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THE BEHAVIOR OF AMIDOHYDROLASES AND L-GLUTAMATE IN SYNCHRONIZED POPULATIONS OF *BLASTOCLADIELLA EMERSONII*

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Abstract—The activities of L-glutamine amidohydrolase, L-asparagine amidohydrolase, and an apparently new enzyme, D-glutamine amidohydrolase, were detected in extracts of the aquatic Phycomycete, *Blastocladiella emersonii*. The D-glutamine amidohydrolase was very unstable in frozen cells, as much as 60 per cent being lost in 4 days and essentially all of it in 2 weeks, while only 20 per cent of the L-glutamine amidohydrolase was denatured during the latter time. L-Asparagine amidohydrolase was stable under these conditions. The total activity per cell of the D-amidohydrolase remained more or less stationary during ontogeny, but both of the L-amidohydrolases increased many fold to maximum levels at 98 per cent of the cell's generation time, and then decreased again just before sporogenesis. During this last stage in cell development, glutamic acid-^{14}C was rapidly consumed. The ^{14}C derived therefrom was incorporated extensively into protein, but very little of it equilibrated with the cell's water-soluble, free, amino acid pool and various other cell components. The relationship between sporogenesis and certain aspects of nitrogen metabolism in *B. emersonii* was discussed in the light of these results.

INTRODUCTION

STUDIES of amino acid metabolism in *Blastocladiella emersonii* have revealed the presence of three enzymes involved in the decomposition of amino acid amides. The ordinary colorless (OC) cells of this aquatic Phycomycete possess an L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1.), an L-glutaminase (L-glutamine amidohydrolase, E.C. 3.5.1.2.) and, in addition, a D-glutaminase (D-glutamine amidohydrolase, E.C. 3.5.1.a). These three amidohydrolases are of the Type I variety;[1] i.e., they do not require an oxo-acid acceptor for release of ammonia. As far as we are aware, this is the first report on the occurrence and activity of D-glutaminase.

L-Glutaminase appears to be ubiquitous, and it has been assigned various metabolic roles in plants, micro-organisms, and animals. Comprehensive reviews on glutamine and glutaminases have appeared, notably those by Meister[1] and Roberts;[2] the latter has also dealt with L-asparagine and the occurrence and distribution of L-asparaginase. And yet, reports on the presence of L-glutaminase in fungi have been surprisingly rare, even though the enzyme was detected in *Neurospora crassa* some ten years ago (Tanenbaum *et al.*[3]). It has occasionally been reported in yeasts;[4] among these, *Saccharomyces cerevisiae* and the yeast-like *Candida utilis* possess demonstrable L-glutaminase activity.[5] On the other hand, L-asparaginase activity has been found in *Piricularia oryzae*, *Aspergillus niger*, and species of

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[1] A. MEISTER, *Biochemistry of the Amino Acids*, Academic Press, New York (1957).
[2] E. ROBERTS, in *The Enzymes*, Vol. 4, p. 285, Academic Press, New York (1960).
[3] S. W. TANENBAUM, L. GARNJOBST and E. L. TATUM, *Am. J. Botany* **41**, 484 (1954).
[4] C. A. ZITTLE, in *The Enzymes*, Vol. 1, Part 2, p. 922, Academic Press, New York (1951).
[5] A. DOMNAS, unpublished data.

Penicillium and Microsporum (Cochrane[6]), in *Rhizopus nigricans*,[7] and in yeasts.[4,5] All three amidohydrolases have also been detected[5] in a new species[8] of *Blastocladiella*, *B. britannica*.

In the following report, we have described the behavior of these amidohydrolases at various stages in ontogeny of synchronous, single-generation populations of OC cells of *B. emersonii*. Emphasis has been placed on the uptake, distribution, utilization, and ultimate disposition of L-glutamic acid, the deamidation product of L-glutamine. Particular attention has been given to the terminal stages in the life cycle of the organism—i.e. from ~ 75–100 per cent of its generation time (GT)—so as to obtain some insight into possible relationships between nitrogen metabolism and spore formation. Finally, we have also discussed the present status of certain aspects of amino acid metabolism in *B. emersonii*. A preliminary abstract of this work has been presented elsewhere.[9]

RESULTS

Enzyme Stability

It has been observed[10] that enzymes of arginine metabolism in *B. emersonii*, when stored either in frozen whole-cell mats or cell-free preparations, were quite labile. We therefore extended these investigations of enzyme instability to include the amidohydrolases. The

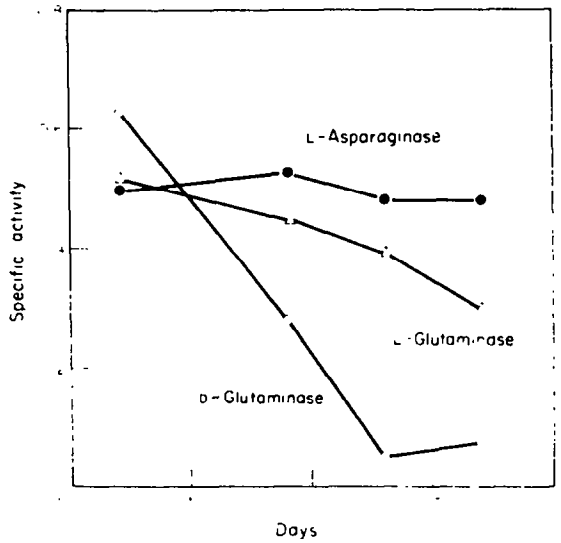

FIG. 1. THE CHANGE IN SPECIFIC ACTIVITY OF L-ASPARAGINASE, L-GLUTAMINASE AND D-GLUTAMINASE DERIVED FROM OC CELLS OF *B. emersonii* WHICH HAD BEEN GROWN TO FULL GT AND THEN FROZEN FOR DIFFERENT PERIODS OF TIME BEFORE HOMOGENIZATION.

results (Fig. 1) revealed that D-glutaminase was the most unstable in frozen intact cells; almost 100 per cent of it was denatured after 13 days of storage at −5°. In later studies, we found that as much as 60 per cent of it was lost after only 4 days. L-Glutaminase, on the other

[6] V. W. COCHRANE, *Physiology of Fungi*. John Wiley, New York (1958).
[7] J. CHURY, *Spisy Lékařské Fak. Masarykovy Univ.* **22**, 1 (1948).
[8] E. A. HORENSTEIN and E. C. CANTINO, *J. Bact.* **84**, 37 (1962).
[9] A. DOMNAS and E. C. CANTINO, *Plant Physiol* (suppl.) **38**, iv (1963).
[10] A. DOMNAS and E. C. CANTINO, *Biochim. Biophys. Acta* (In press).

hand, displayed a perceptible but much slower rate of denaturation; significant activity remained even after 21 days of storage. This, together with the effects of freezing and thawing (Table 1), suggests that the hydrolytic activity of extracts on D-glutamine is due to a distinct enzyme. As for L-asparaginase, no significant loss was detected in intact cells frozen for this same period of time. Cell-free homogenates prepared in water, and in buffers with and without sucrose, rapidly lost activity when frozen; they were devoid of all amidohydrolase activity after 5 days of storage. Successive freezing and thawing also hastened denaturation. In addition, dialysis against water and potassium glutamate had deleterious effects (see Table 1 for representative experiments). Attempts were also made to increase the specific activities of the L-amidohydrolases in cell-free extracts by fractional precipitations with ethanol, n-butanol, $BaCl_2$, Na_2SO_4, and $(NH_4)_2SO_4$, separations on DEAE-cellulose columns, and

TABLE 1. EFFECTS OF VARIOUS TREATMENTS ON THE ACTIVITY OF L-ASPARAGINASE, L-GLUTAMINASE, AND D-GLUTAMINASE IN CELL-FREE PREPARATIONS

Treatment	Specific activity (μM NH_3/hr/mg protein)		
	L-Asparaginase	L-Glutaminase	D-Glutaminase
Crude homogenate*	0·55	0·62	0·21
Frozen, thawed ×1	trace	0·20	0·17
Frozen, thawed ×2	0	0	0
Crude homogenate†	0·57	0·37	0·26
Frozen, thawed ×3	0·30	0·27	0·26
Frozen, thawed ×4	0·13	0·17	0·06
Crude homogenate, lyophilized	0·33	0·36	0·11
Dialyzed‡	0	0	0
Undialyzed control (18 hr at 4°)	0·33	0·20	0
Crude supernatant§	0·46	0·37	—
Dialyzed‖	0	0	—

* In phosphate buffer, 0·1 M, pH 7·05.
† Prepared in 1 M sucrose:0·1 M phosphate (1:1), pH 6·95.
‡ Dialyzed 18 hr against 0·1 M potassium glutamate, pH 7·0 at 4°.
§ Homogenized in 1 M sucrose and centrifuged at 32,000 g, 40 min at 4°.
‖ Dialyzed 18 hr against water at 4°.

homogenizations and dialysis with borateacetamide buffers; increased specific activities were not achieved. Because of these and other observations, we were unable to devise satisfactory procedures for maintaining adequate quantities of crude material long enough to partially purify these enzymes. As a consequence of their cold lability, all extracts were worked up and assayed for enzyme activity within 6 hr of cell harvests.

Behavior of Amidohydrolases during Ontogeny

Figure 2 illustrates the behavior of amidohydrolases during the late stages in the development of OC cells. Data for zero per cent GT are shown as a point of reference; i.e. the spore itself contained measurable quantities of both D- and L-glutaminase, but very low levels of L-asparaginase. The specific activity of D-glutaminase reached its maximum at 95 per cent GT, whereas maxima for L-asparaginase and L-glutaminase appeared at ~ 99 per cent GT.

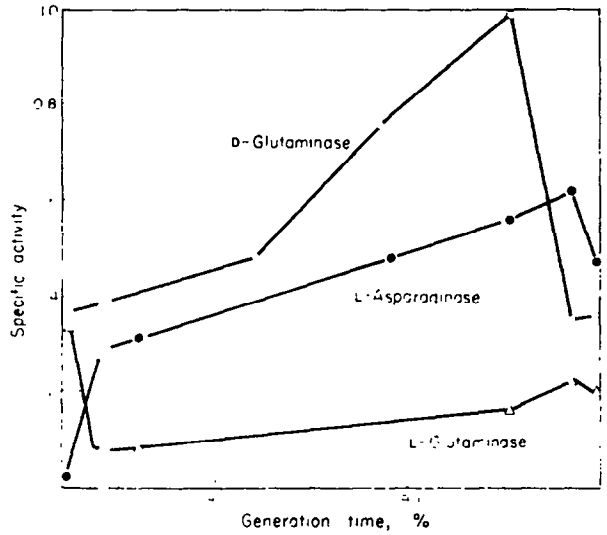

FIG. 2. THE SPECIFIC ACTIVITY OF L-ASPARAGINASE, L-GLUTAMINASE, AND D-GLUTAMINASE IN EXTRACTS OF SPORES (ZERO % GT) AND OF OC CELLS HARVESTED AT DIFFERENT STAGES IN THEIR ONTOGENY.

Population densities during growth were maintained between 2.8×10^5 and 10^6 cells/ml.

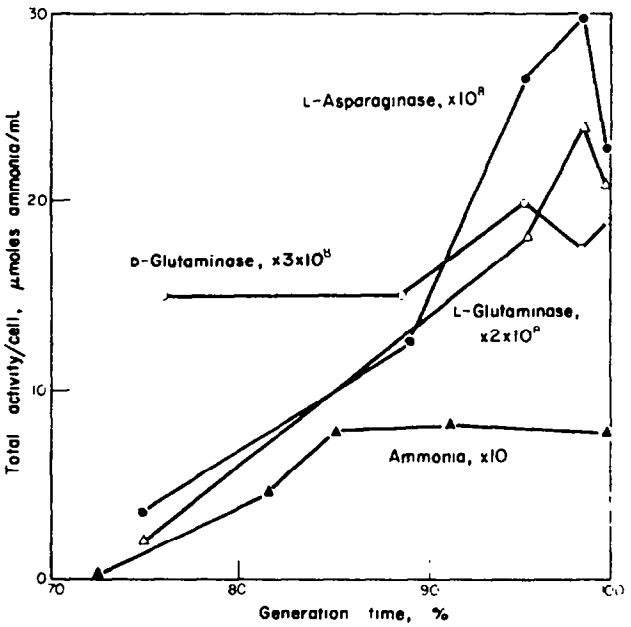

FIG. 3. THE TOTAL ACTIVITY PER CELL OF L-ASPARAGINASE, L-GLUTAMINASE, AND D-GLUTAMINASE, AND THE ACCUMULATION OF FREE AMMONIA IN THE GROWTH MEDIUM, AT DIFFERENT STAGES IN THE ONTOGENY OF OC CELLS

Population density, 10^6 cells/ml.

A common feature was the decrease in specific activities of all enzymes just before GT—a quality also characteristic of L-ornithine transcarbamylase and L-arginase.[10] When specific activities were converted to total units of activity per cell, a different picture emerged (Fig. 3).

Uptake of Glutamic Acid

Uptake of glutamic acid-U-^{14}C from the medium (Fig. 4, curve I) consisted of three phases: (*a*), an initial phase, to ~ 35 per cent of GT, characterized by no uptake of glutamate; (*b*), a second phase, corresponding to the interval between ~ 35 and 95 per cent of GT, with gradual uptake of label; and (*c*), a phase corresponding to ~ 95–100 per cent of GT, with very rapid uptake of label. During this last, very short phase in the life history, the cells took

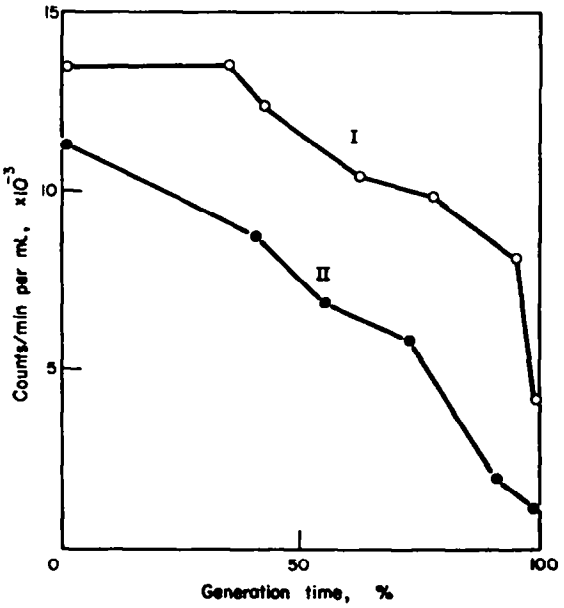

FIG. 4. THE PATTERN FOR NET DISAPPEARANCE OF RADIOACTIVITY FROM THE GROWTH MEDIUM AT DIFFERENT STAGES IN THE ONTOGENY OF OC CELLS FED 10 μCURIES OF GLUTAMIC ACID-U-^{14}C THROUGHOUT THEIR GENERATION TIME.

Curve I, $3{\cdot}56 \times 10^5$ cells per ml of medium; Curve II, $2{\cdot}9 \times 10^6$ cells per ml of medium.

up 3800 counts per minute (counts/min.)/ml of medium as opposed to 5450 counts/min./ml for the much (12-fold) longer gradual phase. It is perhaps self-evident but, none the less, important to emphasize that the nature of an uptake curve of this sort is dependent upon population density. For example, the rapid uptake of label at the end of the generation time was lost when the medium was overloaded with cells of *B. emersonii*. This is illustrated by curve II in Fig. 4, where it can be seen that uptake of glutamic acid-U-^{14}C by an abnormally heavy population took place somewhat more rapidly and resulted in earlier depletion of radioactivity in the medium.

Intracellular Distribution of Label

The data delineated in Fig. 5 illustrate the rise in the soluble amino acid pool per cell which occurred during the life cycle of the organism; a maximum level was reached at

~ 95 per cent of GT. The sharp rise in the intracellular pool of soluble amino acids (including non-protein nitrogen compounds) occurred between 89 and 94 per cent of GT and, once again, this was followed by a decrease just prior to the formation and release of spores. During uptake of glutamic acid-U-^{14}C, the accumulation of total intracellular radioactivity reached a maximum at ~ 90 per cent of GT. This peak did not coincide with that for accumulation of ninhydrin-positive material. During this same period of time, label was rapidly incorporated into the trichloroacetic acid (TCA)-insoluble material found in high-speed supernatants of these cells; it appeared as if incorporation of label into protein began quite early in the life cycle of the organism, starting at least by 40-60 per cent of its GT.

Cells which had been fed glutamic acid-U-^{14}C were fractionated according to established procedures (Table 2). Half of the radioactivity was located in the soluble protein of the

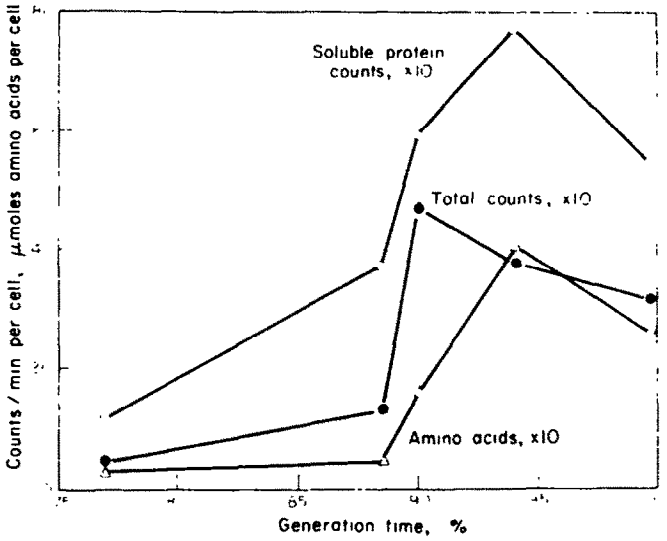

FIG. 5. THE ACCUMULATION OF TOTAL RADIOACTIVITY PER CELL, RADIOACTIVE SOLUBLE PROTEIN PER CELL, AND TOTAL AMINO ACIDS (AS GLYCINE) PER CELL AT DIFFERENT STAGES IN THE ONTOGENY OF OC CELLS

$1{\cdot}75 \times 10^6$ cells per ml of medium (550 ml) were fed 10 μCuries of glutamic acid-U-^{14}C from 0-100% GT

supernatant fraction. But, in contrast to results[10] obtained with arginine-^{14}C, a very large portion (36 per cent) of the radioactivity was found in particulate and otherwise-insoluble matter.

Cells labeled with glutamic acid-U-^{14}C were also processed in a somewhat different manner in order to obtain a more detailed fractionation of their contents (explanatory notes, Table 3). By this procedure, almost all of the ^{14}C in the TCA-soluble fraction was found to be readily soluble in ethyl ether; soluble polysaccharide, free sugars and, in particular, free amino acids were not significantly labeled (Table 3). The major portion (70 per cent) of the radioactivity resided in the TCA-insoluble material, in good agreement with the results in Table 2. It is evident that most of the glutamic acid had been incorporated into protein. A significant fraction of the total count in the TCA-insoluble matter, however, was alcohol soluble, possibly suggesting that labeled glutamate had been converted to lipid moieties of

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN MATURE OC CELLS FED LABELED GLUTAMATE*

Fraction	counts/min ($\times 10^{-3}$)	Total ^{14}C in whole homogenate (%)
Whole homogenate†	92·8	
Supernatant	46·8	49·7
Pellet	33·5	35·6
TCA-soluble compounds‡	13·9	14·7

* $4{\cdot}35 \times 10^9$ cells in 1500 ml of growth medium[10] containing 20 μcuries of glutamic acid-U-^{14}C were grown from 0–100 per cent GT at 24°.

† Cells were homogenized in 0·005 M NaCl in an ice bath and centrifuged at 34,000 *g* at − 5° for 1 hr to yield supernatant and pellet.

‡ TCA-soluble fraction was derived from supernatant by mixing with an equal volume of 12% TCA in the cold for 15 min and then centrifuging.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN MATURE OC CELLS FED LABELED GLUTAMATE*

Fraction	Total ^{14}C in whole homogenate (%)
TCA-insoluble†	
DNA	1·3
RNA	1·3
Chitin	2·0
Ethanol-soluble compounds	18·7
Protein (and other TCA insoluble material)	70·0
Total TCA-insoluble material	93·3
TCA-soluble‡	
Soluble polysaccharide	0·6
Carbonyl compounds	0·2
Amino acids and sugars	0·2
Ether-soluble compounds	5·7
Total TCA-soluble material	6·7

* Cells grown as described in Table 2.

† Cells homogenized in water at 1°. Part of homogenate was treated with an equal volume of cold 20% TCA, centrifuged at 12,000 *g* for 10 min at 2° and the TCA-insol. fraction assayed for RNA,[11] DNA,[11] chitin,[12] and ethanol-sol. components (i.e., dissolved by three successive extractions with 5 vols 100% ethanol).

‡ Part of the TCA-sol. fraction was analyzed for polysaccharide.[13] Ether-sol. compounds were removed by four successive extractions with 5 vols ethyl ether. Amino acids and sugars in both ether-sol. and ether-insol. fractions were chromatographed in one dimension (Whatman No. 1) with n-propanol:ammonium hydroxide:water (6:3:1); carbonyl compounds were converted to their 2,4-dinitrophenylhydrazones and chromatographed with various solvents.[14]

[11] E. C. CANTINO, *Phytochem.* 1, 107 (1961).
[12] J. S. LOVETT and E. C. CANTINO, *Am. J. Botany* 47, 550 (1960).
[13] E. C. CANTINO and A. GOLDSTEIN, *Arch. Mikrobiol.* 39, 43 (1961).
[14] H. D. MCCURDY and E. C. CANTINO, *Plant Physiol.* 35, 463 (1960).

some kind. The distribution of radioactivity in proteins of cell-free extracts was also established with the techniques[10] used previously for studies with arginine-^{14}C. A nucleic acid-protein pattern typical of extracts of *B. emersonii* was obtained (Fig. 6). Radioautography of acid hydrolysates of whole cells, pre-extracted with hot water and washed, revealed that label resided wholly in glutamic and aspartic acids.

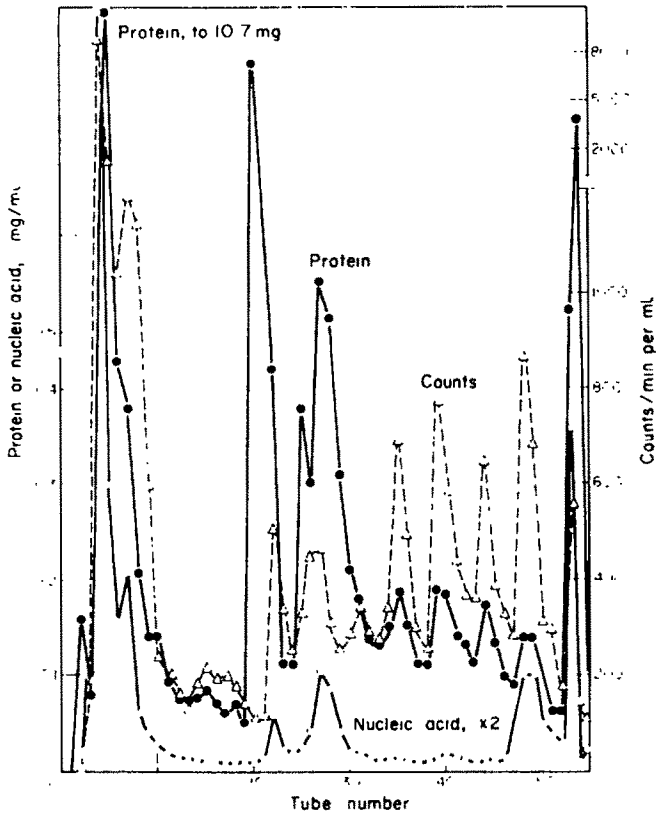

FIG. 6. THE DISTRIBUTION OF RADIOACTIVITY, SOLUBLE PROTEIN AND SOLUBLE NUCLEIC ACID IN HOMOGENATES OF 4·35 × 10⁹ MATURE (100% GT) OC CELLS FED 20 μC URIES OF GLUTAMIC ACID-U-^{14}C FROM 0–100% GT

Fractionations were made on DEAE-cellulose columns using step-wise elutions with NaCl.[10]

DISCUSSION

L-Glutamine participates in many reactions, among the more important being those involved in transaminations and in purine and glucosamine biosynthesis. The presence of an L-glutaminase has generally been invoked, if not explained, as a control device which functions to destroy L-glutamine, or to create more L-glutamic acid and ammonia, or both, according to the presumed requirements of the cell.

Our data show that both the specific activities and the total activities/cell of amidohydrolases rise as the cell approaches the end of its life history. It is instructive to compare the data for the period preceding 95 per cent of GT with those for the period between 95 and 99

per cent of GT, the latter being the period just preceding spore formation. Between 88 and 95 per cent of GT, the total D-glutaminase/cell rose about 35 per cent over its preceding level; the specific activity of this enzyme also rose about 35 per cent. Thus, if enzyme activity was proportional to enzyme protein, it seemed as if enzyme protein per cell was increasing at about the same rate as total protein per cell. However, during this time period, the total L-asparaginase per cell increased about two-fold, but its specific activity rose only ~ 17 per cent. In this case, therefore, enzyme protein per cell appeared to be rising at one-fifth or less of the rate for total protein per cell.

The above can be contrasted with what happened between 95 and 99 per cent of GT; during this time, the total D-glutaminase per cell actually dropped about 13 per cent, while its specific activity decreased approximately 67 per cent; thus, the total enzyme activity per cell apparently dropped sharply relative to the smaller change in protein per cell which occurred. But, the L-asparaginase per cell continued to rise about 13 per cent, and its specific activity also rose about 11 per cent; therefore, in this case, the rise in total enzyme activity per cell was keeping pace with the rise in total protein per cell during the period immediately preceding sporogenesis.

In any case, the rise in L-glutaminase activity could be interpreted to mean that this enzyme helps to meet a need for more glutamic acid, with a consequent release of ammonia. It is tempting to speculate that the L-glutamine required by an OC cell for L-glucosamine synthesis is no longer needed by this time, in as much as the cell is not proceeding along its alternate developmental pathway[15] which leads to a much thicker-walled, melanized, resistant sporangial (RS) cell. Indeed, perhaps L-glutaminase activity would be completely repressed if the organism were induced to proceed along this RS path, for it has been shown[12,16] that formation of RS cells involves greatly increased production of both chitin and glucosamine synthetase, the latter requiring glutamine as its substrate. This question will be answered when we have analyzed RS cells for L-glutaminase at different stages in their development, as has been done here for OC cells.

At the present time, we know of no role for D-glutamine in *B. emersonii*. But, since this amide is synthesized by L-glutamine synthetase,[17] its occurrence in nature can be expected. D-Glutamic acid is needed for construction of the cell walls of certain organisms,[1,18] the formation of certain peptides,[19] etc. During ontogeny of *B. emersonii*, the total units of D-glutaminase per cell remained relatively constant; thus, perhaps no undue stress was laid upon this enzyme for increased production of D-glutamic acid and ammonia. But in any case, an explanation for the presence of D-glutaminase in *B. emersonii* will depend upon the elucidation of the nature of its requirement for D-glutamine.

L-Asparagine is distributed ubiquitously in nature, and it has been shown to be involved in transamination.[1] So far, however, it does not have roles in metabolism analogous to those of L-glutamine. Heretofore, it has often been invoked as an agency for storing nitrogen. *B. emersonii* possesses a potent L-asparaginase, and possibly its function is to produce L-aspartic acid needed for synthesis of protein and/or nucleic acid. An increased requirement for pyrimidines would bring about a demand for increased production of carbamyl aspartate;

[15] E. C. CANTINO and J. S. LOVETT, *Advances in Morphogenesis*, Vol. 3, p. 33, Academic Press, New York (1964).
[16] J. S. LOVETT and E. C. CANTINO, *Mycologia* **52**, 338 (1960).
[17] L. LEVINTOW and A. MEISTER, *J. Am. Chem. Soc.* **75**, 3039 (1953).
[18] M. R. J. SALTON, *Microbial Cell Walls* (CIBA Lectures in Microbial Biochemistry), John Wiley, New York (1960).
[19] W. J. WILLIAMS, J. LITWIN and C. B. THORNE, *J. Biol. Chem.* **212**, 427 (1955).

in this connexion, the available data[20] suggest that synthesis of thymine may be a rate-limiting factor for growth and nuclear reproduction by *B. emersonii*, and it is also known[21] that nucleic acids are synthesized exponentially by OC cells during exponential growth. Evaluation of this possibility will require knowledge about the behavior of L-carbamylaspartate transferase during growth; this is currently under investigation. Finally, since ammonia is liberated by this reaction, a portion of that released into the medium during growth (see Fig. 3) may have been derived from the combined action of the amidohydrolases. The latter have characteristics similar to those of amidohydrolases described from other sources: they are easily denatured, difficult to maintain, cold-labile, and hard to purify (cf. Altenbern and Housewright[22]).

The many roles in which L-glutamate may be involved will not be reviewed now. *B. emersonii* does not grow without exogenous L-glutamic acid.[23] It is apparent from our data that this amino acid is utilized extensively and quite rapidly for synthesis of proteins. The soluble material in hot-water extracts of *B. emersonii* contained but very small quantities of labeled amino acids. While the intracellular content of ninhydrin-positive material peaked at 95 per cent of the cell's GT, these amino acids were not derived to any significant extent from L-glutamic acid-U-^{14}C. The radioactivity in these extracts was largely associated with unknown, heat-labile, ether-extractable substances which were not amino acids. In contrast, the soluble proteins in the supernatants, and the proteins in the insoluble particulates were highly radioactive. Thus, perhaps like the situation described by Steward and Bidwell,[24] protein synthesis in *B. emersonii* may occur at the expense of some small compartmentalized reservoir distinct from the total intracellular "pool" of amino acids. Chromatograms revealed that although glutamic acid was the principle ^{14}C-compound present in these labeled proteins of *B. emersonii*, significant quantities of labeled aspartic acid were also found. In particular, the presence of aspartic acid-^{14}C suggests that some L-glutamic acid may have been decomposed via the tricarboxylic acid cycle to yield oxoglutaric acid. However, the turnover rate for this reaction would then have had to have been very high since protein-free supernatants contained only vanishingly small amounts of labeled oxo-compounds associated with the Krebs cycle. Thus, just as in earlier experiments[25] where labeled intracellular oxoglutarate, succinate, fumarate, isocitrate, and malate apparently did not equilibrate quickly with glutamate and aspartate, so in the present experiments, intracellular labeled glutamate and aspartate did not seem to equilibrate readily with the cell's pool of Krebs cycle intermediates. Finally, it must be mentioned that little if any labeled proline was found, in spite of the fact that *B. emersonii* contains abundant quantities of this acid. In fact, this observation raises the question of a different pathway for synthesis of proline in this fungus; alternatively, of course, it may be that *B. emersonii* has no large requirement for proline during the late stages of its life cycle.

Finally, at this juncture, it is important to re-emphasize the interesting observation made in this study, as well as that in a previous report:[10] namely, that nearly all the parameters we measured rose to a peak at or near 95 per cent of GT, and then decreased again just before the end of GT. Is this final drop related to the formation of spore-wall material, to the

[20] E. C. CANTINO and G. TURIAN, *Arch. Mikrobiol.* **38**, 272 (1961) and references therein.
[21] A. GOLDSTEIN and E. C. CANTINO, *J. Gen. Microbiol.* **28**, 689 (1962).
[22] R. A. ALTENBERN and R. D. HOUSEWRIGHT, *Arch. Biochem. Biophys.* **49**, 130 (1954).
[23] H. D. BARNER and E. C. CANTINO, *Am. J. Botany* **39**, 746 (1952).
[24] F. C. STEWARD and R. G. S. BIDWELL, in *Amino Acid Pools*, p. 667, Elsevier, Amsterdam (1962), and references therein.
[25] E. C. CANTINO and E. A. HORENSTEIN, *Mycologia* **48**, 777 (1956).

incorporation of free amino acids into spore protein, and/or to a transformation of soluble protein into insoluble spore protein? In other words, is it a cause—or is it a consequence of—sporogenesis: the final, manifold division of the whole OC protoplast into its several hundred, uniflagellate, uninucleate, motile, uniquely organized[26,27] spores, with all that this high degree of complexity entails? We suspect that, in part at least, it is the cause; as more information becomes available about this last critical phase in the life cycle of *Blastocladiella*, we hope to obtain a definitive answer.

EXPERIMENTAL

Culture Methods

Synchronous, single generation cultures of the fungus were started with filtered suspensions of motile spores and grown at 24° on Difco PYG media as previously described.[10]

Preparation of Cell-free Extracts and Measurement of Enzyme Activities

Cells were homogenized in phosphate buffer, and extracts were derived therefrom by centrifugation as described previously.[10] Asparaginase and glutaminase were assayed with Obrink's[28] modification of Conway's micro-diffusion technique, activity being determined by measurement of the ammonia released, using Nessler's reagent according to Koch and McMeekin.[29] The end-products of amidohydrolase activity were identified by paper chromatography. Specific activity was defined as μmoles of ammonia produced per hr per mg protein at 36°, in a reaction mixture consisting of 0·5 ml of 0·1 M phosphate, pH 7·05, 0·5 ml of supernatant, and 0·5 ml of 0·1 M substrate (asparagine or glutamine). Reaction time was 60 min. Appropriate enzyme and substrate blanks were included. Protein was measured by the procedure of Lowry *et al.*[30] Enzyme activities were converted to total enzyme per cell by multiplying specific activities by values for total soluble protein per cell, as previously described.[12,21]

Assay for Ammonia and Total Amino Acids

Suitable portions of cell-free spent media were evaporated to dryness under vacuum in a flash evaporator at 40°. The residue was taken up in water, and ammonium salts were analyzed with Nessler's reagent as described above. Two methods were employed for determining total amino acids. The ninhydrin technique of Hagan and Rose[31] was used on hot-water extracts (30 min at 94°) of OC cells. For analyses of cell-free homogenates, the copper-salt modification of Spies[32] was employed.

L-*Glutamic Acid-U-*^{14}*C Experiments*

Uptake of radioactivity, introduced as L-glutamic acid-U-^{14}C, was followed during growth by counting samples of filtered spent media. The distribution of radioactivity in various fractions of the cell (cf. Table 2) was determined by methods used previously;[10] for the

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results reported in Table 3, however, fractionations for various categories of compounds were made according to other methods[11–13] (see footnotes, Table 3). Radioactive samples were counted on planchets with a Tracerlab Versa Matic II scaler and an FD1-PI flow counter or, when present on paper strips, with a Tracerlab 4π scanner. The L-glutamic-U-^{14}C was purchased from Calbiochem Corp. (U.S.A.).

Chromatography

Amino acids were resolved by descending chromatography in two dimensions on Whatman No. 1 paper, using phenol:water (80:20) and butanol:acetic acid:water (60:15:25); papers were sprayed with 0·1% ninhydrin in ethanol and heated at 98° for 10 min. Radioautograms were made with Kodak No-Screen X-ray film.

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