Urease and ATP:Urea Amidolyase Activity in Unicellular Algae

By J. W. LEFTLEY AND P. J. SYRETT

Department of Botany and Microbiology, University College, Swansea

(Received 3 October 1972; revised 12 February 1973)

SUMMARY

Urease or ATP:urea amidolyase activity was detected in extracts of unicellular algae (representing two phyla and five classes) grown with urea as sole source of nitrogen. All the algae examined that are unequivocally classified as Chlorophyceae, contained ATP:urea amidolyase but no urease. All the other algae contained urease but no ATP:urea amidolyase.

INTRODUCTION

Urea can serve as sole source of nitrogen for many unicellular algae (Thomas, 1968; Naylor, 1970). In algae grown on urea, two enzymes that catabolize urea have been described. They are:

(a) Urease (urea amidohydrolase, EC. 3.5.1.5) which catalyses the hydrolytic cleavage of urea:

(b) ATP:urea amidolyase (UALase) which catalyses the ATP-dependent degradation of urea. Although originally reported to be one enzyme (Roon & Levenberg, 1968) UALase appears to be either two separate enzymes (Thompson & Muenster, 1971) or an enzyme complex (Whitney & Cooper, 1972*a, b*). The two enzymes are urea carboxylase (urea:CO₂ ligase (ADP), EC 6.3.4.6) and allophanate hydrolase (allophanate amido hydrolase, EC 3.5.1.13) which catalyse the reactions:

Because the properties of urease and the UALase system differ so markedly, they are readily distinguished in extracts. UALase requires ATP, Mg²⁺, K⁺ and is inhibited by avidin (Roon & Levenberg, 1968; Rognes, Roon & Levenberg, 1969), a specific inhibitor of carboxylating enzymes which acts by binding the biotin co-enzyme. This inhibition is abolished by excess biotin. The purified enzyme has an absolute requirement for CO₂ (Whitney & Cooper, 1970; Roon & Levenberg, 1970*a*) but this is present in sufficient quantities in crude extracts at alkaline pH. Urease does not require cations and is not stimulated by ATP or inhibited by avidin.

The criteria used to distinguish urease and UALase in this study were: stimulation by ATP, inhibition by avidin and the abolition of this inhibition by excess biotin.

Table 1. *Algae used, with details of origin, growth conditions, culture media and references to original report(s) of growth on urca*

Organism	Strain no., freshwater (F) or marine (M)	Growth conditions: temperature (°C), illumination,* aerated (A)† or static (S), magnetically stirred (M), volume of culture (l)	Reference to medium used	References to original report(s) of growth on urca
Chlorophyta				
Chlorophyceae				
<i>Ankistrodesmus braunii</i>	c202/7c (F)	25° 7.5 klx (A)	Syrett & Morris, 1963	—
<i>Asterococcus superbus</i>	c3/3b (F)	25° 7.5 klx (A)	See Methods	Birdsey & Lynch, 1962; Cain, 1965
<i>Chlamydomonas reinhardtii</i>	2192c (F)x	25° 12.0 klx (A)	Thacker & Syrett, 1972	Sager & Granick, 1953; Cain, 1965
<i>Dunaliella primolecta</i>	c11/34 (M)	15° 7.5 klx (A + M)	ASP2-Provasoli, McLaughlin & Droop, 1957	—
<i>Nannochloris coccoides</i>	c251/1b (F/M)	25° 12.0 klx (A + M)	ASP2	—
<i>Nannochloris oculata</i>	s66 (M)	15° 8.0 klx (A + M)	ASP2	Droop, 1955
<i>Stichococcus bacillaris</i>	c379/5 (M)	25° 12.0 klx (A + M)	1/3 ASP2‡	Ryther, 1954
<i>Scenedesmus basillensis</i>	c276/1a (F)	25° 7.5 klx (A)	Syrett & Morris, 1963	Ellner & Steers, 1955
<i>Scenedesmus obliquus</i>	c276/3a (F)	25° 7.5 klx (A)	Syrett & Morris, 1963	Birdsey & Lynch, 1962
Prasinophyceae				
<i>Tetraselmis subcordiformis</i>	c161/1a (M)	15° 2.0 klx (S)	ASP2	—
<i>Tetraselmis</i> sp.	s221 (M)	15° 8.0 klx (A + M)	ASP2	Turner (unpublished)
Chrysophyta				
Xanthophyceae				
<i>Monodus subterraneus</i>	c848/1 (F)	25° 7.5 klx (A)	5 × Chu no. 10, Miller & Fogg, 1958	Miller & Fogg, 1958
Chrysophyceae				
<i>Monochrysis lutheri</i>	s131 (M)	15° 8.0 klx (A)	S 50, Droop, 1958	Droop, 1955
Bacillariophyceae				
<i>Phaeodactylum tricornutum</i>	c1052/6 (M)	15° 2.0 klx (S)	ASP2	Hayward, 1965

c, from Culture Centre for Algae and Protozoa, Cambridge; s, from Scottish Marine Biological Association Collection; x, see Thacker & Syrett, 1972.

* Continuous; warm white fluorescent tubes.

† Sparged with air + 0.5 % CO₂ about 1 l/min.‡ 1/3 strength with respect to NaCl, MgSO₄, CaCl₂ and KCl.

METHODS

Algae. Fourteen axenic cultures were obtained from the sources shown in Table 1. The Table also gives the conditions of culture.

Media. References giving the culture media used are in Table 1. The original major nitrogen sources were replaced with 0.1 % (16.6 mM) urea which was filter-sterilized and added after autoclaving the medium. In the S 50 medium of Droop (1955), glycyl-glycine was retained as the buffer system since it was not utilized. In the Chu no. 10 $\times$ 5 medium used by Miller & Fogg (1958), $\text{Ca}(\text{NO}_3)_2$ was replaced by CaCl_2 . The medium used to culture *Asterococcus* was (mg/l): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 27; K_2SO_4 , 434; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 493; KH_2PO_4 , 136; citric acid, 3; ferric citrate, 3. Trace elements were added as Arnon's A 5 micronutrient solution, 1 ml/l (Arnon, 1938); 1 ml of Vitamin mix 8/l (Provasoli, McLaughlin & Droop, 1957) was also added because the strain of *Asterococcus* used was found to be auxotrophic. The pH of this medium was 4.5.

With the exceptions of *Monochrysis lutheri* and *Nannochloris oculata*, stock cultures were maintained on a solid enrichment medium, designed to promote vigorous growth and to reveal any contaminants. This contained (g/l): Bacto peptone (Difco), 5.0; glucose, 0.25; yeast extract (Oxoid L 21), 0.1; tryptose (Oxoid L 47), 0.1; beef extract (Oxoid L 29), 0.1; casein hydrolysate (Oxoid L 41), 0.1; anhydrous sodium acetate, 0.1; agar, 15.0. For marine algae this mixture was dissolved in ASP 2 medium (Provasoli *et al.* 1957), final pH, 7 to 8. For freshwater strains the mixture was made up in a solution containing (per l): KNO_3 , 0.2 g; K_2HPO_4 , 0.02 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g; Arnon's A 5 micronutrient solution, 0.5 ml; ferric citrate, 3 mg; citric acid, 3 mg; the final pH was 7 to 8.

Sterility tests. To detect the presence of any microbial contaminants, 0.2 ml portions of culture were taken at the time of harvesting and streaked on plates of the appropriate solid enrichment medium and inoculated into broth (non-solidified enrichment medium). Plates and broth were incubated at 25 °C and periodically inspected for signs of growth. Results obtained from any cultures found to be contaminated were disregarded.

Preparation of extracts. Late log-phase organisms were harvested by centrifuging at 5000 g for 1 to 2 min at 4 °C and the pellet resuspended and washed in three changes of cold, sterile nitrogen-free medium.

The algae were then resuspended in 10 ml of the appropriate ice-cold assay buffer and broken in a French pressure-cell at 12000 lb in⁻². *Phaeodactylum* and *Dunaliella* were broken with an M.S.E.-Mullard ultrasonic disintegrator with a nominal output of 60 W at 20 kHz; the temperature was kept below 3 °C. The resulting broken algal suspension was diluted to the required volume with ice-cold assay buffer and centrifuged at 25000 g for 30 min at 4 °C. Portions (2 ml) of the supernatant were assayed for enzyme activity.

Protein in the supernatant was determined by the method of Lowry, Roseborough, Farr & Randall (1951).

Enzyme assays. The reactions were carried out in 25 ml universal bottles by a method modified from Adams (1971). In each bottle a Durham tube, containing 0.1 ml 40 % (w/v) KOH to absorb ¹⁴CO₂, was suspended above the reaction mixture and the vessel sealed with a no. 41 Suba-seal stopper.

Incubation mixtures for the assays contained the following.

Urease: 270 μmol tris (tris(hydroxymethyl) amino ethane)-HCl buffer, pH 8.4; 3.7 μmol dithiothreitol; 2.7 μmol Na₂EDTA. The following were also added as indicated in Table 2: 5 μmol $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10 μmol KCl; 10 μmol ATP; 50 μg avidin. The final vol. in all assays was 2.7 ml.

Table 2. *Distribution of UALase and urease activity in extracts from unicellular algae*

Enzyme activity is expressed as nmol CO₂ liberated/mg protein during 1h. Boiled enzyme controls and reagent blanks showed negligible activity.

Organisms	Buffer used: UALase (A) Urease (U)	Additions to basal assay buffers*								Enzyme present
		No addition	ATP (10)	ATP (10)		ATP (10)		ATP (10)		
				Mg ²⁺ (5) K ⁺ (10)	Avidin (50 µg)	ATP (10) Avidin (50 µg)	ATP (10) Avidin (50 µg)	Mg ²⁺ (5) K ⁺ (10)		
<i>Ankistrodesmus braunii</i>	A	0.01	5.92	—	—	0.28	5.87	—	—	UALase
<i>Asterococcus superbus</i>	A	0.01	15.36	—	—	2.90	17.20	—	—	UALase
<i>Chlamydomonas reinhardtii</i>	A	0.002	1.45	—	—	0.18	1.31	—	—	UALase
<i>Dunaliella primolecta</i>	A	0.50	11.56	—	—	4.25	11.60	—	—	UALase
<i>Nannochloris coccoides</i>	A	0.003	2.09	—	—	0.35	2.08	—	—	UALase
<i>N. oculata</i>	U	1.38	—	—	1.41	—	—	—	1.51	Urease
<i>Scenedesmus basilensis</i>	A	0.005	1.91	—	—	0.98	—	—	1.90	UALase
<i>S. obliquus</i>	A	0.03	6.15	—	—	2.92	5.98	—	—	UALase
<i>Stichococcus bacillaris</i>	A	0.002	0.78	—	—	0.49	0.77	—	—	UALase
<i>Tetraselmis subcordiformis</i>	U	2.83	—	—	3.13	—	—	—	3.51	Urease
<i>Tetraselmis</i> sp.	U	4.22	—	—	4.28	—	—	—	—	Urease
<i>Monodus subterraneus</i>	U	8.55	—	—	9.92	—	—	—	10.79	Urease
<i>Monochrysis lutheri</i>	U	4.53	—	—	4.59	—	—	—	4.80	Urease
<i>Phaeodactylum tricornutum</i>	U	1.84	—	—	1.89	—	—	—	2.22	Urease

* Figures in parentheses in µmoles unless otherwise indicated; —, not tested.

UALase: 265 μmol tris-HCl, pH 8.4; 3.6 μmol dithiothreitol; 2.6 μmol Na_2EDTA ; 6.6 μmol $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 13.2 μmol KCl. The following were added as required: 10 μmol ATP; 50 μg avidin; 50 μg d-biotin. In all assays the total volume was 2.65 ml. For the two *Nannochloris* spp. tricine (*N*-tris (hydroxymethyl) methyl glycine)-KOH, pH 8.4, replaced tris-HCl since the latter appeared to be inhibitory. This buffer was also used to prepare extracts from *Dunaliella*.

Urease or UALase activity was determined by measuring the quantity of $^{14}\text{CO}_2$ liberated during incubation of the crude enzyme with [^{14}C]urea for 1 h at 30 °C; each treatment was duplicated and denatured enzyme controls always included. After pre-incubating the reaction bottles for 10 min, the reaction was started by injecting 0.2 ml of [^{14}C]urea solution (0.2 μCi and 0.05 μmol) through the rubber seal. The reaction was stopped by injecting 0.2 ml of $\text{M-H}_2\text{SO}_4$. The bottles were then shaken vigorously for several hours to allow release and trapping of all $^{14}\text{CO}_2$. Radioactivity was measured with a Beckman LS 200B liquid scintillation counter. The counts, corrected for background and counting efficiency (75 %), were converted to absolute values and enzyme activity expressed as nmol CO_2 recovered/mg protein during the 1 h assay period.

RESULTS AND DISCUSSION

Table 2 summarizes the results of the enzyme assays. No attempt was made to define the optimum pH and temperature for every preparation and the substrate concentration was not necessarily saturating in every assay. Consequently the figures given should not be taken to represent the maximal enzyme activities. Nevertheless, the results are clear cut for comparative purposes.

With one exception, extracts from those algae belonging to the Chlorophyceae liberated little CO_2 from urea unless ATP was added. This ATP-dependent release of CO_2 was inhibited by avidin and this inhibition could be almost completely abolished by excess biotin. It is reasonable to conclude on these criteria that these algae contain UALase.

In contrast, all other algae examined were found to contain urease. Extracts from these organisms readily liberated CO_2 in absence of ATP, Mg^{2+} and K^+ and addition of these co-factors produced no marked stimulation, nor did avidin inhibit CO_2 production.

Extracts from the two species of *Tetraselmis* at first gave anomalous results. These extracts catalysed a substantial release of CO_2 from urea in absence of any co-factors and this release was markedly stimulated on addition of ATP but was not avidin-sensitive. Subsequent work showed that this apparent stimulation by ATP was due to its ability to chelate Mg^{2+} ions. We conclude that the two species of *Tetraselmis* studied contained urease but that crude extracts from these organisms probably contained sufficient Mg^{2+} to give partial inhibition of the enzyme. The urease from these organisms was particularly sensitive to Mg^{2+} . The results presented in Table 2 for *Tetraselmis* spp. were obtained with extracts from well washed algae; with these the stimulation by ATP was much reduced.

To the number of Chlorophyceae here shown to contain UALase can be added several species of *Chlorella*: *Chlorella ellipsoidea*, *C. pyrenoidosa* (Roon & Levenberg, 1968), *C. vulgaris* var. *viridis* (Thompson & Muenster, 1971) and *C. fusca* var. *vacuolata* (Adams, 1971). Roon & Levenberg (1970b, 1972) found UALase in *Chlamydomonas reinhardtii*, as we did.

Nannochloris oculata was the only member of the Chlorophyceae examined which did not contain UALase. However, the taxonomic position of this organism is now in doubt. It was originally placed in the Chlorophyceae (Droop, 1955) but more recent studies by R.

Guillard (personal communication) show that it does not contain chlorophyll b and is therefore not a chlorophyte. Possibly the presence or absence of urease or UALase could be a useful diagnostic characteristic in taxonomic studies of those chlorophytes of uncertain classification, for example, certain strains of *Nannochloris* and *Stichococcus* which are at present placed in the Chlorophyceae.

All the representatives of the other algal classes – Prasinophyceae, Xanthophyceae, Bacillariophyceae and Chrysophyceae – which we surveyed contained urease. Other algae known to contain urease are: *Ochromonas malhamensis* (Chrysophyceae) (Lui & Roels, 1970) and several members of the Myxophyceae: *Nostoc* sp. (Roon & Levenberg, 1972), *Phormidium luridum* and *Plectonema calothricoides* (Berns, Holohan & Scott, 1966). Roon & Levenberg (1972) also found urease in *Monodus*, as we did, and these workers (1970b) suggest that urease is present in *Euglena gracilis* var. *bacillaris* (Euglenophyceae).

Present knowledge of the distribution of UALase in the plant kingdom is limited. Roon & Levenberg (1972) found UALase only in some yeasts and Chlorophycean algae. However, the Chlorophyceae and yeasts are clearly different when other metabolic patterns are considered, e.g. lysine biosynthesis (Vogel, Thompson & Shockman, 1970). UALase has also been obtained and partially purified from spores of the fungus *Geotrichium candidum* (Shorer, Zelmanowicz & Barash, 1972). The distribution of urease is known to be widespread throughout the plant kingdom (Varner, 1960; Roon & Levenberg, 1972). It is found in bacteria, fungi, yeasts, algae and higher plants and may prove to occur much more commonly than UALase.

We thank Dr M. R. Droop of the Scottish Marine Biological Association, Oban, Argyll, for providing some of the algae used in this study and for helpful advice on their cultivation. One of us (J. W. L.) acknowledges the receipt of a N.E.R.C. studentship.

REFERENCES

- ADAMS, A. A. (1971). *The regulation of nitrogen assimilation in Chlorella*. Ph.D. Thesis, University College of Swansea.
- ARNON, D. I. (1938). Microelements in culture solution experiments with higher plants. *American Journal of Botany* **25**, 322–325.
- BERNS, D. S., HOLOHAN, P. & SCOTT, E. (1966). Urease activity in blue-green algae. *Science, New York* **152**, 1077–1078.
- BIRDSEY, E. C. & LYNCH, V. H. (1962). Utilization of nitrogen compounds by unicellular algae. *Science, New York* **137**, 763–764.
- CAIN, BRO. J. (1965). Nitrogen utilization in 38 freshwater chlamydomonad algae. *Canadian Journal of Botany* **43**, 1367–1378.
- DROOP, M. R. (1955). Some new supra-littoral protista. *Journal of the Marine Biological Association of the United Kingdom* **34**, 233–245.
- DROOP, M. R. (1958). Requirement for thiamine among some marine and supra-littoral protista. *Journal of the Marine Biological Association of the United Kingdom* **37**, 323–329.
- ELLNER, P. D. & STEERS, E. (1955). Urea as a carbon source for *Chlorella* and *Scenedesmus*. *Archives of Biochemistry and Biophysics* **59**, 534–535.
- HAYWARD, J. (1965). Studies on the growth of *Phaeodactylum tricornutum* (Bohlin). I. The effect of certain organic nitrogenous substances on growth. *Physiologia Plantarum* **18**, 201–207.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- LUI, N. S. T. & ROELS, O. A. (1970). The nitrogen metabolism of aquatic organisms. I. The assimilation and formation of urea in *Ochromonas malhamensis*. *Archives of Biochemistry and Biophysics* **139**, 269–277.
- MILLER, J. D. A. & FOGG, G. E. (1958). Studies on the growth of Xanthophyceae in pure culture. II. The relations of *Monodus subterraneus* to organic substances. *Archiv für Mikrobiologie* **30**, 1–16.

- NAYLOR, A. W. (1970). Phylogenetic aspects of nitrogen metabolism in the algae. *Annals of the New York Academy of Sciences* **175**, 511-523.
- PROVASOLI, L., McLAUGHLIN, J. J. A. & DROOP, M. R. (1957). The development of artificial media for marine algae. *Archiv für Mikrobiologie* **25**, 392-428.
- ROGNES, S. E., ROON, R. J. & LEVENBERG, B. (1969). Separation and differential sensitivity toward avidin of carbamyl phosphate synthetase and urea amidolyase. *FEBS Letters* **3**, 233-236.
- ROON, R. J. & LEVENBERG, B. (1968). An adenosine triphosphate-dependent avidin-sensitive enzymatic cleavage of urea in yeast and green algae. *Journal of Biological Chemistry* **243**, 5213-5215.
- ROON, R. J. & LEVENBERG, B. (1970a). CO₂ fixation and the involvement of allophanate in the biotin-enzyme-catalyzed cleavage of urea. *Journal of Biological Chemistry* **245**, 4593-4595.
- ROON, R. J. & LEVENBERG, B. (1970b). ATP:urea amidolyase (ADP) (*Candida utilis*). In *Methods in Enzymology* **17** A, 317-324.
- ROON, R. J. & LEVENBERG, B. (1972). Urea amidolyase. I. Properties of the enzyme from *Candida utilis*. *Journal of Biological Chemistry* **247**, 4107-4113.
- RYTHER, J. H. (1954). The ecology of phytoplankton blooms in Moriches Bay and Great South Bay, Long Island, New York. *Biological Bulletin Marine Biological Laboratory, Woods Hole, Massachusetts* **106**, 198-209.
- SAGER, R. & GRANICK, S. (1953). Nutritional studies with *Chlamydomonas reinhardtii*. *Annals of the New York Academy of Sciences* **56**, 831-838.
- SHORER, J., ZELMANOWICZ, I. & BARASH, I. (1972). Utilization and metabolism of urea during spore germination by *Geotrichum candidum*. *Phytochemistry* **11**, 595-605.
- SYRETT, P. J. & MORRIS, I. (1963). The inhibition of nitrate assimilation by ammonium in *Chlorella*. *Biochimica et biophysica acta* **67**, 566-575.
- THACKER, A. & SYRETT, P. J. (1972). The assimilation of nitrate and ammonium by *Chlamydomonas reinhardtii*. *New Phytologist* **71**, 423-433.
- THOMAS, W. H. (1968). Nutrient requirements and utilization: Algae. In *Metabolism*, pp. 210-228. Edited by P. L. Altman and D. S. Dittmer. Bethesda, Maryland: Federation of American Societies for Experimental Biology.
- THOMPSON, J. F. & MUENSTER, A.-M. E. (1971). Separation of the *Chlorella* ATP:urea amidolyase into two components. *Biochemical and Biophysical Research Communications* **43**, 1049-1055.
- VARNER, J. E. (1960). Urease. In *The Enzymes*, 2nd ed., vol. IV, pp. 247-256. Edited by P. Boyer, H. Lardy and K. Myrback. New York: Academic Press.
- VOGEL, H. J., THOMPSON, J. S. & SHOCKMAN, G. D. (1970). Characteristic metabolic patterns of prokaryotes and eukaryotes. *Symposium of the Society for General Microbiology* **20**, 107-120.
- WHITNEY, P. A. & COOPER, T. G. (1970). Requirement for HCO₃⁻ by ATP:urea amidolyase in yeast. *Biochemical and Biophysical Research Communications* **40**, 814-819.
- WHITNEY, P. A. & COOPER, T. G. (1972a). Urea carboxylase and allophanate hydrolase. Two components of adenosine triphosphate: urea amidolyase in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **247**, 1349-1353.
- WHITNEY, P. A. & COOPER, T. G. (1972b). Urea carboxylase and allophanate hydrolase: Two components of a multienzyme complex in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications* **49**, 45-51.