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UTILIZATION AND METABOLISM OF UREA DURING SPORE GERMINATION BY *GEOTRICHUM CANDIDUM*

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Abstract—Urea can serve as a sole N_2 source for spore germination of *Geotrichum candidum* which lacks urease. However, when endogenous urea concentration exceeded 50 μmole/g dry wt., a significant inhibition of protein synthesis and spore germination occurred. At external urea concentration of 5×10^{-3} M, approximately 95% of the urea-^{14}C assimilated by the spores evolved as $^{14}CO_2$. Urea-^{14}C incorporation showed temperature and pH dependence and obeyed saturation kinetics. The uptake of urea was completely inhibited by 10^{-3} M NaN_3. A sharp increase in the accumulation of endogenous urea was detected when extracellular urea exceeded 6×10^{-2} M. The latter increase was proportional to external substrate concentration until it reached about $1{\cdot}5 \times 10^{-1}$ M. Most of the labelled C incorporated into germinated spores during 15 min feeding period with either urea-^{14}C or $NaH^{14}CO_3$ (with ammonia), was located in the cationic fraction. However, whereas citrulline, aspartate and glutamate were concurrently labelled immediately following $NaH^{14}CO_3$ incorporation, citrulline was preferentially labelled when the germinated spores were fed with urea-^{14}C. ATP:urea amidolyase (UALase) was purified approximately 126 fold from germinated spores of *G. candidum*. The enzymes had an optimum pH of 7·8 and the Km values for ATP and urea were $1\text{–}3 \times 10^{-3}$ M and $1{\cdot}4 \times 10^{-4}$ M respectively. Avidin and KF were potent inhibitors of UALase activity. Attempts to demonstrate either enzyme induction by urea or enzyme repression by ammonia gave negative results.

INTRODUCTION

UREA is generally known as an intermediate product in the enzymatic degradation of arginine, purines and pyrimidines.[1] The occurrence of free urea in fungi is well documented,[1,2] and in some fungi (e.g. fruiting bodies of higher basidiomycetes) it constitutes a major component of the soluble N_2 fraction representing about 6% of the total dry wt.[3,4]

Urea can also serve as a utilizable N_2 source for fungi[2] as well as many other microorganisms and plants.[1,5] In most of these organisms, urea metabolism is presumed to involve hydrolysis by urease to CO_2 and NH_3, and subsequent incorporation of the NH_3 into amino acids. However, urea could also function as a sole N_2 source for growth of yeasts and unicellular green algae which lack detectable urease activity.[6–8] Roon and Levenberg[9] have recently demonstrated that some of these organisms possess an inducible biotin enzyme, ATP:urea amidolyase (UALase) which catalyses the cleavage of urea to CO_2 and ammonia. The reaction requires K^+, Mg^{2+}, and bicarbonate.[9,10]

[1] H. REINBOTHE and K. MOTHES, *Ann. Rev. Plant Physiol.* **13**, 129 (1962).
[2] V. W. COCHRANE, *Physiology of Fungi*, p. 278, Wiley, New York (1958).
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[4] N. N. IVANOW, *Biochem. Z.* **192**, 36 (1928).
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[9] R. J. ROON and B. LEVENBERG, *J. Biol. Chem.* **243**, 5213 (1968).
[10] R. J. ROON and B. LEVENBERG, *J. Biol. Chem.* **245**, 4593 (1970).

Preliminary studies[11] have shown that urea could be metabolized by spores of *Geotrichum candidum* without the presence of urease. Urea was also found to increase the specific activities of certain enzymes related to the formation or utilization of carbamyl phosphate.[11] The present report is concerned with the assimilation and metabolism of urea-^{14}C in germinating spores of *G. candidum*.

RESULTS

The Effect of Urea on Germination

Results summarized in Table 1 showed that urea could be utilized as a sole N_2 source for germination of *G. candidum*. The rate of germination in the presence of 0·035 M urea was slightly higher than in the presence of an equivalent-nitrogen amount of NH_4Cl but substantially lower than with 0·3% yeast extract. However, a significant inhibition of germination occurred when the intracellular level of free urea exceeded 0·05 μmole/mg dry wt. (Fig. 1).

TABLE 1. THE EFFECT OF UREA ON GERMINATION OF *G. candidum*

Nitrogen source	Germination %	Mean dry wt. after 3·5 hr (mg/flask)
	0	32·2
Urea	50	57·7
Ammonium chloride	40	51·2
Citrulline	70	59·0
Yeast extract (0·3%)	95	70·7

* Spores (25 mg) were germinated in duplicate culture flasks containing 50 ml of sterile glucose (0·5%) yeast extract (0·01%) medium. Urea, NH_4Cl and citrulline were added as nitrogen sources at nitrogen-equivalent amounts (0·07 M).

The inhibitory effect of urea took place under optimal conditions for germination and was proportional to the amount of endogenous urea until it reached approximately 0·2 μmole/mg dry wt.

The possibility that urea accumulation inhibits protein synthesis was tested in the following experiment. Spores were germinated in 50 ml yeast-extract glucose medium (YEG) for 3 hr in the presence of 0, 0·035 M and 0·2 M urea respectively. After being harvested, the spores were washed with excess of distilled water and resuspended in the same volume of 0·05 M phosphate buffer, pH 7·5, which contained H^3-phenylalanine (0·3 μCi/ml). The incorporation of the labelled amino acid into the protein as a function of time was determined as described elsewhere.[12] Incubation was carried out on a shaker at 30°. The rate of radioactivity incorporated into the spores which were pregerminated with 0·035 M urea or without urea was similar and reached $4·5 \times 10^2$ counts/min/mg protein after 1 hr. In contrast, only a trace of radioactivity was detected after 2 hr in the protein fraction of spores which were preincubated with 0·2 M urea.

[11] I. BARASH and I. ZELMANOWICZ, *Israel J. Chem.* **8**, 140 (1970).
[12] I. BARASH, M. L. CONWAY and D. H. HOWARD, *J. Bacteriol.* **93**, 656 (1967).

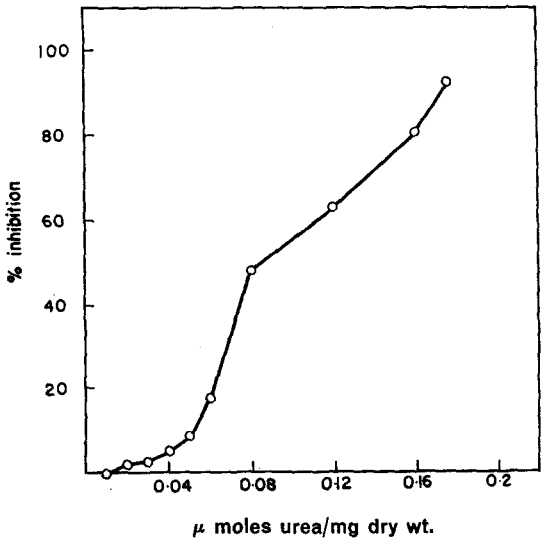

FIG. 1. THE RELATIONSHIP BETWEEN ENDOGENOUS UREA CONCENTRATIONS AND INHIBITION OF GERMINATION. SPORES WERE GERMINATED IN YEG MEDIUM AT 30° IN THE PRESENCE OF VARIOUS UREA CONCENTRATIONS (0–0·2 M). THE AMOUNT OF ENDOGENOUS UREA AND GERMINATION WERE DETERMINED AFTER 3·5 hr AS DESCRIBED IN EXPERIMENTAL.

Uptake of Urea-^{14}C by Spores of G. candidum

Preliminary experiments revealed that feeding of urea-^{14}C (5×10^{-3} M, 1 μCi/ml) to germinating spores results in the release of 90–95% of the labelled carbon as $^{14}CO_2$. In order to follow the fate of the labelled carbon inside the spores, optimal conditions for net uptake (i.e. influx minus efflux of ^{14}C) as well as the nature of urea uptake have been investigated.

The rate of urea-^{14}C incorporation into spore constituents was markedly affected by physiological conditions. Preincubation of conidia with glucose, as described in 'Experimental', caused an increase of about 4 fold in the incorporation of labelled C. Addition of 0·035 M urea to the glucose medium did not induce the rate of incorporation. The effect of pH on the net uptake of urea-^{14}C is illustrated in Fig. 2. Following preincubation with glucose, the cells were washed with distilled water and then suspended in solutions that varied from pH 3–9. The incorporation was started by addition of urea-^{14}C. The pH of each suspension was determined at the beginning and on termination of the experiment. The incorporation was maximal at pH 5 but the rates were still more than half of the maximal at pH values of 2·5 and 6·5. Higher rate of uptake was observed with phosphate ion as compared to citrate or acetate.

Results in Fig. 3 indicate that the rate of urea uptake is highly temperature dependent. The rate increased with increasing temperature to a maximum at 32°. The Q_{10} between 22° and 32° was about 2·8. A typical saturation curve was obtained when the effect of urea concentration on net uptake was investigated. The rate of incorporation approached saturation at the concentration of 6–7 $\times 10^{-2}$ M urea. Further studies were aimed at determining the involvement of metabolic energy in the uptake process. Sodium azide at the concentration of 10^{-3} M blocked both the incorporation of urea-^{14}C into the spores (Fig. 4) and the release of $^{14}CO_2$.

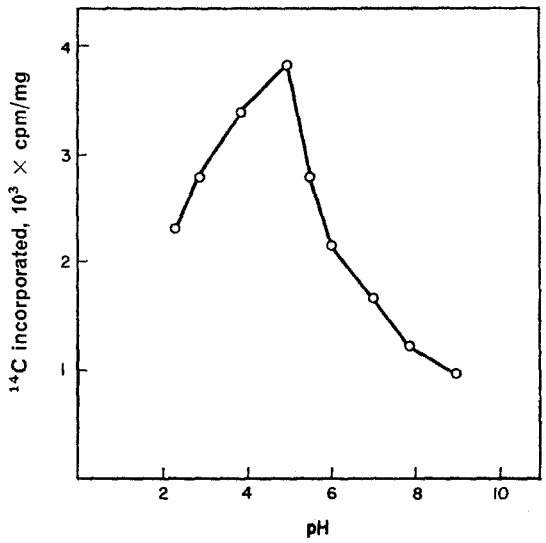

FIG. 2. INFLUENCE OF pH ON THE INCORPORATION OF UREA-^{14}C. PROCEDURES FOR UREA-^{14}C INCORPORATION AND MEASUREMENTS OF RADIOACTIVITY WERE DESCRIBED IN EXPERIMENTAL. THE FINAL TEST SYSTEM CONTAINED 5×10^{-4} M UREA-^{14}C (0·5 μCi/ml), 0·05 M KH_2PO_4–K_2HPO_4, pH 5, and 0·5 mg spores/ml. INCUBATION WAS CARRIED OUT AT 30°.

Metabolism of Urea-^{14}C in Germinating Spores

After feeding urea-^{14}C to germinating spores for various periods of time (Table 2), 73–86 per cent of the radioactivity appeared in the ethanolic extract. Results in Table 2 show that more than 84 per cent of the labelled carbon was recovered in the cationic fraction. Changes with time of the radioactivity distribution in the cationic compounds was followed

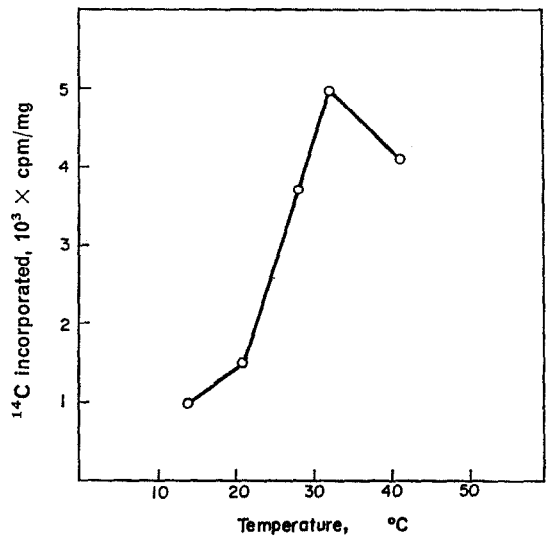

FIG. 3. INFLUENCE OF TEMPERATURE ON THE RATE OF UREA-^{14}C INCORPORATION. UPTAKE CONDITIONS WERE AS IN FIG. 2.

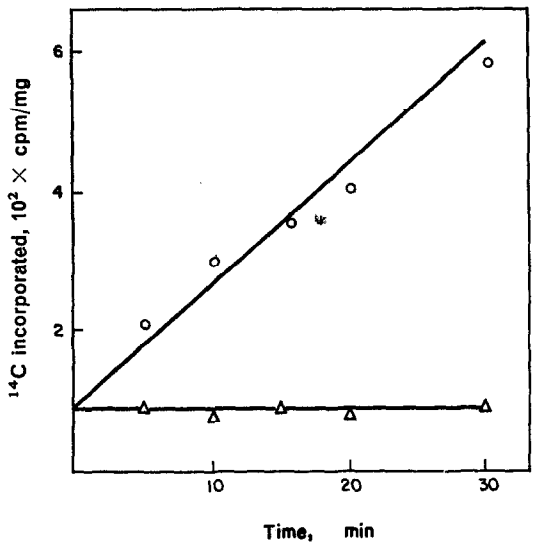

FIG. 4. EFFECT OF NaN_3 (10^{-3} M) ON THE INCORPORATION OF UREA-^{14}C. FEEDING CONDITIONS WERE AS IN FIG. 2.

○—With NaN_3; △—without NaN_3.

by paper chromatography (Fig. 5). Citrulline was the only labelled compound after 1 min of incorporation, citrulline and aspartate appeared labelled after 5 min, and glutamate-^{14}C was detected after 10 and 15 min. The greatest amount of radioactivity was always present in citrulline.

Since $^{14}CO_2$ had been detected within the first min of urea-^{14}C incorporation, it was considered an early product of urea metabolism. Therefore, it was of interest to compare the incorporation pattern of urea-^{14}C to that of $^{14}CO_2$ in the presence of ammonia. Results in Fig. 6 show that citrulline, aspartate and glutamate were concurrently labelled within 2 min exposure to $NaHC^{14}O_3$. No preferential incorporation to one of these compounds was observed. Under conditions used in Fig. 6, the amount of incorporated radioactivity and the distribution of ^{14}C in the different fractions were similar to that of Table 2.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN ETHANOLIC EXTRACTS OF GERMINATING SPORES AFTER FEEDING WITH UREA-^{14}C*

Time (min)	Total radioactivity† Count/min/mg dry wt.	% Recovery in various fractions Cationic	Anionic	Nonionic
1	2200	94·2	1·9	3·9
5	9600	91·6	5·0	3·4
10	10,800	92·0	6·0	2·0
15	7525	84·1	13·1	2·8

* Urea-^{14}C (5×10^{-3} M, 4 μCi/ml) was fed to spores which were pregerminated for 3·5 hr in YEG medium with 0·035 M urea. Germination, feeding and fractionation procedures are described in Experimental.

† Represents approximately 5% of the metabolized urea-^{14}C. The rest of the labelled carbon evolved as $^{14}CO_2$.

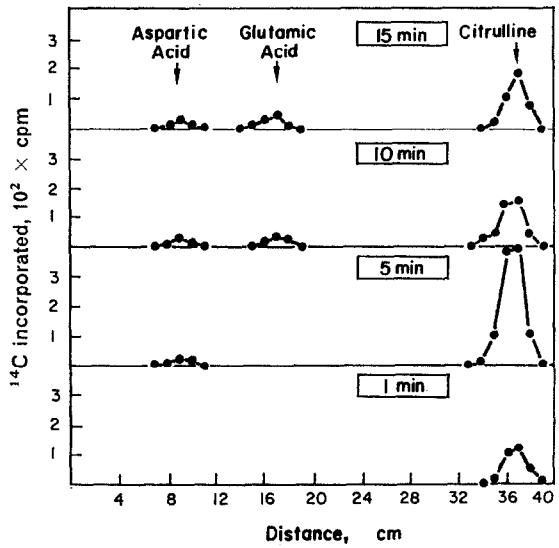

FIG. 5. THE DISTRIBUTION PATTERN OF RADIOACTIVITY IN THE CATIONIC FRACTION AFTER FEEDING OF UREA-^{14}C TO SPORES. CONDITIONS FOR GERMINATION AND INCORPORATION WERE THE SAME AS IN TABLE 2. FOR PROCEDURE OF RADIOPAPERCHROMATOGRAPHY, SEE EXPERIMENTAL. THE SOLVENT USED WAS 80% PHENOL.

Purification and Properties of Urea-amidolyase (UALase) of G. candidum

Previous results[11] have shown that *G. candidum* lacks urease, yet urea-^{14}C was readily metabolized to $^{14}CO_2$. Further experiments revealed activity of UALase in cell-free extracts from this fungus. The following experiments were undertaken to isolate and characterize the enzyme of *G. candidum* which is responsible for the breakdown of urea.

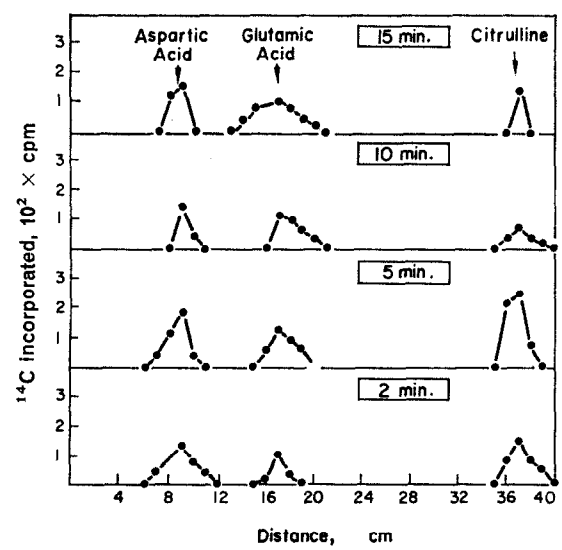

FIG. 6. THE DISTRIBUTION PATTERN OF RADIOACTIVITY IN THE CATIONIC FRACTION AFTER FEEDINGS OF $NaH^{14}CO_3$ TO SPORES. CONDITIONS WERE THE SAME AS IN FIG. 5 EXCEPT THAT THE INCORPORATION MEDIUM CONTAINED 1 μCi $NaH^{14}CO_3$/ml, 0·01 N NH_4Cl and 0·05 M KH_2PO_4–K_2HPO_4 pH 6·5.

TABLE 3. SUMMARY OF ENZYME PURIFICATION

Fraction	Volume (ml)	Protein (mg/ml)	Enzyme activity* (counts/min/ml)	Specific activity
Crude extract	150	20·1	6834	340·0
105,000 *g* supernatant	120	9·0	7140	793·3
$(NH_4)_3SO_4$ (30%–50%)	12	38·8	62,195	1602·9
DEAE-cellulose	10	1·8	76,500	42,500·0

* Activity expressed as $^{14}CO_2$ evolved (counts/min) per 15 min under conditions described in Experimental.

A procedure adopted from Roon and Levenberg[13] resulted in a 126-fold purification of UALase (Table 3). Enzyme recovery was approximately 75 per cent. The greatest purification (26-fold) was achieved by gradient elution from DEAE-cellulose (Fig. 7). The enzyme was eluted from the column when KCl concentration approached 0·2 M. The recovery of the enzyme during the step of column chromatography was 100 per cent.

The optimum pH in Tris–HCl buffer was about 7·8. Enzyme activity was dependent on the presence of ATP in the medium which had a K_m of 1–3 $\times$ 10^{-3} M. The K_m for urea (Fig. 8) was calculated as 1·4 $\times$ 10^{-4} M. Both avidin (50 μg/ml) and KF (10^{-3} M) completely inhibited enzyme activity.

Similar levels of UALase were observed in resting spores as well as in germinating spores (300–800 counts/min of $^{14}CO_2$ evolved/mg protein/15 min). No significant induction or repression could be detected in the presence of urea or NH_4Cl respectively during germination. However, the levels of UALase in the spores were affected by growth conditions. Spores

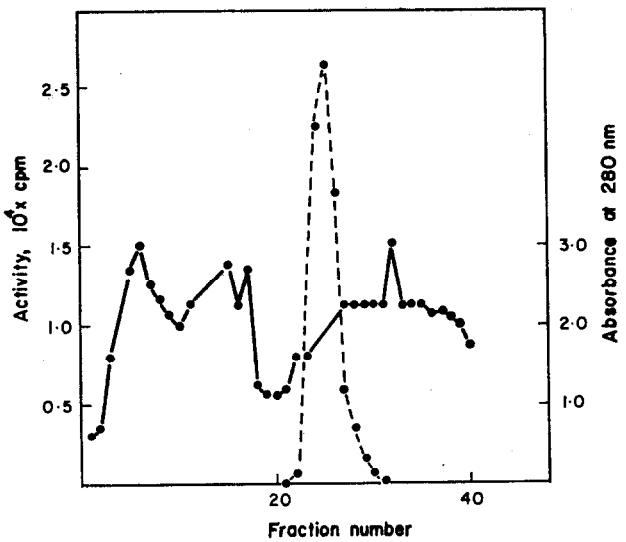

FIG. 7. CHROMATOGRAPHY OF UREA AMIDOLYASE ON DEAE CELLULOSE. FOR PROCEDURE, SEE EXPERIMENTAL.

●– – Enzyme activity; ●— Protein.

[13] R. J. ROON and B. LEVENBERG, *Methods in Enzymology*, Vol. 17, pp. 317–324, Academic Press, New York (1970).

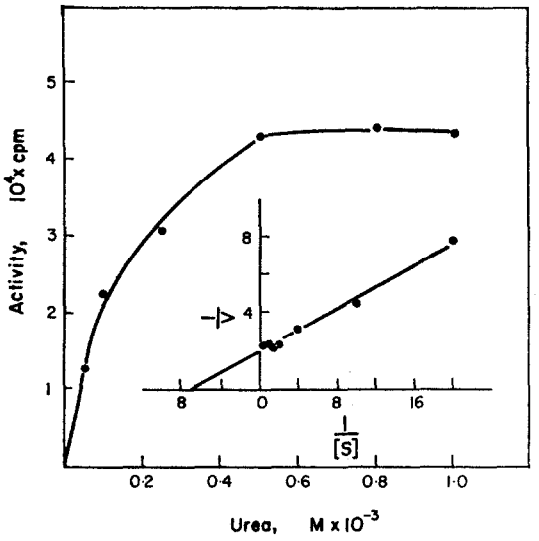

FIG. 8. EFFECT OF UREA CONCENTRATION ON ACTIVITY OF UALASE.

obtained from slow growing cultures (i.e. 0·05 M of urea, NH_4Cl, or glutamine respectively as the sole N_2 source) showed significantly lower levels than spores obtained from rapidly growing YEG cultures.

DISCUSSION

The influx of urea into spores of *G. candidum* has to be considered in respect to the extracellular concentration of urea. Under physiological conditions, where urea did not exert significant inhibition on germination (< 0·06 M), urea transport is assumed to be mediated by a system that has enzyme-like properties. The latter premise was supported by the observations that urea-^{14}C incorporation obeyed saturation kinetics and was dependent on temperature with a Q_{10} value of 2·8 (Fig. 3). The complete inhibition of urea uptake by catalytic amounts of NaN_3 (Fig. 4) and the stimulating effect of preincubation with glucose on the incorporation rate, suggests an involvement of metabolic energy in the above mentioned process. Active transport of urea was reported in animal cells[14,15] and in the alga *Chlorella ellipsoidea*.[8,16] The rate of urea incorporation in *G. candidum* was higher in mild acidic conditions with an optimum at pH 5 (Fig. 2).

When the concentration of extracellular urea exceeded 0·06 M, a sharp increase in the amount of free intracellular urea was detected. The increase in endogenous urea was proportional to the concentration of external urea until approximately 0·15 M substrate was present in the medium. The last phenomenon took place presumably due to the deleterious effect of the high urea concentration on the transport system as it had been observed for protein synthesis and spore germination (Fig. 1).

The present work indicates that spores of *G. candidum* are capable of utilizing urea as a sole nitrogen source (Table 1) by means of UALase. The latter enzyme has been characterized as UALase[9] since it catalysed an ATP-dependent cleavage of urea to CO_2 and NH_3, and

[14] B. SCHMIDT-NILSEN, *Am. J. Physiol.* **170,** 45 (1952).
[15] H. H. USSING and B. JOHANSEN, *Nephron* **6,** 317 (1969).
[16] A. HATTORI, *Plant and Cell Physiol.* **1,** 107 (1960).

showed remarkable sensitivity to inhibition by avidin. The partially purified enzyme (126-fold) of *G. candidum* showed a pH optimum at 7·8, a $K_m = 1{\cdot}4 \times 10^{-4}$ M for urea, and was inhibited by 10^{-3} M of KF. The investigated properties as well as the behaviour of the enzyme during the purification procedure, (Table 3) were similar if not identical to those reported by Roon and Levenberg[9,13] for *C. utilis*.

In contrast to the UALase of *C. utilis*[13,17] the enzyme of *G. candidum* was neither inducible nor repressible by urea or ammonia respectively. The amount of enzyme during germination was affected by growth conditions and was generally higher under optimal germination. Relatively high levels of UALase could be detected also in resting spores. In this connection, it is of interest to note that we could detect free urea in resting spores under the conditions employed for sporulation. The last observation and the fact that urea accumulation exerts a toxic effect on germination (Fig. 1) suggests that the primary function of UALase in spores of *G. candidum* might be the removal of excessive metabolic urea rather than its utilization as an external nitrogen source.

Based on the action pattern of UALase and the early detection of $^{14}CO_2$ during urea-^{14}C assimilation (Table 2), CO_2 might be considered as the first product of urea catabolism in *G. candidum*. Therefore, the observed preferential incorporation of urea carbon to citrulline (Fig. 5) as compared to $NaH^{14}CO_3$ in the presence of ammonia (Fig. 6) was unexpected. Similar incorporation results had led Kating[7] to implicate carbamyl phosphate (CAP) as an intermediate of urea cleavage in *Torulopsis utilis*, however, later work[5,9] conclusively eliminated such a possibility. Formation of allophanate (*N*-carboxyurea) from urea and bicarbonate as an intermediate product of UALase activity has been recently demonstrated by Roon and Levenberg.[10] This compound could also be detected free in the reactions mixture during the catalysis of urea breakdown by UALase.[10] Although the exact mechanism of allophanate decomposition to CO_2 and ammonia is not yet known, carbamate can be postulated as a reasonable intermediate. Urea was previously reported[11] to induce the synthesis of CAP in *G. candidum* spores by an ammonium-dependent reaction. The latter enzyme was identified as carbamate kinase[18] (CPKase) (Barash, unpublished results). Thus the utilization of carbamate for citrulline formation by means of CPKase and ornithine transcarbamylase can be postulated in *G. candidum*. The above pathway implies that urea carbon could be channelled to citrulline via carbamate before its release as CO_2. Since the incorporation of urea-^{14}C to aspartate and glutamate apparently involves $^{14}CO_2$ fixation,[19] its channelling to citrulline might precede the other two compounds. $^{14}CO_2$ can also be incorporated to CAP by means of glutamine-dependent CAP synthetase[11] and thus contribute to the higher incorporation rate observed for citrulline (Fig. 5).

Because most of the assimilated urea-^{14}C is evolved as $^{14}CO_2$, it is difficult to attribute any metabolic significance to the above mentioned pathways, as far as urea carbon is concerned. Perhaps they play a more efficient role in the reutilization of the urea N_2. In *T. utilis*, for instance, which also lacks urease, the NH_3 was not liberated during urea utilization.[7]

EXPERIMENTAL

Germination procedure. Conidia of *G. candidum* (culture No. 205) were harvested from a liquid culture containing 0·5% yeast extract and 1% glucose as described previously.[20] The spores were washed 2 × H_2O by resuspension in 50 vol. H_2O following centrifugation for 5 min at 6000 *g*. Unless otherwise stated,

[17] R. J. Roon and B. Levenberg, *Fed. Proc.* **28**, 668 (1969).
[18] S. M. Kalman and P. H. Duffield, *Biochem. Biophys. Acta*, **92**, 498 (1964).
[19] R. V. F. Lachica, *Enzymologia* **34**, 281 (1968).
[20] I. Barash, *Phytopathology* **58**, 1364 (1968).

spores were germinated in an autoclaved medium (YEG) containing 0·3% yeast extract, 0·5% glucose and 0·05 M phosphate buffer (pH 7·5). The spores were adjusted to a final concentration of approximately 0·5 mg dry wt./ml. Germination was carried out in Erlenmeyer flasks (1–4 l., depending upon the experiment) in a reciprocal shaker at 30°. After about 3·5 hr incubation period during which approximately 90% germination was reached, the spores were harvested in a Buchner funnel with Ederol No. 15 filter paper and washed thoroughly with H_2O without being allowed to dry. The germinated spores were used either immediately for urea-^{14}C or $^{14}CO_2$ incorporation experiments, or were maintained frozen until used for the preparation of cell free extracts.

Incorporation of urea-^{14}C and $NaH^{14}CO_3$ into spores. Prior to incorporation of labelled substances, spores were either germinated as described earlier, or shaken in 0·5% glucose solution for 60 min at 30°. After several washings with H_2O, the conidia were resuspended (0·5 mg/ml) in 10 ml of 0·05 M KH_2PO_4–K_2HPO_4, pH 5·0, and equilibrated at 32° for 15 min before introducing the labelled compound. Incorporation took place on a shaker at 32° for 20 min. When feeding urea-^{14}C, the incorporation was stopped by adding 20 ml of cold NaN_3 solution (0·1 M) followed by rapid centrifugation at 0° (35,000 *g* for 10 min). The spores were carefully washed with H_2O and 1 ml of suspension (0·5–1 mg/ml) was then filtered through Whatman glass fibre paper, CF/C. For measuring radioactivity, the dried filter papers were introduced into counting vials with toluene counting solution. Under these conditions, urea-^{14}C uptake was a linear function of time. Similar procedure was also used for $NaH^{14}CO_3$ except that the incorporation was carried out at pH 6·5 and was terminated by adding 1 ml of 6 N HCl with a few pieces of solid CO_2.

Preparation of cell free extract and purification procedure. The spores were taken in 50 mM tris–HCl buffer (pH 7·8) which contained 10%, v/v glycerol and 10 mM $MgSO_4$. The cells (40–60 mg/ml) were disrupted with a precooled Aminco French Pressure Cell at 15,000 lb/in^2. All the following procedures were performed at 4°. Cell debris were removed by centrifugation at 27,000 *g* for 15 min. The purification steps were essentially according to Roon and Levenberg.[13] The supernatant fluid was centrifuged at 105,000 *g* for 45 min and the proteins were precipitated by $(NH_4)_2SO_4$ between 30–50% saturation. The precipitate was dissolved in 10–13 ml of 50 mM Tris–HCl buffer, pH 8·0 containing 10% (v/v) glycerol and 1 mM mercaptoethanol, and dialysed overnight against the same buffer. The enzyme preparation was then placed on a column (2 × 15 cm) of DEAE-cellulose (Whatman DE-52) previously equilibrated with the latter buffer. Enzyme solution was carried out by gradient increase in KCl concentration from 0 to 0·5 M, each reservoir containing 250 ml of KCl–buffer–glycerol solution.

Determination of UALase activity. Enzyme assay was based on the method described by Roon and Levenberg.[13] Incubation was carried out for 15 min at 30° in 15 ml Warburg vessels. The main compartment contained the following components for a final volume of 0·7–0·8 ml; Tris–HCl pH 7·8, 30 μmole; KCl, 60 μmole; $MgSO_4$, 3 μmole; ATP, 1 μmole; Urea-^{14}C, 2 μmole (0·5 μCi). The side arm contained 0·2–0·3 ml enzyme preparation, and the centre well contained 0·2 ml of 15% KOH on 2 × 1 cm^2 filter paper. Reaction mixture of heat denaturated enzyme served as a control. Reaction was started by tipping the enzyme to the main compartment, and stopped by the addition of 0·2 ml of 4 N HCl. After shaking the closed vessel gently for another 30 min, the filter paper was dried and kept for counting radioactivity as described earlier.

Extraction and fractionation procedures. Spores (2 mg/ml) were extracted with boiling 80% EtOH for 10 min. The suspension was centrifuged and the precipitate was re-extracted with EtOH. The supernatant fluids were combined and labelled as the soluble fraction. The soluble fraction was concentrated under reduced pressure (30–40 mm) at 50° to 10 ml. The solution was first passed through a column (1 × 10 cm) of Dowex 50 resin (H^+ form) and then through Dowex 1 resin (formate form). The effluent of the latter column was labelled as the neutral fraction. Compounds adsorbed by the Dowex 50 column were eluted with 6 N NH_4OH to obtain the cationic fraction, and the Dowex 1 column was eluted with 4 N HCOOH to obtain the anionic fraction. All fractions were taken to complete dryness by procedures described above and the residue from each fraction was taken up in 2–3 ml H_2O. The solutions were stored at −10° until used for paper chromatography and counting radioactivity.

Chromatographic and analytical procedures. The solvents used for paper chromatography of the cationic fraction were: (1) *n*-BuOH–HOAc–H_2O 4:1:5 v/v; and (2) 80% phenol. Amino acids were detected on chromatograms by ninhydrin after 16-hr incubation period. For radioactivity determination, the sprayed and dried chromatograms were cut into strips of 1 × 1·5 cm and introduced into vials containing 10 ml of toluene solution. The identification of each spot was performed by co-chromatography with authentic samples. Radioactivity was measured with a Packard Model 3375 Liquid Scintillation Spectrometer. All counts were corrected for quenching by an automatic external standard method.

Protein was determined by the method of Lowry *et al.*[21] For determination of endogenous urea, the spores were extracted twice in boiling H_2O and the supernatant fluids were combined and brought to a final concentration of 0·1 M tris–HCl buffer, pH 8·5. The urea, in aliquotes of the extract, was estimated by measuring carbamyl derivatives spectrophotometrically[22] both prior to and after complete hydrolysis of the urea by urease.

[21] D. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[22] D. HUNNINGHAKE and S. GRISOLIA, *Anal. Biochem.* **16**, 200 (1966).

Urea-^{14}C (58 mCi/mM), $NaH^{14}CO_3$ (56·9 mCi/mM), and DL-phenylalanine-^{3}H (100 mCi/mM) were purchased from Radiochemical Centre. ATP disodium salt was obtained from Fluka Chemische Fabrik, and Urease (Jack bean meal) from Calbiochem. All other chemicals used were of analytical grade.

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Key Word Index—Geotrichum candidum; Fungi; urea utilisation; spore germination