tr>
<td>5</td>
<td>NH₄NO₃ (2.0)</td>
<td>39</td>
<td>0</td>
</tr>
</tbody>
</table>

* Induction of cultures 2 to 5 was for 6 h with 10 mM urea.
  * A, Absorbance measured in Klett units.
  * Urea amidolyase was assayed in the complete system listed in Table 1. Each incubation received the extract of 4 ml of culture. [¹⁴C]urea specific activity, 1,763 disintegrations/min per nmol. One microunit: 1 pmol of [¹⁴C]urea formed per min at 25 °C.

---

**Fig. 5. Urea amidolyase activity and extracellular ammonia concentration during growth of *Chlamydomonas* with urea. (A) Culture started on 0.5 mM NH₄NO₃ and induced with 3 mM urea at time zero. (B) Culture started on 3 mM urea and no more urea added. The enzyme assay components were as given in the footnote to Table 3; [¹⁴C]urea specific activity was 1,738 disintegrations/min per nmol. Incubation was for 60 min at 25 °C.**
urea carboxylase does not begin to accumulate before lyase (Fig. 6A). The variable ratio may be an artifact due to underestimation of carboxylase activity at the lower extreme of carboxylase concentrations. However, the temporal relationship of accumulation appears to be real because both enzymes were detected even in fully repressed cells. This means that changes in either enzyme activity could be detected even though the enzyme assay may not have measured maximum activity. We conclude that carboxylase and lyase are not induced sequentially in the order that they act in the biochemical pathway.

**Repression kinetics with Chlamydomonas.** Early experiments, such as those illustrated in Fig. 5 and Table 4, suggested that ammonia or a product of ammonia depressed or prevented the accumulation of urea amidolyase even if inducer was present in the medium. This could be due to exclusion of inducer from the cell (30) or to repression (32). Permeases have been demonstrated in fungi for ammonia (4, 15) and urea (10), and inhibition of urea uptake by ammonia has been shown for a diatom (14). To determine whether ammonia caused exclusion of urea from the cell, ammonia-grown cultures were pulsed with [14C]urea in the presence and absence of exogenous ammonia, and the amount of [14C]urea uptake and intracellular [14C]urea in the two cultures was compared to the amount of allophanate lyase induced in companion cultures receiving unlabeled urea (Table 5). During the first 30 min, [14C]urea uptake and the intracellular pool of [14C]urea were unaffected by the presence of ammonia in the medium. However, only the culture without ammonia showed induction of lyase. These results indicate that Chlamydomonas can accumulate inducer (or precursor of the inducer) in the presence of ammonia and, therefore, that ammonia acts by an intracellular mechanism.

The second possibility, that of repression, was tested by the classical kinetic approach (32). A culture growing with 3 mM urea was divided; one portion received 5 mM NH4NO3 and the other was left with urea. Ammonia caused an essentially immediate depression of carboxylase and lyase accumulation per milliliter of culture (Fig. 7). The rate of carboxylase accumulation was depressed to essentially zero, whereas lyase was depressed to about 20% of the fully induced rate. This differential effect of ammonia on the rate of carboxylase and lyase accumulation per milliliter of culture is also reflected in the ratio of enzyme activity (Fig. 7, right inset). The “escape” of lyase from complete repression by ammonia may account for the finding that more lyase than carboxylase is found in repressed and derepressed (nitrogen starved) cultures. These findings are reminiscent of carbon catabolite repression without transient repression in bacterial systems (32), and of nitrogen metabolic repression described for Aspergillus nidulans (3). It is not known whether Chlamydomonas possesses nitrogen metabolite repression, a process described as affecting several ammonia-repressible pathways simultaneously (2, 3).

**Regulation of urea amidolyase activities in Chlorella and Chlamydomonas compared.** Thompson and Muenster reported (53) that the
Table 5. Comparison of urea uptake and allophanate lyase induction of ammonia-grown Chlamydomonas in the absence and presence of exogenous ammonia

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>$[^{14}C]$urea uptake (nmol/ml of suspension)</th>
<th>Intracellular $[^{14}C]$ urea (nmol/ml of suspension)</th>
<th>Allophanate lyase activity (mU/ml of suspension)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{-NH_4^+}$</td>
<td>$^{+NH_4^+}$</td>
<td>$^{-NH_4^+}$</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0.013</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>60</td>
<td>0.312</td>
<td>0.021</td>
<td>0.080</td>
</tr>
</tbody>
</table>

* Culture was grown with 5 mM NH$_4$NO$_3$ to 180 Klett units, washed, and suspended to 150 Klett units in nitrogen-free medium ($^{-NH_4^+}$ treatment) or 10 mM NH$_4$NO$_3$ medium ($^{+NH_4^+}$ treatment). Preincubated 15 min.

$[^{14}C]$urea (16,990 disintegrations/min per nmol) added at 0 min to 0.3 mM. Cultures shaken at 27 to 28°C with 500 ft-c (ca. 5,000 lx) of white fluorescent light.

$[^{14}C]$urea (16,990 disintegrations/min per nmol) added at 0 min to 0.3 mM. Cultures shaken at 27 to 28°C with 500 ft-c (ca. 5,000 lx) of white fluorescent light.

$[^{14}C]$urea (16,990 disintegrations/min per nmol) added at 0 min to 0.3 mM. Cultures shaken at 27 to 28°C with 500 ft-c (ca. 5,000 lx) of white fluorescent light.

$[^{14}C]$urea (16,990 disintegrations/min per nmol) added at 0 min to 0.3 mM. Cultures shaken at 27 to 28°C with 500 ft-c (ca. 5,000 lx) of white fluorescent light.

At times indicated, 2.5 ml of suspension was filtered and cell radioactivity was counted. Data are means of duplicates, adjusted for nonspecific binding of $[^{14}C]$urea to freeze-killed cells and for $[^{14}C]$ lost from the cells as $[^{14}C]$CO$_2$.

At times indicated, 2.5 ml of suspension was filtered, the cells were lysed by freeze-thawing in phosphate buffer, and the $[^{14}C]$urea content of lysates was determined from the $[^{14}C]$CO$_2$ released by jack bean urease. Data are means of duplicates, adjusted for $[^{14}C]$CO$_2$ present in minus urease controls.

$[^{14}C]$urea added (to 0.3 mM) at 0 min. Allophanate lyase activity assayed in the reaction system listed in Table 3. Extract from 2 ml of cell suspension and 1 mM $[^{14}C]$allophanate (150 disintegrations/min per nmol) were incubated 60 min at 30°C. Data are means of four assays.

Fig. 7. Repression kinetics of urea carboxylase (UC, left) and allophanate lyase (AL, right) upon addition of ammonia to one-half of a Chlamydomonas culture growing with 3 mM urea. Symbols: ●, no addition; ▲, addition of 5 mM NH$_4$NO$_3$ at the arrow. Left, Growth. Right, Ratio of enzyme activities. UC was assayed with the reaction components given for urea amidolyase in the legend to Table 3, but with $[^{14}C]$urea (1,549 disintegrations/min per nmol) and 3 mU of Chlamydomonas allophanate lyase (brushite fraction I). AL was assayed with the reaction components given in the legend to Table 3, but with 2.5 μmol of $[^{14}C]$allophanate (121 disintegrations/min per nmol). Incubations were for 45 min at 30°C.
specific activity of urea carboxylase in *C. vulgaris* var. *viridis* was greater in urea-grown compared to ammonia-grown cells, whereas the specific activity of allophanate lyase remained unchanged. This response to nitrogen source by *Chlorella* is dramatically different from the *Chlamydomonas* system, and it prompted a comparison between representatives of the two species.

Cultures of *Chlorella* and *Chlamydomonas* were grown organophototrophically to mid-exponential phase, with urea and ammonia as separate nitrogen sources. Since cultures of the two species have different apparent absorbancies, specific activities are expressed on the basis of trichloroacetic acid-precipitable protein left in the extracts after centrifugation. Both species had depressed urea amidolase activity when they were grown with ammonia, and growth with urea induced this activity (Table 6). The induction potential for *Chlamydomonas* was about 200-fold greater than that in *Chlorella*. This difference is due both to the greater level of urea amidolase in repressed *Chlorella* and to the greater level of induced activity in *Chlamydomonas*. A further difference in regulation between the two species is found in the individual enzyme activities. Urea amidolase in *Chlorella* is inducible by virtue of only the carboxylase; the lyase appears to behave as a constitutive enzyme. In *Chlamydomonas*, as shown above, both enzymes are inducible.

These results with *Chlorella* confirm a previous observation (53) and indicate that regulation of urea catabolism in *Chlorella* may be fundamentally different from that in *Chlamydomonas*. It is indeed unfortunate that genetic recombination has not been demonstrated with *Chlorella*, so that the genetic basis for these differences in regulation can be explored only with *Chlamydomonas* at this time.

**DISCUSSION**

There are two potential endogenous sources of urea in algal metabolism (29). One comes from the action of arginase (EC 3.5.3.1) in the arginine catabolic pathway, and the other comes from allantoicase (EC 3.5.3.4) in purine degradation. Sussenbach and Strijkert (47) found that *Chlamydomonas* does not possess arginase and, instead, degrades arginine to citrulline and ammonia. The enzyme involved, arginine deimidase (or desimidase, EC 3.5.3.6), was also described earlier as being responsible for arginine catabolism in *Chlorella* (42). Thus, two representatives of different major taxons within the green algae are not able to form urea from arginine. It remains to be seen if this is a general feature of chlorophycean algae. With respect to urea from purines, Amman and Lynch (1) showed in nutritional studies that a strain of *C. pyrenoidosa* could utilize adenine, hypoxanthine, xanthine, and uric acid as sole nitrogen sources for growth. We have observed in preliminary work (60) that *Chlamydomonas* can utilize xanthine but not adenine; hypoxanthine and uric acid were not tested by us. The usual pathway of uric acid to urea involves the sequential action of uricase (EC 1.7.3.3), allantoinase (EC 3.5.2.5), and allantoicase (EC 3.5.3.4).

**Table 6. Specific activities of enzymes of urea hydrolysis pathway from Chlamydomonas and Chlorella grown with urea or ammonia**

<table>
<thead>
<tr>
<th>Alga</th>
<th>Nitrogen source for growth</th>
<th>Enzymatic activity (μU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urea amidolase</td>
</tr>
<tr>
<td>Chlamydomonas reinhardyi (y-1)</td>
<td>Urea</td>
<td>11,000</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃</td>
<td>15 (2)</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa (3E)</td>
<td>Urea</td>
<td>1,400</td>
</tr>
<tr>
<td></td>
<td>NH₄Cl</td>
<td>370 (10)</td>
</tr>
</tbody>
</table>

*The amounts of culture assayed were: Chlamydomonas, 1 ml at 115 Klett units (urea grown) and 24 ml at 119 Klett units (NH₄⁺ grown); Chlorella, 8 ml at 234 Klett units (urea grown) and 8 ml at 166 Klett units (NH₄⁺ grown). Crude homogenates of Chlamydomonas were prepared by sonic treatment; those of Chlorella were prepared by using a French pressure cell. All incubations were for 60 min at 25°C. Urea amidolase and allophanate lyase were assayed with the reaction components given in footnote a to Table 3. Urea carboxylase was assayed as for amidolase plus either 6 μU (for Chlorella extract) or 9 μU (for Chlamydomonas extract) of allophanate lyase. Substrate specific activities: [1⁴C]urea, 1,417 disintegrations/min per nmol; [1⁴C]allophanate, 456 disintegrations/min per nmol. One microunit: 1 pmol of [1⁴C]CO₂ formed per min.

*Concentrations: Urea, 3 mM; NH₄NO₃, 5 mM; NH₄Cl, 10 mM.

Numbers in parentheses are standard errors of the mean; they are omitted where smaller than the last significant digit.
3.5.3.4). Villeret detected allantoinase activity in tolenized cells of *Chlamydomonas* and *Chlorella*, but did not detect allantoicase in either strain (54). Until the absence of allantoicase is confirmed with other enzyme extraction methods, it remains possible that *Chlorella* and *Chlamydomonas* have the usual pathway for purine catabolism and that the urea amidolyase system plays a role in recycling endogenous purine nitrogen. Substrates for urea amidolyase other than urea have been sought in yeast and *Chlorella*, but none has been found (38, 53, 58).

If urea amidolyase is necessary, or important, in the recovery of nitrogen from endogenous urea, then it is curious that we have not observed conditions other than culturing on urea that give rise to significant levels of the enzymatic activity in *Chlamydomonas*, i.e., autoinduction. A common ploy with *Chlorella* to obtain rapid urea catabolism is to starve the organism of nitrogen for several days (19). Since *Chlamydomonas* is capable of a reproductive cycle involving gametic differentiation and zygote formation in addition to the usual vegetative cycle shared with *Chlorella*, it is conceivable that urea amidolyase in *Chlamydomonas* might be confined to events surrounding sexual differentiation. Nitrogen starvation promotes gametogenesis, and sexual competency is achieved in asychronous cultures during the period of starvation employed here (5 to 8 h [31]), so it is likely that we have already included the early period of the reproductive cycle in our studies. It is also possible that urea amidolyase is significant when *Chlamydomonas* is grown on a nitrogen source other than urea or ammonia, such as nitrate. Unfortunately, the y-1 strain is unable to utilize nitrate.

The urea amidolyase system in *Chlamydomonas*, as in *Chlorella* (51), is shown to consist of two discrete enzymatic activities that carboxylate urea and then hydrolyze it to ammonium ion and bicarbonate. The enzymes, urea carboxylase and allophanate lyase, are readily separated and may be assayed individually in the presence of each other. Urea carboxylase probably requires biotin because it is inhibited by egg white avidin, and it also requires ATP and Mg²⁺. These requirements are consistent with an energy-dependent carboxylation of urea. Allophanate lyase does not appear to have cofactor requirements. These properties of amidolyase systems from two different unicellular algae are similar to those of yeast, except that yeast carboxylase and lyase have not been physically separated from each other with retention of enzymatic activity.

All of the amidolyase systems examined to date are metabolically regulated. Of these, *Chlamydomonas* seems to have one of the largest potentials for response to nutritional conditions. Growth on ammonia allows only a trace of carboxylase and lyase activity, and derepression by nitrogen starvation increases both by less than a factor of two. But in the presence of urea, both enzymatic activities are induced to levels as much as 100 times over the basal or derepressed levels. In addition, ammonia overrides urea when the two are presented to *Chlamydomonas* at the same time. Thus, urea amidolyase is inducible rather than derepressible, and is also subject to repression. This type of dual control has been shown for urea amidolyase in *Candida* (39) and *Saccharomyces* (59), and for nitrite reductase in *Neurospora* (12) as well as for other enzyme systems in euakaryotes, and it has inspired an interesting model (8).

One of the perhaps less interesting ways in which apparent repression can occur is by inducer exclusion (30, 32). In our case this has been made unlikely by showing that [¹⁴C]urea uptake by repressed cells is not reduced by ammonia. A more likely means for repression control is found in recent work on *Aspergillus nidulans* (3, 4), where several ammonia-producing enzyme systems are simultaneously repressed by ammonia, aptly called nitrogen metabolic repression. It may be significant for this argument that, in *Chlamydomonas*, ammonia represses nitrate and nitrite reductases (16) as well as arginine deimidase (45). It remains to be seen whether there is evidence for a common mechanism, perhaps a single kind of macromolecule, coupling these various repressions.

The true inducer may not be urea but a metabolic product of urea. Whitney and Cooper (59) have proposed that yeast urea amidolyase is induced by allophanate, the product of the first enzyme activity.

Control mechanisms operating at the enzyme level have been considered here only briefly. We have no evidence for feedback inhibition of urea carboxylase by ammonia or for inactivation by ammonia such as described by Herrera et al. (16) for nitrate reductase. Ammonium sulfate up to 0.1 M has not been inhibitory to urea carboxylase or allophanate lyase in cell-free systems (Hodson, unpublished observations). It is possible that ATP or the "energy charge" may regulate carboxylase activity. Roon and Levenberg (39) reported that adenosine 5'-diphosphate inhibited the carboxylase in *Candida*, but this has not been investigated further.
One of the questions that has intrigued investigators of controlled systems in eukaryotes is whether the genes specifying those systems occur in operons (33). The kinetic evidence we now have on repression of urea carboxylase and allophanate lyase in Chlamydomonas does not fit the bacterial model (36). Repression quenches the accumulation of carboxylase per milliliter of culture, whereas the lyase continues to accumulate, albeit at a much reduced rate. On the other hand, induction is not obviously sequential in the order that the enzymes function in the pathway, as has been suggested for nitrate and nitrite reductases in the duckweed Lemna minor (44).

ACKNOWLEDGMENTS

We wish to thank John F. Thompson, Cornell University, and R. J. Ellis, Bucknell University, for helpful suggestions, and David E. Sheppard, University of Delaware, for critical discussions concerning the organization of the manuscript. The assistance of Nancy Lomax, Teresa Della-Volpe, Susan Wilson, and Ann Siberski is gratefully acknowledged. This work was supported by grants from the University of Delaware Research Foundation.

LITERATURE CITED


