ISOCITRITASE, GLYCINE-ALANINE TRANSAMINASE, AND DEVELOPMENT IN BLASTOCLADIELLA EMERSONII [1, 2, 3]

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The discovery (9) that the development of a new aquatic fungus, *Blastocladiella emersonii,* along either of two distinct morphogenetic pathways leading to ordinary colorless (OC) or resistant sporangial (RS) plants could be determined by a simple manipulation of the external environment, presented an excellent opportunity for studying the relationship between metabolism and morphogenesis. Even with this very simple, two-celled organism, however, almost a model system in fact, the relationship is a complex one, and the progress of our understanding inevitably slow. The comparatively recent finding (14) that light plays a role in development has added an interesting but complicating aspect to the story.

Studies during the past decade have led us (11, 13, 18, 58) to postulate mechanisms for the morphogenetic and light phenomena in Blastocladiella. An integral part of these is the key role of an enzyme, isocitritase; its presence was implied but not explicitly demonstrated. In the work reported here, the presence of isocitritase in Blastocladiella has been confirmed, the enzyme partially purified, and its properties studied. A second enzyme, glycine-alanine transaminase, presumably involved in the in vivo role of isocitritase, has been similarly examined. After the development of new culture techniques, the relationship of these enzymes to differentiation was investigated in detail.

MATERIALS AND METHODS

B. emersonii was grown on Difco CANTINO PYG AGAR and BROTH media. Resistant sporangial (RS) plants were cultivated in PYG broth containing 0.75 g $NaHCO_3$/liter (38). For partial purification of enzymes, 1.5 l of PYG were inoculated with heavy spore suspensions, continuously aerated, and harvested just before the population produced one generation of plants. The latter were washed with 2 l of water and sucked dry. Additional details are found in the text.

Biochemicals were obtained as follows: amino, ketoglutaric, and oxalacetic acids from Nutritional Biochemical Corp.; triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), sodium glyoxylate monohydrate, L-isocitric acid lactone (allo free), and pyridoxal-5-phosphate, from Sigma Chemical Co.; pyruvic acid from California Corp. for Biochemical Research.

Isocitric acid was prepared from its lactone by hydrolysis with KOH; the hydrolysate contained 50 % D-isocitric acid (by assay with pig heart isocitric dehydrogenase (43). Sodium pyruvate was prepared from the acid (46) and recrystallized from a water-alcohol solution. Inorganic chemicals and organic solvents were reagent grade. Glass beads (No. 100) used for grinding plant tissue in the Servall Omnimixer were from the Minnesota Mining and Manufacturing Co.

SPECIFIC METHODS FOR STUDIES OF ISOCITRITASE. Glyoxylic acid was assayed as its 2,4-dinitrophenylhydrazone (DNPH) colorimetrically (25), and spectrophotometrically by an adaptation of the method of Smith and Gunsalus (55). Keto acids were chromatographed as DNPH derivatives according to Cavallini (19) in butanol-ethanol-water (BEW; 4:1:5), butanol-acetic acid-water (BAW; 4:1:5), 1-propanol-ammonia-water (PAW; 6:3:1), and two-dimensionally in phenol-water (PW; 2.5:1). Protein was determined turbidimetrically with trichloroacetic acid (TCA) according to Stadtman et al (57). Estimates of protein concentration on purified enzyme preparations were made at 215 and 225 mμ (60). Succinic acid was determined manometrically with succinoxidase from pig heart according to Krebs as described by Umbreit et al (59). Isocitric acid was determined with pig heart isocitric dehydrogenase (43).

Isocitritase activity was determined at 30° C in 3.1 ml reaction mixtures containing, in μM: D,L-isocitrate, 40; cysteine · HCl, 5; $MgCl_2$, 10; phosphate, pH 7.4, 200. The reaction was initiated by adding substrate and stopped by adding 0.3 ml *2N* HCl. When necessary, protein was precipitated with 0.02 ml 10 % $Na_2WO_4 \cdot 2H_2O$ and removed by centrifuging. After diluting to 10 ml, 1 ml of 0.1 % dinitrophenylhydrazine in *2N* HCl was added to 1 ml of reaction mixture. After 6 minutes at room temperature, 2 ml 95 % ethyl alcohol was added, followed by

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4 ml of 1.5 *N* NaOH. After 3.5 minutes, the color was read at 490 and 540 mμ. Under our conditions, the optical density (O.D.) ratio (490 mμ: 540 mμ) of the DNPH derivatives of glyoxylate, pyruvate, and ketoglutarate were about 1.5, 1.0, and 0.8 respectively; thus, the O.D. ratio revealed the presence of keto acids other than glyoxylate, and derivation of simultaneous equations permitted calculation of the individual concentrations of the acids present. One unit of isocitritase activity was defined as the amount of enzyme catalyzing formation of 1 μM glyoxylate/10 minutes; and specific activity, the number of units/mg protein.

Specific Methods for Studies of Glycine-alanine Transaminase. Glycine and alanine were determined quantitatively (31), following their separation with PAW on acid-washed Whatman No. 1 paper. For qualitative studies, amino acids were resolved with BEW, PAW, and BAW, and detected with 0.1 % ninhydrin in 95 % ethyl alcohol. Pyruvic acid was determined according to Straub, as outlined by Green et al (27), with slight modification. Mixtures of glyoxylate and pyruvate were assayed as the DNPH derivatives (as described previously). Glycine-alanine transaminase was determined at 37° C in 1 ml assay mixtures containing, in μM: sodium glyoxylate monohydrate, 10; L-alanine, 10: phosphate, pH 8.0, 40; $MgCl_2$, 5; and pyridoxal-5-phosphate, 10 μg. After 10 minutes the reaction was stopped with Straub test Reagents (a control is necessary to correct for a small amount of non-enzymatic transamination which occurs between glyoxylate and alanine in the strong alkali used in the test). A unit of transaminase was defined as the amount of enzyme catalyzing production of 1 μM pyruvate/10 minutes; specific activity, the number of units/mg protein.

Specific Methods for Studies of Physiology of Development. Uniform spore inocula, with known total dry weight and viable counts, were standardized turbidimetrically. Large Petri plates containing 40 ml PYG agar were inoculated with about 10^5 viable spores; the latter were spread evenly over the surface and incubated at 27° C. After germination of the mature plants had begun, plates were flooded with 10 to 15 ml of sterile water. After about 30 minutes, a spore suspension was generally obtained containing from 10^6 to 10^7 spores/ml.

After a density determination, the suspension was centrifuged at 400 × G for exactly 1.5 minutes in 40 ml conical tubes, and the supernatant removed by pipette, care being taken not to disturb the loosely packed sediment. The spores were resuspended in the small amount of residual medium, transferred to 12 ml centrifuge tubes, and cooled to 10° C. The suspension was centrifuged at 1,600 × G for exactly 30 seconds, the supernatant decanted, and the sediment resuspended in 10^{-2} M phosphate, pH 7.0. Finally, the resuspended spores were washed by centrifugation as before, the supernatant decanted, and the spores resuspended again.

Careful adherence to this procedure was essential; when followed carefully, it resulted in zoospore suspension exhibiting 80 to 100 % mobility. Any deviation, however, generally led to the fragile zoospores rounding up and losing their flagella. Repeated centrifugation in distilled water or hypertonic solutions (10^{-1} M), or high-speed centrifugation, generally led to loss of motility, deformation, and sometimes complete disintegration.

For preparation of homogenates, swarmers were centrifuged at 1,600 × G for 3 minutes. The pellet was resuspended in five volumes of phosphate (10^{-1} M, pH 7.4) containing 10^{-4} M versene plus 10^{-3} M cysteine · HCl) and homogenized in a 3 ml glass homogenizer or ground with a carborundum-glass mixture in a mortar and pestle.

Results

Isocitritase. Incubation of crude homogenates of Blastocladiella with isocitric acid led to the identification of glyoxylic acid as the product of an enzymatic reaction. However, ketoglutarate and pyruvate were always produced as well when long incubation periods were used; this was later shown to be due to enzymatic and chemical transaminations between glyoxylate and endogenous amino acids. The demonstration of the stoichiometry and properties of the isocitritase reaction were therefore dependent upon preliminary purification. In all cases where attempts were made to obtain active, particle-free preparations by means of high speed (22,000 × G) centrifugation of homogenates (prepared with glass homogenizer) most of the activity was always retained in the sediment. However, soluble preparations from OC plants were finally obtained from extracts of acetone-dried powders and extracts obtained by homogenization in a Servall Omnimixer.

Preparation of Isocitritase from Acetone Powders with Glass Homogenizer. A 5-day culture was harvested and homogenized in an ice bath for 10 minutes, (5.0 ml 2 × 10^{-2} M phosphate/gm wet wt). The homogenate was centrifuged at 22,000 × G, the supernatant discarded, and the sediment resuspended in the same volume of buffer. The centrifugation was repeated, the sediment resuspended in one-fifth volume of buffer, the latter treated with 10 volumes of acetone (−10° C), allowed to stand 10 minutes, filtered, washed, with two volumes acetone, and sucked dry. The mat was air dried, ground to a powder, and stored at −18° C in a vacuum desiccator.

Active extracts were prepared as follows: 1 g acetone-dried powder was stirred vigorously with 20 ml 10^{-1} M phosphate, pH 7.4, containing 10^{-3} M cysteine for 15 minutes, centrifuged at 22,000 × G, and the sediment discarded. To 10 ml of supernatant, which contained the enzyme, 3.2 g $(NH_4)_2SO_4$ was added in the cold, and the precipitate centrifuged off and resuspended in one-fourth the original volume of buffer.

PARTIAL PURIFICATION OF ISOCITRITASE FROM OMNIMIXER HOMOGENATES. Results of a typical fractionation are shown in table I.

Step 1: *Preparation of the crude extract.* Plants from 14 hour cultures were homogenized in 10^{-1} M phosphate, pH 7.4, containing 10^{-3} M cysteine and 10^{-4} M versene (5 ml buffer/g wet wt) with glass beads in the Omnimixer at 15,000 rpm for 3 minutes at −5° C. The homogenate was centrifuged in the cold at 22,000 × G and the supernatant used for the subsequent fractionation.

Step 2: *First* $(NH_4)_2SO_4$ *Fractionation (fraction A).* Twenty-two g $(NH_4)_2SO_4$ was added to 100 ml supernatant and the precipitate removed by centrifugation and discarded. To the supernatant was added 10 g $(NH_4)_2SO_4$/100 ml, the precipitate recovered by centrifugation, and redissolved in a fourth the original volume of 2×10^{-2} M phosphate, pH 6.0.

Step 3: *Protamine sulfate treatment* (cf. Colowick and Kaplan (20)). Protamine sulfate (20 mg/ml 2×10^{-2} M phosphate, pH 6.0) was added to the solution from step 2 such as to yield 0.27 mg protamine/mg protein. The precipitate was removed by centrifugation.

Step 4: *Second* $(NH_4)_2SO_4$ *Fractionation (fraction B).* The protamine sulfate supernatant was brought to 27 % saturation with $(NH_4)_2SO_4$, centrifuged, and the inactive sediment discarded. An additional 5.4 g/100 ml of original volume was introduced, the precipitate collected by centrifugation, and redissolved in one-fifth volume of 2×10^{-2} M phosphate, pH 5.6.

Step 5: *Adsorption of impurities with* $Ca_3(PO_4)_2$ gel (cf. Keilin and Hartree (32). Sufficient gel was added to give a gel/protein ratio of one, the suspension equilibrated by stirring, and the gel removed by centrifugation and discarded.

Step 6: *Third* $(NH_4)_2SO_4$ *Fractionation (fraction C).* To 10 ml of supernatant was added 6.4 ml saturated $(NH_4)_2SO_4$, pH 7.5, the solution centrifuged, and the sediment discarded. An additional 3.1 ml of saturated $(NH_4)_2SO_4$ per 10 ml was added, the sediment recovered, and redissolved in 10^{-4} M phosphate, pH 7.4, containing 10^{-4} M cysteine and 10^{-4} M versene.

IDENTIFICATION OF GLYOXYLATE. Glyoxylate. was identified as a product of the enzymatic reaction by chromatography of its DNPH in four different solvents, and by 2-dimensional co-chromatography in PW and BPW. It was further identified by means of the absorption spectrum of its DNPH (max., 455 mμ) and the alkali-instability of the latter; thus it was readily distinguishable from the DNPH of pyruvate and ketoglutarate, neither of which exhibits marked alkali instability.

STOICHIOMETRY AND REVERSIBILITY OF ISOCITRITASE REACTION. Stoichiometry was established with $(NH_4)_2SO_4$ preparations from acetone extracts and $(NH_4)_2SO_4$ fraction A. The results (table II) are in agreement with the established reaction:

D-isocitrate ⟵⟶ Glyoxylate + succinate

The reversibility of the reaction was confirmed by incubating 20 μM of glyoxylate and succinate with the enzyme; 2.5 μM isocitrate/hour was produced.

PROPERTIES OF ISOCITRITASE. *Stability.* Frozen mats and acetone powders of Blastocladiella may be stored for several weeks without appreciable loss of activity, but crude extracts stored at 4° C may lose as much as 30 to 40 % of their activity in 4 hours. Partially purified enzyme solutions will also lose about 50 % activity on overnight storage at 4° C, but they may be stored for some weeks at −18° C with little loss.

Reaction rate and effect of pH. The rate of glyoxylate formation is directly proportional to en-

TABLE I

PARTIAL PURIFICATION OF ISOCITRITASE*

STEP	FRACTION**	PROTEIN (MG/ML)	SPECIFIC ACTIVITY	% RECOVERY***	PURIFICATION (-FOLD)
1	Crude extract	4.1	0.31	100	1
2	$(NH_4)_2SO_4$ Fraction A	7.6	0.60	98	2
3	Protamine Supernatant	2.4	1.70	91	5
4	$(NH_4)_2SO_4$ Fraction B	3.0	4.00	55	13
5	Gel Supernatant	1.0	8.30	44	27
6	$(NH_4)_2SO_4$ Fraction C	0.8	16.20	26	52

* Reaction mixture: 5 μM cysteine. HCl; 10 μM $MgCl_2$; 200 μM phosphate, pH 7.4; 40 μM D,L-isocitrate; about one unit isocitritase; in 3.1 ml.; 30° C.

** All steps carried out at 0 to 2° C.

*** (Total units/total number units in original extract) × 100.

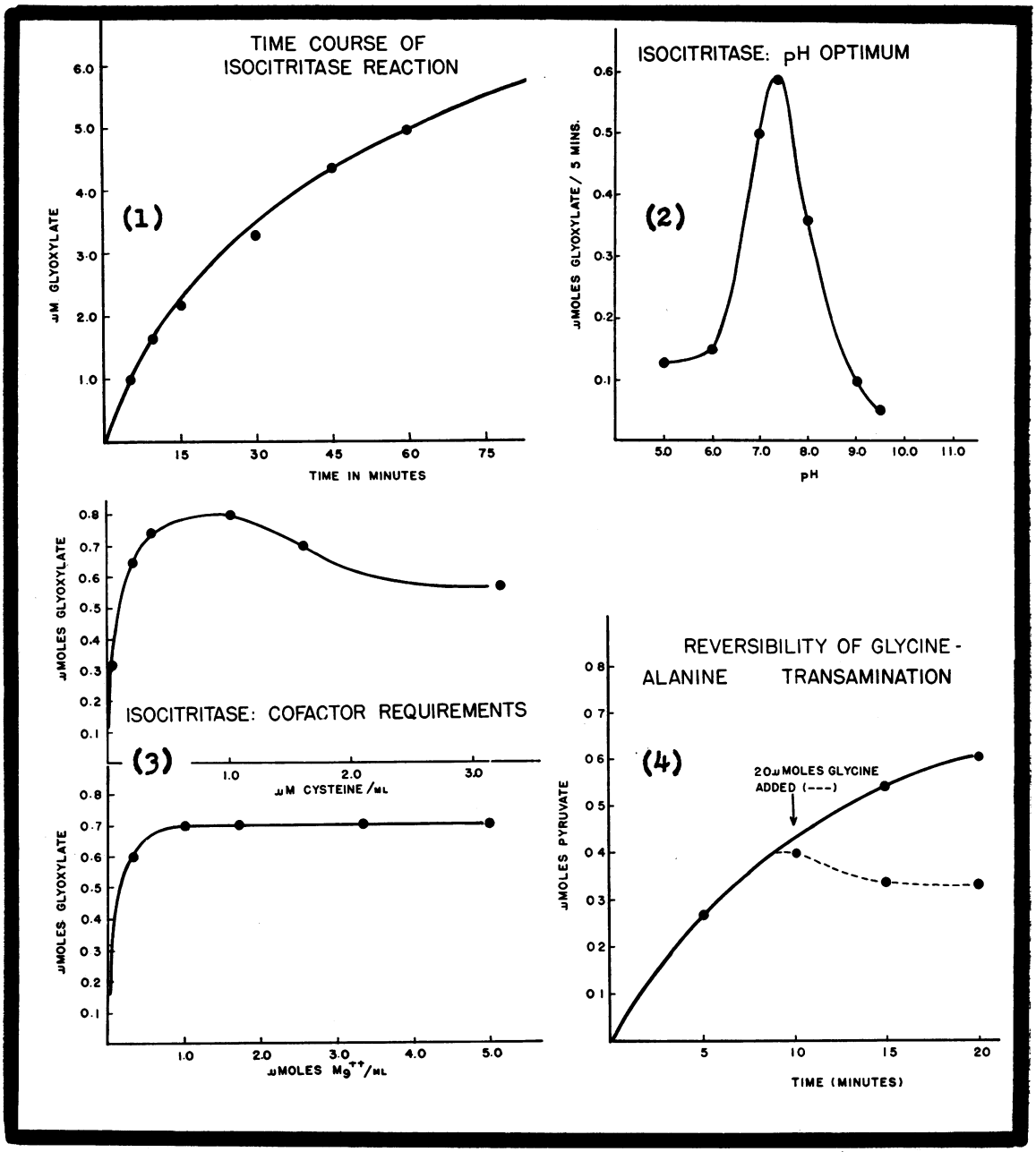


FIG. 1. Time course of the isocitritase reaction. Conditions as described for isocitritase assay (table I), but with incubation times as indicated.

FIG. 2. Isocitritase pH optimum. Conditions as for isocitritase assay (table I) except that a series of 5 minute incubations was used at the indicated pH. Enzyme: $(NH_4)_2SO_4$ fraction C.

FIG. 3. Isocitritase co-factor requirements. Conditions as for isocitritase assay (table I). Enzyme: $(NH_4)_2SO_4$ fraction C.

FIG. 4. Reversibility of glycine-alanine transaminase. Two parallel series of four reaction tubes were set up using the conditions described for the transaminase (table III). The reaction was initiated by addition of substrates and a tube from each set stopped at 5 minute intervals up to 20 minutes. As shown in one set, 20 μM glycine in 0.1 ml was added to the 15 and 20 minute tubes. Enzyme: Gel Supernatant B.

TABLE II

ISOCITRITASE STOICHIOMETRY*

ENZYME	D-ISOCITRATE μM	GLYOXYLATE μM	SUCCINATE μM
$(NH_4)_2SO_4$-acetone fraction			
Expt. 1	4.9	5.0	5.4
Expt. 2	4.0	5.3	4.1
$(NH_4)_2SO_4$-fraction A			
Expt. 1	3.8	4.0	...
Expt. 2	5.2	4.9	...

* Reaction mixture as in table I, but 1 hour incubation; enzyme, about two units.

zyme concentration up to about two units. The time course of the reaction is shown in figure 1. The enzyme exhibits a sharp pH optimum at 7.4 (fig 2); thus, assays were carried out at this pH and with about one unit of enzyme.

Specificity. Though neither pure D-isocitrate nor L-isocitrate was available, specificity for the D-isomer is implied by the stoichiometry data; that is, while the isocitric dehydrogenase used for determination of isocitrate is specific for the D-isomer, both isomers were always present in the isocitritase reaction mixture in equal concentrations.

Crude extracts did not produce DNPH-forming compounds when incubated with citrate or cis-aconitate. This is of particular interest since various workers (e.g. 21, 56, 61) have reported an enzyme from bacteria (citritase) which cleaves citrate to oxaloacetate and acetate. In particular, oxaloacetate was never detected as a product of isocitrate utilization by crude extracts or acetone powders of OC plants, though conditions were such that it would have been detected, and the presence of aconitase activity in such preparations has been demonstrated (17). Neither did they produce oxaloacetate from citrate as judged by measurements at 280 mμ (28).

COFACTOR REQUIREMENTS. Isocitritase requires a metallic ion for maximum activity as well as a sulfhydryl group. Glutathione was only 60 % as effective as cysteine. Though Mg was routinely employed as cofactor, Mn, at the same concentration, was equally effective (when tried with tris buffer systems). Cofactor saturation curves for both Mg and cysteine are shown in figure 3; both gave maximum activity at ca. 10^{-3} M.

EFFECT OF SUBSTRATE CONCENTRATION, TEMPERATURE, AND LIGHT. When isocitrate concentration (as the D-isomer) was plotted against reaction velocity, a typical substrate concentration curve was obtained; rearrangement of the data according to Lineweaver and Burk (37) permitted estimation of the Michaelis constant as 4.8×10^{-4} M. Determination of reaction rates at several temperatures allowed graphical estimation of the activation energy according to the Arrhenius equation (42, 2) as E = 10, 700 calories/mole. The Q_{10} of the reaction was 1.8. Finally, it was established that glyoxylate production in white light (300 ft-c.) and dark was identical.

GLYCINE-ALANINE TRANSAMINASE. During the early studies on isocitritase using crude preparations and long incubation periods, pyruvate was a frequent product of side reactions involving glyoxylate. Because it seemed likely that transamination between glyoxylate and endogenous amino acids was responsible, glyoxylate was incubated with a number of amino acids (L-glutamate, L-glutamine, L-aspartate, L-alanine, L-tyrosine, L-lysine, L-proline, L-leucine, L-histidine, L-arginine, and L-threonine) in the presence of boiled and unboiled crude enzyme and appropriate controls. Enzymatic transamination occurred only in those reaction mixtures containing L-alanine. Since it seemed likely that an alanine-glyoxylate transamination might constitute an important pulling reaction for removal of glyoxylate produced by isocitritase, further studies were carried out.

PARTIAL PURIFICATION OF GLYCINE-ALANINE TRANSAMINASE (see table III).

TABLE III

RESULTS OF TRANSAMINASE PURIFICATION*

FRACTION	PROTEIN (MG/ML)	SPECIFIC ACTIVITY	% RECOVERY**	PURIFICATION
1. Crude extract	5.3	0.68	100	1
2. Protamine sulfate	2.7	1.40	103	2
3. Acetone I	2.2	11.00	96	16
4. Gel supernate B	0.7	29.0	100	43
5. Acetone II	0.9	55.0	60	80

* Reaction mixture: 10 μM sodium glyoxylate; 10 μM L-alanine; 40 μM phosphate, pH 8.0; 5 μM $MgCl_2$; 10 μg pyridoxal-5-phosphate; enzyme, under 0.7 units; in 1 ml, 37° C. Reaction stopped at 10 minutes with Straub reagents.

** (Total units/total No. units in original extract) × 100

TABLE IV

STOICHIOMETRY OF GLYOXYLATE-ALANINE TRANSAMINATION*

TIME, HR	μM			GLYOXYLATE
	GLYCINE	PYRUVATE	ALANINE	
1	2.9	2.7**	2.5	2.8
2	3.5	3.6**	3.3	3.2

* Reaction mixture as in Table III, but incubation times as indicated; reaction stopped with 0.09 ml. 2 *N* HCl; enzyme: Gel supernatant B.

** Determined by calculation from O.D. at 490 and 540 mμ, and Straub test. (averaged).

Step 1: *Preparation of crude extract.* Plants from 14 hour cultures were homogenized in water (4 ml/g) containing 10^{-4} M versene and glass beads (4 g/g plants) in an Omnimixer (16,000 rpm; −5° C) for 3 minutes. The homogenate was centrifuged at 22,000 × G, and the supernatant used for fractionation. Endogenous amino acids in the crude extract prevented direct estimation of initial transaminase activity. Therefore, the extract was dialyzed 20 hours against 2 × 10^{-1} M phosphate, pH 7.4, at 4° C, after which it was assayed for activity. Since dialysis lowered the protein concentration through apparent denaturation of inactive protein, the initial, specific activity was expressed as μM pyruvate produced by the dialyzed preparation/mg protein in the original undialyzed material (prolonged dialysis of enzyme does not affect transaminase activity).

Step 2: *Protamine sulfate treatment.* Three-fourths ml of protamine sulfate (20 mg/ml) was added for every 100 mg crude extract protein at 0° C, and the precipitate removed by centrifugation at 30,000 × G.

Step 3: *First acetone fractionation.* Acetone (−10° C) was added until its concentration reached 47 % and the precipitate was removed by centrifugation and discarded. The active fraction was obtained by bringing the acetone concentration to 57 % at −10° C, the solution centrifuged, and the sediment resuspended in one-fifth the original volume of water. The solution was dialyzed 20 hours at 1° C against three changes of 100 volumes of water containing 10^{-4} M versene.

Step 4: *Negative adsorption with* $Ca_3(PO_4)_2$ gel. Enough gel solution (18.6 mg/ml) was added to the dialyzed fraction at 0° C to give a gel-protein ratio of 2:1. After adsorption equilibration (15 min), the gel was removed by centrifugation and discarded. The process was repeated once, and the supernatant used in the next step.

Step 5: *Final acetone fractionation.* To further concentrate the transaminase, enough acetone (−10° C) was added to give a concentration of 62 % at −5 to 0° C. The precipitate was recovered by centrifugation, resuspended in one-fourth volume water, and dialyzed 20 hours against three changes of 500 volumes of water.

STOICHIOMETRY, REVERSIBILITY, AND IDENTIFICATION OF PRODUCTS. Pyruvate was established as the product of the transamination between glyoxylate and alanine by paper chromatography of its DNPH in BEW, and by Straub's quantitative salicylaldehyde reaction. Glycine was identified by paper chromatography in PA, BEW, and BAW. Determinations of reactants and products after incubations of glyoxylate and alanine with enzyme (table IV) defined the reaction as:

Glyoxylate + L-alanine ⇄ glycine + pyruvate

To demonstrate reversibility, pyruvate and glycine were incubated in the usual reaction mixture. No disappearance of pyruvate was detected nor was alanine produced, in spite of the fact that the forward reaction had proceeded to only one-third conversion. Thus, it seemed likely that pyruvate or glycine was inhibitory. With this in mind, modified experiments were set up which did demonstrate the reversibility of the reaction (fig 4); glycine brought about apparent reversal. This was confirmed in another experiment in which 2.0 μM pyruvate and 20 μM glycine were incubated for 1 hour in the usual reaction mixture; this led to disappearance of 0.28 μM pyruvate and appearance of a corresponding amount of alanine.

PROPERTIES OF TRANSAMINASE. *Stability, effect of pH, and reaction rate.* The enzyme may be stored for at least a week at 4° C, or 2 months at −18° C, without loss of activity; it is also stable to prolonged dialysis against various buffers. Highest activity is obtained at pH 8.5. However, since considerable non-enzymatic transamination occurs here (fig 5), routine assays were carried out at pH 8.0. The amount of pyruvate produced was directly proportional to the amount of enzyme up to 0.7 units. The reaction proceeds to ca. one-third conversion (fig 6).

Activators and inhibitors. Neither prolonged dialysis against water, phosphate, nor 50 % saturated $(NH_4)_2SO_4$ caused the enzyme to exhibit stimulation by addition of pyridoxal-5-phosphate. Precipitation of the enzyme by dialysis against saturated $(NH_4)_2SO_4$ followed by dialysis against 2 × 10^{-2} M acetate, pH 4.0 (cf. 64) caused complete, irreversible loss of activity which could not be restored by prolonged incubation with pyridoxal phosphate and Fe (+3), Mg, Cu (+2), etc. (41). In common with other pyridoxal phosphate enzymes, glycine-alanine transaminase was inhibited (table V) by KCN and hydroxylamine (20); the latter was overcome by 10^{-3} M pyridoxal phosphate. Isoniazid (22, 64), reportedly an inhibitor of vitamine B_6 enzymes, did not inhibit even at 10^{-2} M. So far, requirements for a metal ion have not been demonstrated for any transaminase; our glycine-alanine transaminase was no exception. Dialysis against water and various buffers containing versene failed to reveal the need for the metallic cofactor.

Substrate specificity. Incubations were carried

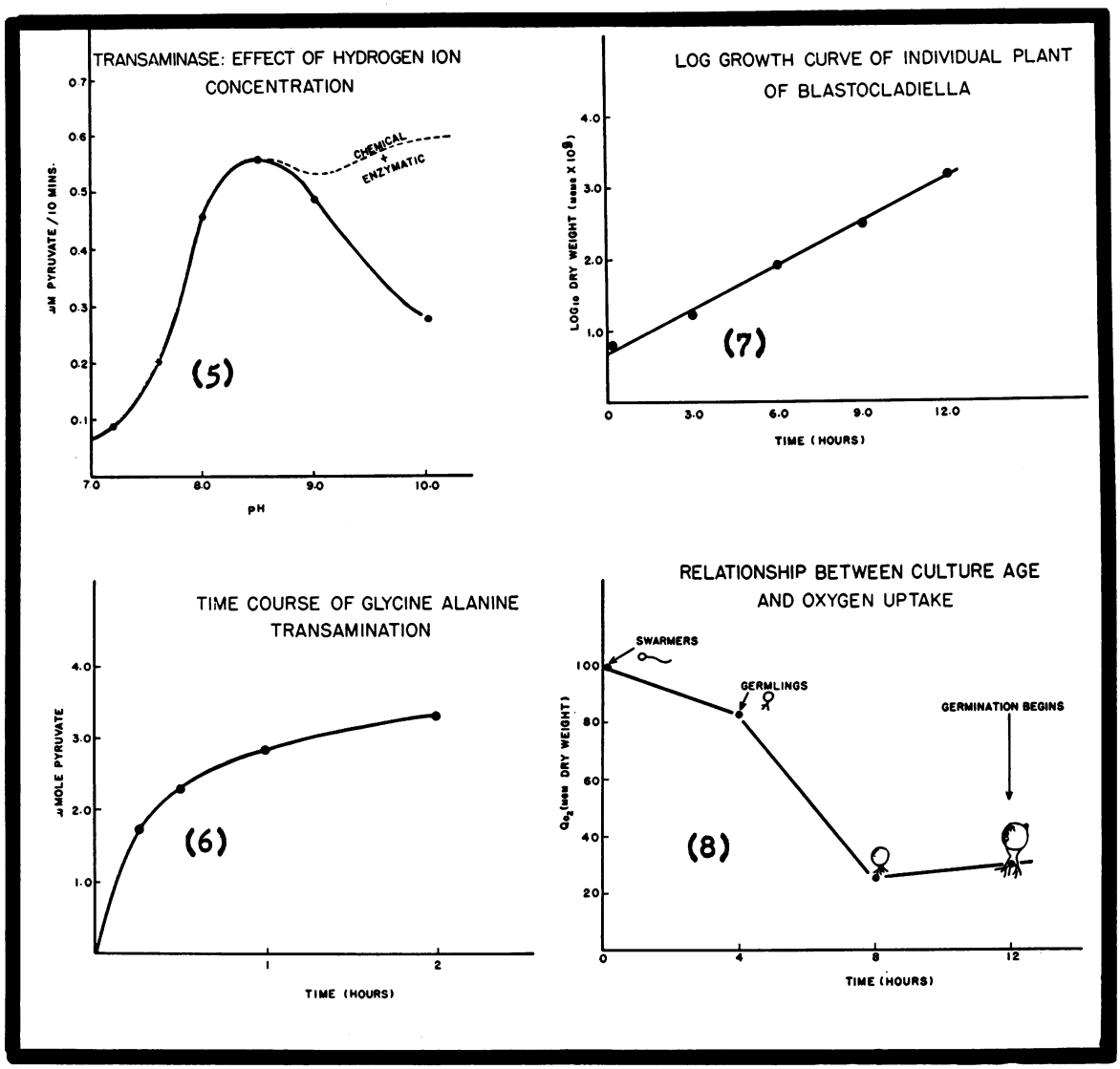


FIG. 5. Transaminase pH optimum. Conditions as for transaminase assay (table III) except 40 μM tris buffer used from pH 7.2 to 8.4, and 40 μM borate buffer from pH 8.4 to 10.0. In addition to the usual boiled enzyme controls, chemical controls were incubated for each pH, and pyruvate determined using the pH 7.2 reaction mixture as control. Enzyme: Gel supernatant B.

FIG. 6. Time course of glycine-alanine transamination. Conditions as for transaminase assay (table III) except that incubation times were as indicated; reaction stopped with 0.09 ml 2 N HCl. Enzyme: Gel Supernatant B.

FIG. 7. Log-growth curve of individual plant of Blastocladiella. The plotted data are the logs of the values obtained by dividing the dry weights obtained at times indicated, by the corrected number viable swarmers in the inoculum.

FIG. 8. Relationship between culture age and oxygen uptake. Qo_2, μl O_2/mg dry wt/hr

TABLE V

INHIBITION OF TRANSAMINASE*

ADDITIONS	μM PYRUVATE	% INHIBITION
Control	0.65	0
10^{-3} M KCN	0.63	0
10^{-2} M KCN	0.05	92
10^{-3} M hydroxylamine	0.00	100
10^{-4} M hydroxylamine	0.38	42
10^{-4} M hydroxylamine and 10^{-3} M pyridoxal phosphate	0.65	0

* Reaction mixture as in Table III, but with additions as indicated; enzyme: Gel supernatant B.

out with a number of substrate pairs, including the keto acids pyruvate, glyoxylate, oxaloacetate, alpha-ketobutyrate, and alpha-ketoglutarate, and the amino acids alanine, glycine, isoleucine, serine, valine, leucine, lysine, phenylalanine, proline, glutamine, asparagine, cysteine, arginine, tryptophane, threonine, aspartate, and glutamate. Products were detected by paper chromatography. L-Alanine could not be replaced by any other amino acid in systems containing glyoxylate; D-alanine was inactive. An enzymatic transamination between pyruvate and asparagine, however, was detectable, but further work is necessary to determine if this is due to the same or a different enzyme; structural considerations, however, suggest that a separate enzyme is involved. Finally, assays of transaminase activity were carried out under the conditions used previously for light and dark assays of isocitritase. No stimulation by light occurred.

ENZYMOLOGY AND ONTOGENY IN BLASTOCLADIELLA: *Cultivation of synchronous single generation cultures of OC plants.* Using heavy spore suspensions and continuous aeration, up to 4 g/1.5 l PYG of single-generation populations were obtained in 14 hours. It seemed likely that a time-course analysis of such cultures might reveal further clues to the function of isocitritase and glycine-alanine transaminase in ontogeny. In preparation for these studies, a growth curve was established for single generation cultures of OC plants, their protein content at various ages determined (table VI), and their overall morphology recorded photographically (fig 9).

TABLE VI

SOLUBLE PROTEINS DURING ONTOGENY

AGE, HRS	SOLUBLE PROTEIN (MG/GM)*
0 (Zoospores)	371
3	393
6	350
9	380
12	312

* Protein determinations on 22,000 × G supernatants obtained from Omnimixer homogenates

Growth was exponential, as indicated by a log-plot of the dry weight data (fig 7). Microscopic examination revealed that at all stages, plants were very uniform in morphology and size (fig 9), except toward the very end of the generation time (ca 12 hours) when some plants began to germinate. It was clear that such cultures resulted from very nearly synchronous growth of single generations of OC plants.

ENDOGENOUS RESPIRATION OF ZOOSPORES AND DEVELOPING PLANTS. Most of the enzymatic activities associated with the Krebs cycle had been found in mature, OC plants (17). Because of this and the close association of isocitritase and CO_2 fixation with the tricarboxylic acid cycle, we looked for some indication of the type of metabolism exhibited by zoospores. The endogenous Qo_2 of spores (at 30° C. and pH 6.5–7.0) was 102. Addition of numerous substrates did not affect the rate. Arsenite (4×10^{-4} M) inhibited O_2 uptake 45 %. At pH 5.5, malonate inhibited completely at 2×10^{-3} M, and 50 % at 4×10^{-4} M. Succinate, at 10 times the malonate concentration, returned the Qo_2 to endogenous levels (table VII). Thus presumptive evidence was obtained for a functioning tricarboxylic acid cycle in zoospores. However, since addition of various substrates failed to stimulate the endogenous Qo_2, further investigations will be necessary.

Since Brown and Cantino (4) had found a much lower Qo_2 (about 9.0) for mature OC plants than reported here for spores, Qo_2 determinations were done at various developmental stages of a single generation culture. The Qo_2 decreased greatly as the plants developed to maturity (fig 8). However, as was to be expected, it was somewhat higher than the values reported for multiple generation cultures which had contained appreciable numbers of dead plants.

TABLE VII

EFFECT OF INHIBITORS ON ZOOSPORE RESPIRATION*

ADDITIONS	CONCENTRATION	O_2 UPTAKE (μL/HR)	% INHIBITION
None	...	56	...
Arsenite	4×10^{-4}	31	44
None	...	46	...
Malonate	4×10^{-4} M	24	48
Malonate Succinate	4×10^{-4} 4×10^{-3} M	37	20
Malonate Succinate	4×10^{-4} M 8×10^{-3} M	46	None

* Flask contained 100 μM glycyl glycine, pH 5.5, 0.4 ml swarmer suspension, 0.2 ml 10 % KOH in center well, and additions as indicated, at 30° C. Glycyl glycine itself did not induce oxygen consumption.

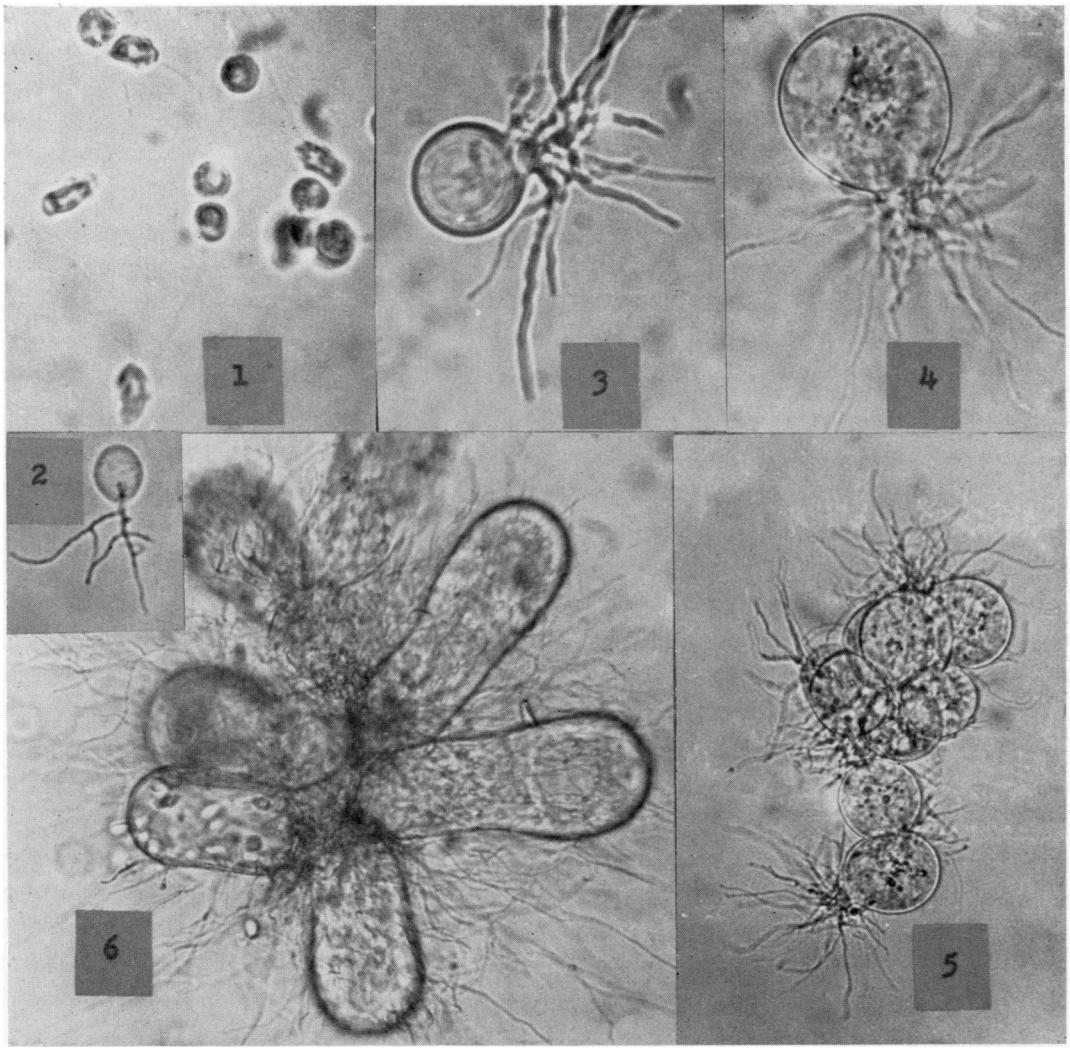


FIG. 9. (1), Zoospores from OC plants; note posterior flagella. (× 500). (2) to (6), plants derived from single-generation synchronous cultures. (2), 3-hour germling (× 500); (3), 6-hour OC plant (× 500); (4), 9-hour OC plant (× 500); (5), several 9-hour OC plants illustrating degree of uniformity of synchronous cultures (× 240); (6), 12-hour OC plants; note that one plant has almost completed release of zoospores (× 240).

RELATIONSHIP OF ISOCITRITASE AND TRANSAMINASE TO DEVELOPMENT AND MORPHOGENESIS. Isocitritase and transaminase activities were established for RS plants, OC plants, and spores. Because of high levels of endogenous amino acids, particularly alanine (38), it was not possible to assay zoospores directly for isocitritase; in this case, assays were made on spore extracts previously fractionated with 0.65 volumes of saturated $(NH_4)_2SO_4$. Both enzymes were detectable at all stages of the life cycle (table VIII).

Enzyme synthesis during growth of single generation cultures of OC plants was then traced in both light and dark. Enzyme activities per plant were calculated from conversion factors obtained from the growth curve data. Isocitritase (units/g dry wt and units/mg protein) decreased during the first 3 hours of growth, remained low until 9 hours, and then rose again to initial levels. But, the number of units *per plant* remained relatively constant and then increased almost exponentially during the last half of the generation time, thus demonstrating that *net synthesis of isocitritase does not occur until relatively late in development.* Although glycine-alanine transaminase drops somewhat (in units/g dry wt, and units/mg protein), it is apparently synthesized exponentially *from the very beginning of plant development!* The relationship between enzyme synthesis and over-all genesis of plant material is shown in figure 10.

TRANSAMINASE AND ISOCITRITASE IN DEVELOPING

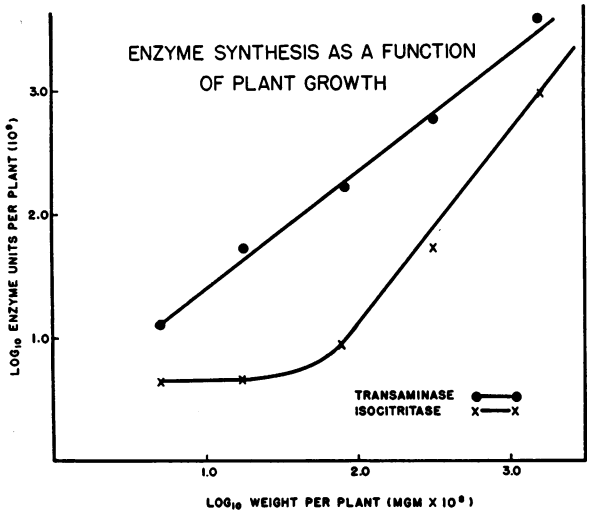
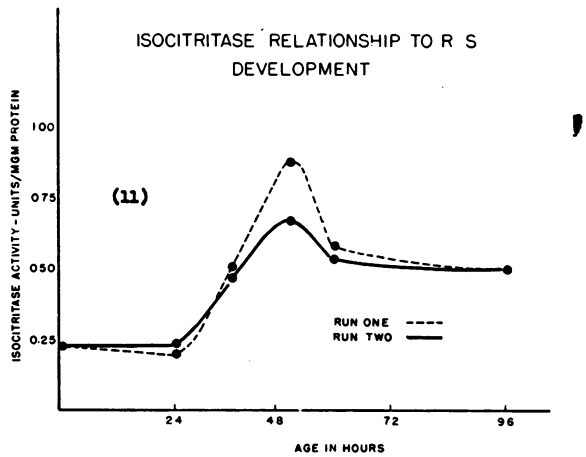


FIG. 10. Enzyme synthesis as a function of OC plant growth in the dark (data for light-grown plants were not significantly different). The plotted values were obtained from the log-data used in figure 7, and the specific activities of isocitritase and transaminase at different stages of development.

RS PLANTS. Finally, synchronous cultures of RS plants (38) were assayed as before for isocitritase and transaminase activities. The specific activity of isocitritase remained fairly constant during the first 24 hours (fig 11). However, beginning at 36 hours, there was a sharp increase in activity which peaked at 48 hours and then slowly declined. The specific activity at 48 hours was higher than that at any other stage in the life cycle (see table VIII). Transaminase activity behaved similarly (fig 12). Since RS plants mature (38) under these conditions at about 84 hours, the levels of activity of both enzymes reach their maxima at about 3/5 of the generation time!

DISCUSSION

The purpose of this work was the study of growth and differentiation in terms of the underlying biochemical mechanisms involved. Enzymological studies of aquatic fungi have been rare, and have never before involved the study of enzymes at the degree of purity achieved for the glycine-alanine transaminase and isocitritase of *B. emersonii*. Therefore, before considering their possible roles in light-stimulated growth and morphogenesis, a brief discussion of the properties of these enzymes is appropriate.



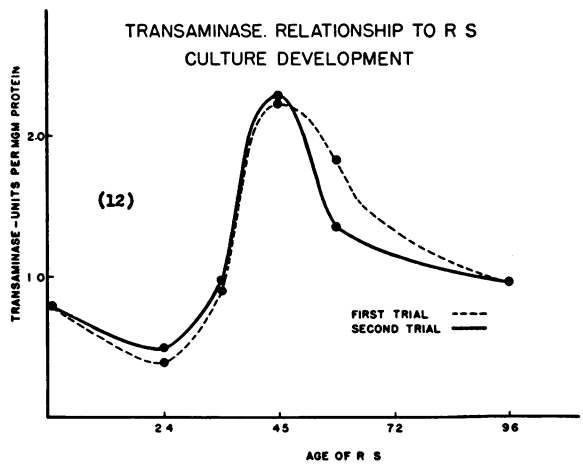


FIG. 11. Relationship between isocitritase activity and RS development.

FIG. 12. Relationship between transaminase activity and RS development, (age, in hrs).

TABLE VIII

DISTRIBUTION OF ISOCITRITASE AT VARIOUS STAGES OF LIFE CYCLE*

STAGE OF DEVELOPMENT	ISOCITRITASE		TRANSAMINASE	
	UNITS/G DRY WT	UNITS/MG PROTEIN	UNITS/G DRY WT	UNITS/MG PROTEIN
Zoospores	82	0.23	271	0.79
Mature OC plants	55	0.18	193	0.58
4-day RS plants	...	0.50	...	0.99

* Conditions as for assays of respective enzymes described previously.

ISOCITRITASE. Evidence for a deviation from the Krebs cycle involving formation of glyoxylate and succinate from tricarboxylic acids was provided by Campbell et al (8) using *Pseudomonas aeruginosa* (Schroeter) Migula, but they were unable to demonstrate the specific substrate for the reaction. However, the results of others (53, 50, 44) have since shown D-isocitrate to be the substrate for the reaction catalyzed by isocitritase; its activity has been identified in aerobic and facultative bacteria (54, 63), certain fungi (45, 35), and the castor bean (34). It has been partially purified from Pseudomonas (55), and yeast (45). Aerobic conditions (54, 36) were required for optimum enzyme production by bacteria grown in acetate and mineral salts media. Glucose repressed isocitritase formation in facultative bacteria, and decreased its activity in aerobic organisms; therefore, it appeared to be adaptively formed in these organisms.

The isocitritase of Blastocladiella closely resembles that from other organisms where similar levels of purification have been achieved. Thus, though it differs slightly in its pH optimum (pH 7.4 vs 8.2 for Pseudomonas) and $(NH_4)_2SO_4$ solubility, its Michaelis constant (4.8×10^{-4}) is almost identical to that of the enzyme from *Pseudomonas aeruginosa* (55). The Michaelis constant of the yeast enzyme (45), determined under somewhat different conditions, is 1.2×10^{-3} M. Like the reaction catalyzed by the enzyme from other sources, Blastocladiella's isocitritase mediates a reversible reaction specific for the D-isomer. The isocitritases from Blastocladiella and Pseudomonas exhibit similar saturation curves for both Mg and cysteine, with maximum activities at 10^{-3} M. Worthy of mention is the fact that the response curve for cysteine exhibits a maximum followed by decreasing activity with increasing cysteine concentration, a phenomenon probably attributable to a chemical reaction between cysteine and glyoxylate. Brunel-Chapelle (5) reported such a reaction at pH 9.0 and above. We have found considerable disappearance of glyoxylate even at physiological pH in the presence of sufficiently large excess of cysteine. Olson (45) reported a similar observation in studies on yeast isocitritase.

TRANSAMINASE. Transaminases catalyzing amino group transfer from all natural amino acids have been described (40), but relatively few have been purified. Only recently has a highly purified glutamate-aspartate enzyme been obtained (29, 30). Reports of transaminases catalyzing interconversion of glyoxylate and glycine have been rare and, except for transamination-deamination enzymes (discussed by Meister, 40), none has been purified or thoroughly studied. Cammarata and Cohen (6) and Awapara and Seale (3) reported an enzymatic transamination between glyoxylate and glutamate catalyzed by rat tissue preparations. Wilson et al (62) demonstrated similar activity in plant tissues. More recently, an extract of Pseudomonas was observed (7) to catalyze transamination of glyoxylate to glycine from glutamate, aspartate, alanine, asparagine, and glutamine. It was not established, however, whether one or several enzymes were involved, and no fractionation was attempted.

Though alanine participates in a number of transaminations (49, 3, 47, 48, 1), there are no reports other than that of Campbell (7) of a glyoxylate-alanine transaminase.

Extracts of *B. emersonii* mediated the rapid conversion of glyoxylate to glycine by amino group transfer from alanine. Since this might have constituted a pulling reaction in the S.K.I. cycle (14), and since there have been no previous reports of attempts to purify a glycine-alanine transaminase, further studies were carried out. The enzyme, purified 80-fold, was quite stable and resembled other transaminases in exhibiting maximum activity at alkaline pH. Attempts to demonstrate direct stimulation by pyridoxal phosphate were unsuccessful. However, it seems likely, in view of its almost-universal role in transamination, that pyridoxal phosphate is involved in the glyoxylate-alanine transamination as well: inhibition of the reaction by hydroxylamine and its reversal by pyridoxal phosphate indicates that this is so. We have concluded that the coenzyme is probably very tightly bound to the holoenzyme, though Meister (40) pointed out the theoretical possibility of enzymatic transaminations involving glyoxylate not requiring a cofactor.

It was expected that the equilibrium of the transamination would be far in the direction of glycine production (41). However, conversion to glycine and pyruvate never exceeded about one-third conversion, while the reverse reaction yielding alanine was only detectable under certain conditions; therefore, a true equilibrium was never attained. Clearly, pyruvate had an inhibiting effect upon the enzyme. Nevertheless, there is no reason, a priori, to believe that the equilibrium constant of the enzymatic reaction should be different than that for chemical transamination involving glyoxylate.

ENZYMES AND DEVELOPMENT IN BLASTOCLADIELLA. Our primary interest in isocitritase was based upon indirect evidence (14, 15, 16) that it was involved in a mechanism for CO_2 fixation as the pulling reaction which removed isocitrate from the site of reductive carboxylation of ketoglutarate. The S.K.I. (14) mechanism had been thought to operate as follows: in the presence of bicarbonate, light energy helped to push or pull the reductive carboxylation of ketoglutarate to isocitrate and thence, via an enzyme (presumed to be isocitritase), to succinate and a C_2 fragment (presumed to be glyoxylate). It was thought that glyoxylate and some of the succinate produced were then integrated into the biosynthetic machinery which yielded increased growth under conditions of illumination. In morphogenesis leading to RS plants, if a similar mechanism is assumed to operate, the high concentration of bicarbonate involved (ca 10^{-2} M) must upset a critical, internal

balance so that instead of increased growth, an entirely new course of development is pursued culminating in the production of resistant sporangia. That this is the case now seems all the more likely in view of the observation (12) that the bicarbonate trigger mechanism is also stimulated by light. Moreover, because of the high specific activity of the transaminase as compared to isocitritase and the probable equilibrium (40, 41) of the glyoxylate-glycine conversion towards glycine production, it now seems a reasonable assumption that transamination constitutes one of the important reactions responsible for the further metabolism of glyoxylate. Therefore, experiments were carried out to elucidate the roles of these enzymes in Blastocladiella. Because the specific locus of the light effect had not been established, any reaction presumed to be involved in light stimulated growth was suspect. Tests on the purified enzymes themselves, however, established unequivocally that neither was the specific locus for the light effect.

A method was then developed for cultivating massive OC cultures which, on the basis of their synchronous nuclear division (58), morphological uniformity and the logarithmic nature of their growth curves, were very nearly synchronous for most of the generation time. Meanwhile, conditions were also established for growing synchronous cultures of RS plants (38). In addition, a new technique was developed that permitted for the first time, physiological studies of the motile spores of aquatic fungi. Thus, while previous studies of developmental problems were limited to the use of heterogeneous liquid cultures containing several generations of plants, now it has become possible to study much more precisely, biochemical changes as they occur from the germination of the tiny zoospores to the development of the mature plant along either of the alternative, ontogenetic paths.

Armed with these new techniques, we followed the behavior of isocitritase and glycine-alanine transaminase during the development of a single generation of OC plants. On a protein basis, the specific activity of isocitritase was high in the spores, dropped sharply as development began, remained low for about half the generation time, and then rose again to initial levels. Transaminase activity, on the other hand, changed relatively little during plant growth; it dropped only one-third from starting levels in the spores to those in mature plants. But, for purposes of interpretation, a much different and more meaningful picture evolved by considering the synthesis of the two enzymes per individual plant. Thus, glycine-alanine transaminase was synthesized exponentially throughout the generation time as a linear function of plant growth, while isocitritase synthesis exhibited a definite lag extending to between the 3rd and 6th hours after germination of the spores.

The possible significance of the lag in isocitritase synthesis during ontogeny may best be appreciated on the basis of comparative biochemistry. It is axiomatic that the cell must have some way of controlling its metabolism, and one means whereby this is accomplished is by control of enzyme synthesis. A great deal of work has been done on adaptive enzyme formation in microorganisms as a possible mechanism for control of enzyme synthesis. One significant finding has been the remarkable correlation between the need for particular enzyme functions and the synthesis of the enzymes involved. For example, facultatively anaerobic bacteria (24) and yeast (52), when cultivated anaerobically, apparently synthesized Krebs cycle enzymes at a very low rate; but aerobically, rapid synthesis of the whole array of respiratory enzyme activities was brought about.

Thus, on the basis of past studies and the preceding discussion, we have evolved the following working hypothesis: lack of synthesis of isocitritase during the early stages in development of an OC plant reflects a situation in which the reaction it catalyzes is not required. During the early phases of the generation time, increased nucleic acid synthesis (58) and other reactions associated with light-stimulated growth take place at the expense of a readily available source of metabolites which, at least in part, may be alternatively supplied by isocitritase if it is present. After 3 to 6 hours of growth, this pool of precursors becomes depleted or insufficient, necessitating either initiation of, or a shift to, an alternative mechanism of supply. Since this period in ontogeny corresponds to the sharp decrease in endogenous respiratory activity (fig 8), it is possible that the deficiency is due to decreased production of substances associated with a functioning Krebs cycle. In any case, in response to the requirement for an alternative supply route, synthesis of isocitritase is begun thus bringing into operation the S.K.I. cycle, with a consequent increase in CO_2 fixation, which is now pulled by light. The glyoxylate produced is at least in part transaminated with alanine to yield glycine which, in turn, may be used to produce thymine (23) and purines (26) for nucleic acid synthesis.

It is probably premature to try to discuss in great detail, with the comparative biochemical literature as a frame of reference, the possible metabolic fates of glyoxylate, succinate, and glycine in relation to differentiation and growth. However, the finding (16) that glyoxylate and succinate together, direct products of the isocitritase cleavage, will replace the light effect in vivo, and our discovery of a direct metabolic route from glyoxylate to glycine are strongly suggestive of one particularly likely route of metabolism. Thus, Kikuchi et al (33) reported an enzymatic reaction yielding delta-aminolevulinic acid from succinate and glycine which was stimulated by light. Moreover, studies with radioactive tracers have incriminated delta-aminolevulinic acid as an intermediate in the conversion of glycine to thymine and purines (51), as well as carotene synthesis (39); and this brings us to the observation (58) that thymine (but not uracil) will substitute for light in DNA synthesis in Blastocladiella. From these findings, we cannot help but regard as very attractive, the possibility that in Blastocladiella, delta-aminolevulinic acid

is an important link connecting glyoxylate, glycine, and succinate with light stimulated growth and, in RS morphogenesis, at least carotene synthesis.

Finally, in a preliminary attempt to elucidate the roles of isocitritase and transaminase in the alternate developmental pathway, synchronous cultures of RS plants were also studied. The results showed an extraordinary correlation between the specific activities of the two enzymes and the morphological and biochemical changes which occur during the genesis of a resistant sporangium. Beginning at about the 24th hour of ontogeny, the specific activities of both glycine-alanine transaminase and isocitritase increased sharply, reaching maximum levels at 48 hours; these levels were 3 to 4 times higher than at any other stage of the entire life cycle. Simultaneously, there is a dramatic appearance of gamma carotene and melanin, and increased synthesis of lipid and chitin, as well as other structural and physiological changes (38). All of these events occur at approximately three-fifths of the generation time, the very point of no return in Blastocladiella (10) beyond which differentiation is irreversible!

These results lend significant support to our contention that isocitritase and glycine-alanine transaminase are key components of both the light-stimulated growth of OC plants and the bicarbonate trigger mechanism in morphogenesis of RS plants.

Summary

The enzyme, isocitritase, which had been postulated to play an important role in the development of *Blastocladiella emersonii,* has been purified about 50-fold from extracts of this organism and some of its properties have been studied. A second enzyme, glycine-alanine transaminase, which has not been previously described, was purified about 80-fold and its properties similarly examined. New methods were developed which permitted, for the first time among aquatic fungi, precise, enzymological studies at all stages of the synchronized growth of populations of Blastocladiella, from zoospores to the mature, ordinary colorless plants. The synthesis of isocitritase and glycine-alanine transaminase was followed during the development of both colorless and resistant-sporangial plants, and an attempt was made to integrate the results into an interpretation of the function of these enzymes in morphogenesis and lumisynthesis (light-stimulated growth) in *B. emersonii.*

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