ISOCITRITASE, GLYCINE-ALANINE TRANSAMINASE AND DEVELOPMENT IN *BLASTOCLADIELLA EMERSONII*

By

Howard D. McCurdy Jr.

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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Pat

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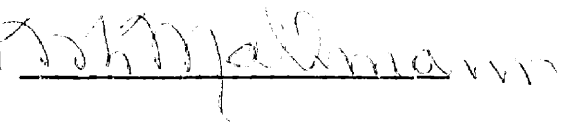
Approved 

ABSTRACT

The enzyme isocitritase, which had been postulated to occur in Blastocladiella, has been purified from extracts of this organism ca. fifty-fold and some of its properties studied. The purified enzyme exhibits maximum activity at pH 7.4 in the presence of 10^{-3} M concentrations of both cysteine and Mg^{++} and has a Michaelis constant of 4.8×10^{-4} M for its specific substrate d-isocitric acid.

A second enzyme glycine-alanine transaminase, not previously purified from any source, has been purified ca. eighty-fold. Stoichiometric determinations show the following to be the reaction catalyzed by the enzyme

glyoxylate + alanine $\rightleftharpoons$ pyruvate + glycine

The reaction was found to be reversible only with difficulty, a true equilibrium was never attained, because of an inhibition of the enzyme by pyruvate. Indirect evidence indicated that pyridoxal-5-phosphate is the prosthetic group. Maximum activity was obtained at pH 8.5. None of the seventeen amino acids tried replaced alanine in systems with glyoxylate.

New methods were developed which permitted enzymological studies on cell free preparations from zoospores as well as plants at various stages of development from near-synchronous, single generation cultures of ordinary (O.C.) colorless plants.

The syntheses of isocitritase and glycine-alanine transaminase, as reflected in activity measurements, were followed during the development of both ordinary colorless and resistant sporangial (R.S.) plants. In O.C. cultures transaminase synthesis was a linear function of plant growth throughout the generation time, whereas isocitritase exhibited a pronounced lag extending to ca. one-third of the generation time. Transaminase and isocitritase activities paralleled each other during R.S. development, reaching maximum activities at ca. three-fifths of the generation time--the point of no return in ortogeny--much higher than at any other stage of the life cycle.

An attempt was made to integrate these results into an interpretation of the function of these enzymes in morphogenesis and light stimulated growth in Blastocladiella.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - Continued

FIGURE Page

INTRODUCTION

The discovery (Cantino, 1951) that the development of the primitive aquatic fungus, Blastocladiella emersonii, along two different morphogenetic pathways could be determined by a simple manipulation of the external environment, has presented an excellent opportunity for studying the relationship between metabolism and morphogenesis. However, even with this relatively simple organism, almost a model system in fact, the relationship is a complex one and the progress of our understanding inevitably slow. But progress is being made and each new experiment and every new finding has served only to intensify the interest of those engaged in Blastocladiella research. The comparatively recent discovery that light plays a role in development has added a new aspect to an already fascinating story which perhaps will have broad implication in our understanding of life itself.

In several papers (see list of references), Cantino and co-workers have postulated mechanisms for the morphogenetic and light phenomena in Blastocladiella. An integral part of these has been the key role of an enzyme, isocitritase, the presence of which was implied but not explicitly demonstrated. In the work reported in this thesis the presence of isocitritase in Blastocladiella has been confirmed, the enzyme partially purified and its properties and relationship to development studied. A second enzyme, glycine-alanine transaminase, perhaps involved in the in vivo role of isocitritase has been similarly

examined. These studies have also involved the elaboration of new techniques for the culture and study of the organism which will be of importance in future research.

REVIEW OF THE LITERATURE

Morphogenesis and Light Stimulated Growth (Lumisynthesis*) In Blastocladiella emersonii

The uniflagellate zoospores of Blastocladiella emersonii (Cantino and Hyatt, 1953a) are capable of embarking on two alternative pathways leading into the development of mature plants of quite different morphology. Cantino (1951) found that the incorporation of ca 10^{-2} M sodium bicarbonate in growth media containing peptone always led to the growth of essentially only one of the two plant types--the resistant sporangium (R.S.)--whereas in the absence of bicarbonate only ordinary colorless (O.C.) plants were produced in the first generation.

Both kinds of thalli consist of a basal stalk bearing a rhizoidal system and an upper globose sac--the sporangium. In R.S. plants, however, the sporangium has a very thick chitinous wall (Cantino, Lovett, and Horenstein, 1957) impregnated with melanin (Cantino and Horenstein, 1955) enclosing a protoplasm containing appreciable quantities of lipoidal particles and gamma carotene (Cantino and Horenstein, 1956b). Ordinary colorless sporangia on the other hand do not produce melanin or gamma carotene, have comparatively thin chitinous walls, and bear several prominent discharge papillae through which zoospores are released at maturity.

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*Light stimulated growth in the absence of chlorophyll.

In experiments designed to determine the mode of action of bicarbonate (Cantino, 1951) it was found that bicarbonate alone, in contrast to its behavior on peptone media, did not induce R.S. formation on basal media containing casein hydrolysate as the nitrogen source. Subsequent studies in which various intermediates and inhibitors were incorporated with 10^{-2} M bicarbonate led to the working hypothesis on which, with some modifications, all work since that time has been based. Thus, if α-ketoglutarate or citrate were provided or the respiratory poisons arsenite or semicarbazide added to the basal medium with bicarbonate the ability to lnduce R.S. was regained. These and related observations, led Cantino to postulate that the increased concentrations of bicarbonate tended to decrease the rates of the decarboxylations catalyzed by isocitric dehydrogenase and α-ketoglutarate dehydrogenase, thereby inducing an accumulation of intermediates between α-ketoglutarate and its precursors in the Krebs' cycle. Somehow this was supposed to initiate various shunt mechanisms leading to the synthesis of gamma carotene, melanin and the other ingredients related to the genesis of a resistant sporangium.

In later studies (Cantino, 1952) it was found that the bicarbonate effect was related to critical periods in the development of both R.S. and O.C. plants. Beyond three-fifths of the generation time of both, the course of development could be altered neither by the addition nor removal of bicarbonate. A change in permeability was thought to be at least partially responsible though the operation of more fundamental processes was not excluded.

The discovery of a mutant (Cantino and Hyatt, 1953b) bright orange in color by virtue of its content of large amounts of gamma carotene and which did not produce resistant sporangia, offered further opportunity for the study of the metabolic basis of R.S. formation. Studies of the comparative physiology of the mutant and the wild type were carried out (Cantino and Hyatt, 1953c). These revealed the presence in the latter of the enzymatic activities associated with cytochrome oxidase, aconitase, a TPN-specific isocitric dehydrogenase, a TPN (and/or DPN) dependent α-ketoglutarate dehydrogenase, succinoxidase, fumarase and a DPN-specific malic dehydrogenase. On the other hand, the mutant exhibited all of these activities except aconitase and α-ketoglutarate dehydrogenase. Thus, the mutant appeared unable to carry out the two oxidative decarboxylations associated with the presumed trigger mechanism of R.S. morphogenesis as a functional unit. Moreover enzymatic activities of a Krebs' cycle, previously only presumed to be present in Blastocladiella on the basis of indirect evidence, was confirmed.

Subsequent experiments concerned with finding a causative relationship between the action of bicarbonate and new biosynthetic pathways, then suggested a mechanism whereby bicarbonate fixation may be coupled to melanin production. First, it was found that melanin synthesis by R.S. could be uncoupled in vivo from the rest of the morphogenetic process by means of incorporating phenylthiourea into the growth medium without affecting the generation of otherwise structurally normal and viable R.S. (Cantino, 1953). In vitro studies demonstrated that the appearance of melanin formation was associated with a cell wall bound

polyphenoloxidase system, not present in O.C. plants, which was also inhibited by phenylthiourea (Cantino and Horenstein, 1955). The enzyme was found to mediate electron transport not only between substrate and oxygen but TPN as well (but not with DPN). Moreover, it appeared that α-ketoglutarate functioned as an essential factor for activity by virtue of its reductive carboxylation and concomittant oxidation of reduced TPN. Thus, it appeared that an important link had been established.

Other studies (cf. Cantino, 1960, for review) have been concerned with elaborating in more detail the biosynthetic and metabolis changes accompanying morphogenesis. The relationship between the findings derived therefrom and the bicarbonate effect is however, in most cases, still obscure.

In studies on nitrogen metabolism (Cantino, Lovett and Horenstein, 1957) it has been found that whereas the total nitrogen content of O.C. and R.S. plants were comparable, certain shifts in distribution did occur. The formation of resistant sporangia was accompanied by a sharp reduction in the soluble amino acid pool and an acid soluble nitrogen fraction, the loss of two electrophoretically separable protein fractions and the appearance of a new one not present in O.C. Furthermore, the decrease in the acid soluble nitrogen fraction was equivalent to the increase in chitin nitrogen. A change in the balance of two enzymes concerned with chitin synthesis was also discovered which may be related to the net increase in chitin in R.S. Both R.S. and O.C. were found to have comparable enzymatic activity associated with the acetylation

of glucoscamine, a reaction involved in the synthesis of chitin. But activity of chitinase, an enzyme catalyzing the conversion of chitin back to acetyl glucosamine, was decreased greatly as compared to O.C.

Studies were also carried out which revealed certain changes in aerobic metabolism associated with the genesis of a resistant sporangium. Corresponding to a decrease in endogenous respiratory activity (Q_{O_2} of O.C. = 90; Q_{O_2} of R.S. = 0.1) (Brown and Cantino, 1955; Cantino and Horenstein, 1955) is a pronounced decrease in enzymatic activity associated with the Krebs' cycle. Thus the terminal cytochrome oxidase is lost and aconitase and other enzymes of the cycle leading from α-keto-glutarate to malate either disappear or are rendered inoperative. However, the isocitric dehydrogenase needed for reversing the cycle by the reductive carboxylation of α-ketoglutarate remains active (Cantino and Horenstein, 1955).

The studies of the effect of bicarbonate on morphogenesis were temporarily interrupted by the discovery of another facet of the role of CO_2 in the life history of Blastocladiella emersonii. It was observed by Cantino and Horenstein (1956a), that cultures of ordinary colorless plants grew more rapidly and produced greater yields of plant material in the light than in the dark. Moreover, bicarbonate, albeit at lower concentrations (10^{-4} M) than for morphogenesis, was found to be involved. In a series of experiments with labelled and unlabelled bicarbonate it was found that illumination induced a large increase in CO_2 fixation by whole plants over dark levels and a corresponding decrease in labelled α-ketoglutarate and increase in labelled succinate within the organism.

In addition a compound appeared which, on the basis of paper chromatography, was thought to be oxalate. Both labelled and unlabelled glucose were also consumed more rapidly in the light while depressing bicarbonate fixation.

During in vitro studies, it was found that cell free preparations mediated a reduction of TPN via isocitric dehydrogenase which was inhibited by light. Conversely the same preparations mediated a light stimulated oxidation of TPNH which was accelerated by α-ketoglutarate and bicarbonate. Moreover succinate was found to induce a detectable amount of CO_2 fixation provided TPNH and light were present and this, too, appeared to be a TPN-specific reaction. When unlabelled α-ketoglutarate and labelled bicarbonate were incubated with cell-free extracts in the presence of TPNH the radioactivity was recovered in isocitrate, succinate and α-ketoglutarate.

Thus studies on the light effect, interesting and of wide implication in their own right, had served to supply direct corroborative evidence for the role of α-ketoglutarate as a locus for the fixation of bicarbonate in Blastocladiella. Moreover, in an attempt to integrate the observed facts from the light experiments, Cantino and Horenstein have extended the original concept of the mechanism of bicarbonate fixation to include the operation of an S.K.I. (succinate-ketoglutarate-isocitrate) cycle which may play a role in morphogenesis as well.

The S.K.I. cycle was thought to operate as follows: In the presence of bicarbonate light energy helps drive the reductive carboxylation of α-ketoglutarate to isocitrate and thence via an enzyme presumed to be

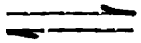
isocitritase to succinate and a C_2 fragment. Some of the succinate somehow yields more α-ketoglutarate (some of which is apparently derived from the nitrogen pool) thus completing the cycle, while the C_2 fragment and excess succinate are integrated into the rest of the metabolic machinery to yield increased growth. In morphogenesis, if a similar mechanism is assumed to operate, the high concentrations of bicarbonate involved (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 recent observations (Cantino, 1957) that the R.S. trigger mechanism is also stimulated by light.

Isocitritase

One of the critical reactions in the hypothetical S.K.I. cycle for Blastocladiella was the C_2-C_4 cleavage of isocitric acid thought to be mediated by the enzyme isocitritase. Evidence for such a deviation from the Krebs' cycle involving the formation of glyoxylate and succinate from the tricarboxylic acids was first provided by Campbell et al. (1953). using extracts of Pseudomonos aeruginosa. The specific substrate of the reaction was at first obscured by the finding of those workers that, while citrate and aconitate were active, isocitrate was not. However, the results of others (Smith and Gunsalus, 1954; Saj and Hillary, 1956; Olson, 1955) have since shown d-isocitrate to be the actual substrate for the reaction catalyzed by the enzyme isocitritase (Smith and Gunsalus. 1954). The activity has been identified in a number of aerobic and

facultative bacteria (Smith and Gunsalus, 1955; Wong and Ajl, 1955; Madsen and Hochster, 1959) certain fungi (Olson, 1959; Kornberg and Collins, 1958), and the castor bean (Kornberg and Beevers, 1957). It has been partially purified from pseudomonas (Smith and Gunsalus, 1957) and Bakers' yeast (Olson, 1959). Growth conditions (Smith and Gunsalus, 1955; Kornberg et al., 1958) for optimum enzyme production by bacteria have been shown to be aerobic in acetate and mineral salts media. Glucose completely represses isocitritase formation in facultative bacteria and decreases activity somewhat in aerobic organisms; therefore, it appears to be adaptively formed in these organisms.

Smith and Gunsalus (1954, 1957) have studied the specificity, stoichiometry, equilibrium and energetics of the reaction catalyzed by the partially purified pseudomonas enzyme. The enzyme catalyzes an aldol cleavage of d-isocitrate:



As indicated, the reaction is reversible with an equilibrium constant in the direction of the condensation of 34 (pH 7.6, 27°C) corresponding to a free energy change of -2100 calories. The authors point out, however, that, in view of the first order kinetics of the cleavage and the second order kinetics of the condensation, the distribution of reactants at low substrate concentrations is far towards glyoxylate and succinate.

Requirements for a sulfhydyl compound and a divalent metal ion have been demonstrated by various investigators (Wong and Ajl, 1955; Smith and Gunsalus, 1957; Olson, 1959). Cysteine and magnesium

respectively have been found to best satisfy these needs for the *Pseudomonas* and yeast enzymes.

Glycine-alanine Transaminase

Previous workers (Campbell, Smith and Eagles, 1954; Smith and Gunsalus, 1957; Wong and Ajl, 1955; Saj and Hillary, 1956) have discussed the production of glycine as one of the possible functions of isocitritase. Since glycine is a very active compound metabolically, such a reaction might connect the tricarboxylic acids to a number of important intermediates in biological syntheses. Thus besides its role as a component of protein, it has been implicated in the production of thymine (Elwyn and Sprinson, 1954) purines (Elwyn and Sprinson, 1950), carotene (MacKinney *et al.*, 1955) as well as purine synthesis and active C_1 unit metabolism (Shemin, 1956). Glycine is readily converted to serine by many organisms (Sakami, 1955) through which it might enter carbohydrate synthesis by way of hydroxypyruvate and 3-phosphoglyceric acid (Sallach, 1956).

A number of studies have shown that glyoxylate is rapidly converted to glycine *in vivo* in both plants and animals (Weinhouse and Friedmann, 1951; Weissbach and Sprinson, 1953; Kolesnikov, 1954; Tolbert and Cohan, 1953). The source of the amino group for the conversion is probably derived from transamination reactions with endogenous amino acids.

Transaminases catalyzing amino group transfer from all of the natural amino acids have been described (Meister, 1955) but relatively few purified. A great deal of effort has been expended during the past two decades in attempts to purify the glutamate-alanine and glutamate-

aspartate transaminases (Green et al., 1945; Cammarata and Cohen, 1951; O'Kane and Gunsalus, 1947; Lis, 1958). Only recently has a highly purified glutamate-aspartate-enzyme been obtained (Jenkins and Sizer, 1957; Jenkins et al., 1959). Other transaminases for which partial purifications have been reported are: gamma-aminobutyric-glutamate (Scott and Jakoby, 1959); tyrosine-glutamate (Canellakis and Cohen, 1956); serine-alanine (Sallach, 1955) and enzymes mediating simultaneous transamination-deamination reactions between asparagine and glutamine and a number of α-keto acids (Meister and Tice, 1950; Meister, et al., 1952; Meister, 1951, 1954) including glyoxylate.

Reports of transaminases catalyzing the interconversion of glyoxylate and glycine have been rare and, except for the transamination-deamidation enzymes, none has been purified or thoroughly studied. Cammarata and Cohen (1950) and Awapara and Seale (1952) have reported an enzymatic transmination between glyoxylate and glutamate catalyzed by rat tissue preparations. Wilson et al. (1954) demonstrated similar activity in various plant tissues. More recently a cell-free extract of Pseudomonas has been observed by Campbell (1956) to catalyze the transamination of glyoxylate to glycine from glutamate, aspartate, alanine, asparagine and glutamine. However, it was not established whether one or several enzymes were involved and no fractionation was attempted.

Metzler et al. (1954) and Nakada and Weinhouse (1953) have reported chemical transaminations between glycxylate and several amino acids including alanine without the intermediate participation of an organic

catalyst. It is of interest to note that the equilibrium of the chemical reaction:

L-Alanine + glyoxylate ⟷ glycine + pyruvate

strongly favors glycine formation with a calculated negative free energy change of two Kcal or more. Metzler (1954) has pointed this out as a possible explanation for the rarity of reports of enzymatically catalyzed transaminations in the direction of glyoxylate formation.

Though alanine has been reported to participate in a number of enzymatic transaminations (Sallach, 1955; Awapara and Seale, 1952; Rowsell, 1956; Rudman and Meister, 1953; Altenbern and Housewright, 1954), to the writer's knowledge there are no reports, other than that of Campbell (1956), of a glyoxylate-alanine transamination.

Much of the study of transaminases has been concerned with the role of vitamin B_6 and its analogues. Pyridoxal-5-phosphate has been established as a coenzyme for almost all of the transaminases examined critically for a requirement (Campbell, 1956; Meister and Peterson, 1954; Baxter and Roberts, 1958; Fincham and Boulter, 1956; Schlenk and Fisher, 1947; Jenkins et al., 1959), and it seems likely that it is involved in all transaminase reactions (Meister, 1955). That the coenzyme is tightly bound, may explain the observation that certain enzymes do not require the addition of pyridoxal phosphate for activity (Fincham and Boulter, 1956; Scott and Jakoby, 1956; cf. also Jenkins et al., 1959, Meister, 1955).

Of particular interest in our studies of glycine-alanine transaminase is the fact that Meister (1955) has pointed out the possibility

of transaminations involving glyoxylate not requiring the intermediate participation of this cofactor. This possibility is suggested by the findings of Metzler et al. (1954) and Nakada and Weinhouse (1953) with chemical transaminations.

The Development of Single Generation, Near-Synchronous Cultures of Blastocladiella

In attempting to obtain suitable material for studying the properties of isocitritase and glycine-alanine transaminase and elucidating their possible functions in growth and development, it was necessary to devise new culture techniques.

Previous studies of enzymes in B. emersonii have involved the use of five to ten day liquid cultures initiated by means of relatively light suspensions of zoospores from agar plate colonies of resistant sporangia (Cantino and Horenstein, 1955) or ordinary colorless plants (Cantino and Hyatt, 1953c). The harvests from such cultures are actually the result of several generations of plants and as such contain varying amounts of dead plant material and thalli at diverse stages of development. In the initial experiments on isocitritase it was found that results obtained by the use of such cultures were variable. More significant results, it seemed, might be forthcoming if means were devised for obtaining, as the result of a single generation of plants, sufficient material with which to carry out physiological studies.

Clearly, the problem of single generation cultures was one of developing a means of getting sufficiently heavy suspensions of zoospores. Barner and Cantino (1952) has already described means of inducing

germination of mature ordinary colorless plants on agar and obtaining zoospore suspensions therefrom. A logical extension of this technique on a very large scale not only permitted the growth of single generation cultures of ordinary colorless plants but it was found that such cultures exhibited essentially synchronous growth for most of the generation time. Furthermore, in the course of developing these culture methods the large scale preparation of zoospore suspensions permitted physiological studies on zoospores in a way not previously attempted in the aquatic fungi. It therefore became possible, for the first time, to study the enzymology of development, using both cell-free preparations and whole plants, at every stage of the life history of Blastocladiella.

GENERAL METHODS AND MATERIALS

The media used for the cultivation of B. emersonii were as follows: Medium PYG (now available as Difco, Cantino PYG Broth or Agar) contained 3.0 gm glucose, 1.25 gm Bacto Peptone (Difco), 1.25 gm yeast extract (Difco), 0.25 ml of 0.04 per cent brom cresol purple and 1000 ml distilled water. For cultivation of resistant sporangia 0.75 gm sodium bicarbonate per liter was added to medium PYG. Solid medium for the maintenance of stock cultures and the production of zoospores contained 20 gm Difco Bacto Agar per liter of PYG.

For the early studies on isocitritase, the methods for the cultivation of liquid cultures were as described by Cantino and Hyatt (1953a). All other studies were made using cultures grown according to methods developed during the course of this study and are described in detail in Part III of the Experimental Work. Material for the partial purification of isocitritase and transaminase was obtained by inoculating two-liter flasks, containing 1.5 liters PYG and equipped with aeration devices, with heavy suspensions of zoospores. The cultures were harvested at ca. thirteen hours by vacuum filtration, washed with ca. two liters distilled water and sucked "dry." Specific details about homogenization etc., are to be found in the appropriate sections.

The biochemicals used during the course of this work were obtained as follows: sodium pyruvate (reagent grade), amino acids, α-ketoglutaric acid, and oxalacetic acid from Nutritional Biochemicals Corporation; triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN),

sodium glyoxylate monohydrate, d,l-isocitric acid lactone (allo free), and pyridoxal-5-phosphate from Sigma Chemical Co.; pyruvic acid from California Corporation for Biochemical Research.

Isocitric acid was prepared from the lactone by hydrolysis with a slight excess of 2N potassium hydroxide in a boiling water bath. For use, the solution was adjusted to pH 7.4. Such preparations contained 50 per cent d-isocitric acid by assay with pig heart isocitric dehydrogenase (Ochoa, 1952).

Sodium pyruvate was prepared from the acid by the method of Robertson (1942) and recrystallized from a water-alcohol solution.

All inorganic chemicals and organic solvents were commercially available, reagent grade compounds.

The glass beads used for grinding plant tissue in the omnimixer were size No. 100 made by the Minnesota Mining and Manufacturing Company.

EXPERIMENTAL WORK

PART I

Isocitritase

1) Analytical Methods

Chemical: Glyoxylic acid was assayed as its 2,4-dinitrophenylhydrazone (DNPH) colorimetrically by the methods of Friedemann and Haugen (1943) and spectrophotometrically by an adaptation of the method of Smith and Gunsalus (1957). Keto acids were demonstrated by chromatography of their DNPH derivatives according to the method of Cavallini (1950) in butanol-ethanol-water (4:1:5), butanol-acetic acid-water (4:1:5), propanol-ammonia-water (6:3:1) and two dimensionally in phenol-water (2.5:1) and butanol-propionic acid-water (4.5:2:3).

Protein was determined turbidometrically by trichloroacetic acid precipitation (Stadtman et al., 1951) using crystalline bovine serum albumin as a standard. Readings were made using a Klett-Summerson colorimeter with a No. 54 filter. Estimates on more highly purified enzyme preparations were done by means of U.V. absorption measurements at 215 and 225 mu according to the method of Waddell (1956).

Enzymatic: Succinic acid was determined monometrically using a succinoxidase preparation from pig heart according to the method of Krebs as described by Umbreit et al. (1957). The method was found to be specific for succinate, no other compound elicited oxygen uptake.

Isocitric acid was determined enzymatically using the isocitric dehydrogenase prepared from pig heart by measuring TPN reduction spectrophotometrically at 340 mu (Ochoa, 1952).

Isocitritase activity was determined in an assay system containing in micromoles: d,l-isocitrate, 40; cysteine hydrochloride, 5; magnesium chloride, 10; phosphate buffer, pH 7.4, 200. The total volume was 3.1 ml, temperature, 30°C. The reaction was initiated by the addition of substrate and stopped by the addition of 0.3 ml 2N hydrochloric acid. When necessary, protein was precipitated by the addition of 0.02 ml ten per cent sodium tungstate and removed by centrifugation. After bringing to 10 ml, 1.0 ml of a 0.1 percent of 2,4-dinitrophenylhydrazine in 2N hydrochloric acid was added to 1.0 ml of the reaction mixture and allowed to react six minutes at room temperature. Two ml of 95 per cent ethanol was then added, followed by 4.0 ml of 1.5 N sodium hydroxide. After 3.5 minutes the color was read at 490 and 540 mu with a glyoxylate standard. Micromoles of glyoxylate per sample = 0.435 x optical density at 490 mu. Under the conditions of the assay, the optical density ratios, 490:540 of the DNPH derivatives of glyoxylate, pyruvate and α-ketoglutarate are ca. 1.5, 1.0 and 0.8 respectively. The optical density ratio, therefore, gave an indication of the presence of keto acids other than glyoxylate.

One unit of isocitritase activity = that amount of enzyme catalyzing the formation of 1.0 uM of glyoxylate per ten minutes in the protocol given above.

Specific activity = the number of units of activity per milligram protein.

2) Preliminary Observations

The incubation of crude homogenates prepared from six day old cultures of Blastocladiella with isocitric acid, led to the identification of glyoxylic acid as the product of an enzymatic reaction by means of paper chromatography of the 2,4-dinitrophenylhydrazone. However, α-ketoglutarate and pyruvate were always produced as well when longer incubation periods were used. In experiments in which succinate, succinate and glyoxylate, succinate and sodium bicarbonate, and glyoxylate were incubated with Blastocladiella homogenates, it was found that only glyoxylate gave rise to these two other compounds. This was later shown to be due to enzymatic and chemical transaminations between glyoxylate and endogenous amino acids.

The demonstration of the stoichiometry, and the study of 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 centrifugation of homogenates prepared using a motor driven glass homogenizer, activity was low or absent. In experiments where either 22,000 x G supernatants, low speed (500 x G) supernatants, or crude homogenates were incubated with isocitrate, it was found that most of the activity had been retained in the high speed sediment.

However, soluble preparations from ordinary colorless plants suitable for ammonium sulfate fractionation were finally obtained from extracts of acetone dried powders. Most of the determinations on stoichiometry were done using preparations obtained by this procedure.

It was later found that this method was unsuitable for use with resistant sporangia because of their very thick walls which resisted breakage using the glass homogenizer. The acquisition of a Servall Omnimixer permitted the preparation of soluble extracts from both R.S. and O.C. without preliminary treatment so that the use of acetone powder extracts were then abandoned.

3) Preparation of Enzyme from Acetone Powders

A five day culture was harvested in the usual manner and homogenized for exactly ten minutes in a motor driven homogenizer consisting of a Teflon pestle and glass tube, using 5.0 ml of 0.02M phosphate buffer for each gram (wet weight) of plant material in an ice bath.

The homogenate was then centrifuged at 22,000 x G for thirty minutes, the supernatant discarded and the sediment resuspended in the same volume of buffer. The centrifugation was repeated and the sediment resuspended in one-fifth volume of buffer.

To the mixture was added quickly, with stirring, ten volumes of acetone previously chilled to $-10^{\circ}C$. The mixture was allowed to stand ten minutes in the cold, after which it was filtered by suction through a Buchner funnel, washed with two volumes of acetone and sucked dry. The mat was then dried by passing air over it at room temperature or by evacuation in a vacuum desiccator over paraffin. After drying, the mat was ground to a powder by means of a mortar and pestle and stored at $-18^{\circ}C$ in a vacuum desiccator until ready for use.

Active extracts were prepared as follows: One gram of the acetone dried powder was stirred vigorously with 20 ml of 0.1M phosphate

buffer pH 7.4 containing 10^{-3} M cysteine for fifteen minutes. The preparation was then centrifuged at 22,000 x G for 30 minutes and the sediment discarded. The supernatant contained the enzyme; 3.2 gm powdered ammonium sulfate was then added slowly, with stirring, to each 10 ml of extract in the cold (ca. 45 per cent saturation). The precipitate which formed was centrifuged and resuspended in one-fourth the original volume of the same buffer.

4) Partial Purification

The results of a typical fractionation are shown in Table I. All steps were carried out at 0-2°C and continuously.

Step One: Preparation of the Crude Extract. The plant material from several fourteen hour cultures was homogenized in 0.1M phosphate buffer, pH 7.4, containing 10^{-3} M cysteine and 10^{-4} M versene (5 ml buffer per gm wet weight) by grinding with glass beads (see General Methods and Materials) in a Servall Omnimixer at 16,000 rpm, for three minutes. The Omnimixer was cooled by means of a salt-ice mixture at ca. -5°C.

After decanting from the beads the homogenate was centrifuged in the cold at 22,000 x G for thirty minutes in a Servall high speed centrifuge. The somewhat straw colored, slightly turbid supernatant was used for the subsequent fractionation.

Step Two: First Ammonium Sulfate Fractionation (Ammonium sulfate fraction A in table). Twenty-two gm of finely powdered ammonium sulfate was added slowly, with mechanical stirring, to each 100 ml of crude extract. The precipitate which formed was removed by

centrifugation and since it was always found to be without activity, discarded. The supernatant was then treated with an additional 10 gm per 100 ml, the precipitate collected by centrifugation and redissolved in one-fourth the original volume of 0.02M phosphate buffer pH 6.0.

Step Three: Protamine Sulfate Treatment (Colowick and Kaplan, 1955). A solution of protamine sulfate (20 mgm per ml of 0.02M phosphate buffer, pH 6.0) was added slowly, dropwise, with stirring to the solution from Step Two such as to give a concentration of 0.27 mgm protamine per milligram protein. The heavy, flocculent precipitate was removed by centrifugation. Most often a relatively clear supernatant was obtained. Unless the solution does clear, poor resolution is obtained in the following step. When it did not, additional small quantities of protamine sulfate (0.1 ml per 10 ml) were added followed by centrifugation until it did.

Step Four: Second Ammonium Sulfate Fractionation (Ammonium sulfate B in table). The protamine sulfate supernatant was brought to 27 per cent saturation by the addition of 19.5 gm of solid ammonium sulfate per 100 ml centrifuged and the inactive sediment discarded. An additional 5.4 gm per 100 ml of the original volume was then introduced and the precipitate collected by centrifugation and redissolved in one-fifth volume of 0.02M phosphate buffer pH 5.6.

Step Five: Negative Adsorption with Calcium Phosphate Gel. Calcium phosphate gel was prepared as described by Keilin and Hartree (1938). Small aliquots of the second ammonium sulfate fraction were

first treated with various quantities of the gel solution (18.6 mgm/ml) to determine what ratio of gel to protein would give maximum adsorption of inactive protein without appreciable adsorption of isocitritase. It was found that, in every trial made, 1.0 mgm of gel per mgm protein was optimal.

The standard procedure then, was to add slowly, with stirring, enough of a solution of calcium phosphate gel to give a gel to protein ratio of one. The suspension was then equilibrated by stirring by hand for fifteen minutes. At the end of that time the gel was removed by centrifugation and discarded.

Step Six: Third Ammonium Sulfate Fractionation (Ammonium sulfate C). To the gel supernatant from Step Four was added 6.4 ml of a saturated solution of ammonium sulfate (adjusted to pH 7.5) to each 10 ml of extract. The solution was then centrifuged and the sediment discarded. An additional 3.1 ml of the ammonium sulfate solution per 10 ml was then added and the sediment containing the activity redissolved in 0.1M phosphate buffer, pH 7.4, containing 10^{-3} M cysteine and 10^{-4} M versene.

5) Identification of Glyoxylate

Glyoxylate was identified as the keto acid product of the enzymatic reaction by chromatography as its 2,4-dinitrophenylhydrazone (DNPH) according to the method of Cavallini (1950), in four different solvents, as well as by two dimensional co-chromatography in phenol and butanol propionic acid. Glyoxylate 2,4-dinitrophenylhydrazone gave two spots in butanol-ethanol-water and in phenol and butanol-propionic acid.

TABLE I

PARTIAL PURIFICATION OF ISOCITRITASE

Fraction	Mgm P/ml	Specific Activity	Recovery* (per cent)	Purification (fold)
1 Crude Extract	4.1	0.31	100	1
2 Amm. S. (A) 31-45% Sat.	7.6	0.60	98	2
3 Protamine Supernatant	2.4	1.70	91	5
4 Amm. S. (B) 27-35% Sat.	3.0	4.00	55	13
5 Gel Supernatant	1.0	8.30	44	27
6 Amm. S. (C) 31-40%	0.8	16.20	26	52

Conditions--Reaction mixture contained: 5 uM cysteine HCl; 10 uM $MgCl_2$; 200 uM phosphate buffer pH 7.4; 40 uM d,l-isocitrate; isocitritase, ca. one unit. Total volume 3.1 ml; Temperature, 30°C.

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

It generally gave only one spot in butanol-acetic acid but occasionally gave evidence of two. The mobilities of the 2,4-dinitrophenylhydrazones of α-ketoglutarate, glyoxylate, pyruvate and oxalacetate are listed in Table II.

Glyoxylate was also identified as the product by means of the absorption spectrum of its 2,4-dinitrophenylhydrazone as well as the alkali instability of the latter. The spectrum of the glyoxylate derivative exhibits an absorption maximum at 455 mu and is readily distinguishable from those of the dinitrophenylhydrazones of pyruvate and α-ketoglutarate which do not exhibit any marked alkali instability. The absorption spectra of the DNPH derivatives of the unknown, glyoxylate, α-ketoglutarate and pyruvate are shown in Figures 1a, 1b, 1c and 1d.

6) Stoichiometry and Reversibility

Determinations on stoichiometry were carried out using the ammonium sulfate preparation from acetone extracts. The results are given in Table III and are in agreement with the established reaction:

$$\text{d-isocitrate} \rightleftharpoons \text{glyoxylate} + \text{succinate}$$

Also in the same table are the results of determinations of isocitrate disappearance and glyoxylate production obtained in runs using ammonium sulfate fractions of extracts from fresh material.

The reversibility of the reaction was confirmed by incubating 20 uM each of glyoxylate and succinate for one hour in the usual incubation mixture containing the acetone enzyme. Two and one-half micromoles of isocitrate were produced.

TABLE II

RF'S OF 2,4-DINITROPHENYLHYDRAZONES

Keto Acid		Solvent				
		Phenol	Butanol-Propionic	Butanol-Ethanol	Butanol-Acetic	Propanol-Ammonia
Glyoxylate	1	.65	.39	.40	.77	.76
	2	.73	.43	.57		.83
Pyruvate	1			.48	.88	.80
	2			.61	.95	.85
α-Ketoglu-turate				.24		.61
Oxalacetate				.30		.48

Phenylhydrazones were prepared and chromatographed according to the method of Cavallini (1950).

The isocitritase reaction product had the same mobilities as glyoxylate in all solvents.

TABLE III

ISOCITRITASE-STOICHIOMETRY

Enzyme	d-Isocitrate	Glyoxylate	Succinate
Ammonium Sulfate-Acetone			
1	4.9	5.0	5.4
2	4.0	5.3	4.1
Ammonium Sulfate A	3.8	4.0	---
	5.2	4.9	---

Conditions: As for isocitritase assay except that incubation was for one hour; enzyme ca. 2 units.

7) Enzyme Properties

Stability: Frozen mats and acetone powders of Blastocladiella may be stored for several weeks without appreciable loss of activity. Crude extracts stored at 4°C lose activity with variable rapidity (often as much as 30-40 per cent in four hours). This may be due to proteolytic activity in the extracts for there is some accumulation of ninhydrin positive substances.

Partially purified enzyme solutions may be stored for some weeks at -18°C with relatively little loss in activity. However, about 50 per cent is lost on overnight storage at 4°C.

Reaction Rate: As delineated in Figure 2 the rate of glyoxylate formation is directly proportional to enzyme concentration up to about two units. Consequently, all assays of enzymatic activity were kept in the range of about one unit. The time course of the isocitritase reaction is shown in Figure 3.

Effect of pH: The isocitritase of Blastocladiella has a rather sharp pH optimum at pH 7.4 as shown in Figure 4. All enzymatic assays were carried out at this pH.

Specificity: Though neither pure d-isocitrate nor l-isocitrate were avilable for testing the specificity of the purified enzyme, specificity for the d-isomer is implied by the stoichiometry data. This follows from the fact that the enzyme preparation used for the determination of isocitrate is specific for the d-isomer while both isomers were always present in the reaction mixture in equal concentrations.

Crude extracts from R.S. and O.C. plants prepared using the Omnimixer did not produce any 2,4-dinitrophenylhydrazone-forming compound when incubated with citrate or cis-aconitate. This is of particular interest since various workers (Dagley and Dawes, 1955; Smith et al., 1956; and Wheat and Ajl, 1955) have reported an enzyme from bacteria, citritase, which cleaves citrate to oxalacetate and acetate. Moreover, oxalacetate was never detected as a product of isocitrate utilization by crude extracts prepared using the glass homogenizer or from acetone powders of ordinary colorless plants though our conditions were such that it would have been detected and Cantino (1953c) demonstrated the presence of aconitase activity in such preparations. Neither did they produce oxalacetate from citrate as determined by light absorption measurements at 280 mμ (Greenwood and Greenbaum, 1953).

Co-factor Requirements: Isocitritase requires a metallic ion for maximum activity as well as a sulfhydryl group. Glutathione was found to be only ca. 60 per cent as effective as cysteine at the concentration generally used (Table IV). Though Mg^{++} was routinely employed as the metallic cofactor throughout these studies Mn^{++} was found equally effective when used with tris buffer at the same concentration.

The cofactor saturation curves for both Mg^{++} and cysteine are shown in Figures 5a and 5b. Both gave maximum activity at ca. 10^{-3} M concentration.

Effect of Substrate Concentration: When isocitrate concentration (as the d-isomer) was plotted against reaction velocity a typical substrate concentration curve was obtained. Rearrangement of the data

TABLE IV

ISOCITRITASE: COFACTOR REQUIREMENTS

Sulfhydryl Compound	A Cysteine	B Glutathione
uM Glyoxylate per 10 minutes	1.23	0.77

Conditions: As for isocitritase assay; enzyme, acetone extract dialyzed 4 hours against 0.1M phosphate buffer, pH 7.4.

TABLE V

IN VITRO EFFECT OF LIGHT ON ISOCITRITASE

	Light	Dark
uM Glyoxylate produced in 10 minutes*	1.26*	1.27

*Avg. of two determinations.

Conditions: As for isocitritase assay; except that the light tubes were subjected to light of intensity 300 foot candles. Dark were shielded with aluminum foil. Enzyme, Ammonium Sulfate C.

in the following form of the Michaelis equation (Lineweaver and Burk, 1934) permitted a graphical estimation of the Michaelis constant as 4.8×10^{-4} M (Figure 6).

$$\frac{[S]}{v} = \frac{1}{V} \cdot [S] + \frac{Km}{V}$$

Effect of Temperature: Determinations of the reaction rates at several temperatures permitted a graphical estimation of the activation energy of the enzyme catalyzed reaction according to the Arrhenius equation (Neilands and Stumpf, 1958; Arrhenius, 1915)

$$\log K = \frac{-E}{2.3\,R} \left(\frac{1}{T}\right) + C \qquad \text{(Figure 7)}$$

E = 10,700 calories per mole. The Q_{10} of the enzyme reaction is 1.8.

Effect of Light: The intensity of the incident light on the illuminated tubes was ca. 300 foot candles. Light was excluded from the dark tubes by wrapping in aluminum foil. As shown in the table there was no difference between the quantities of glyoxylate produced in the light and the dark.

FIGURE LEGENDS

Figure 1a-1d. Adsorption Spectra of the 2,4-dinitrophenylhydrazones of Pyruvate, α-Ketoglutarate, Glyoxylate and the Product of the Isocitritase Reaction. The measurements were made after treatment of the keto-acids with 2,4-dinitrophenylhydrazine as described in Analytical Methods.

Figure 2. Isocitritase: Enzyme Assay Curve. Conditions as described for the isocitritase assay. See Table I.

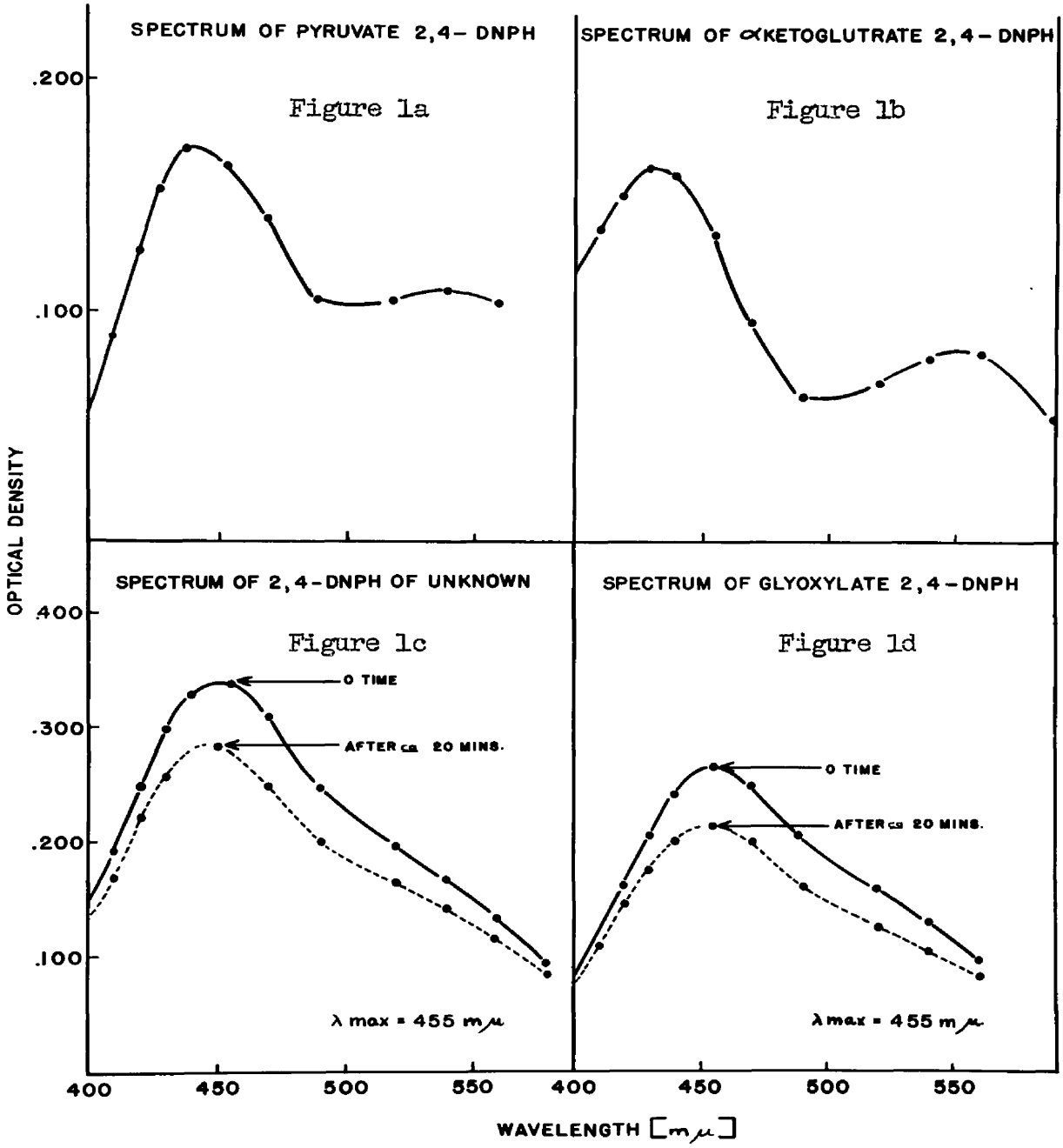
Figure 3. Time Course of Isocitritase Reaction. Conditions as described for the isocitritase assay except that incubation was as indicated.

Figure 4. Isocitritase: pH Optimum. Conditions as for isocitritase assay except that a series of 5 minute incubations was used at the indicated pH's. Enzyme, Ammonium Sulfate C.

Figure 5a-5b. Isocitritase: Cofactor Requirements. Conditions as for isocitritase assay. Figure 5a, cysteine-HCl varied; Figure 5b, magnesium chloride varied. Enzyme, Ammonium Sulfate C.

Figure 6. Isocitritase: Effect of Substrate Concentration. Conditions as for isocitritase assay except that substrate concentrations were as indicated. Enzyme, Ammonium Sulfate C.

Figure 7. Isocitritase: Activation Energy. Conditions as for isocitritase assay except that temperatures were as indicated. Enzyme, Ammonium Sulfate C.


SPECTRUM OF PYRUVATE 2,4- DNPH
Figure 1a
SPECTRUM OF αKETOGLUTRATE 2,4- DNPH
Figure 1b
SPECTRUM OF 2,4-DNPH OF UNKNOWN
Figure 1c
0 TIME
AFTER ca 20 MINS.
λ max = 455 mμ
SPECTRUM OF GLYOXYLATE 2,4-DNPH
Figure 1d
0 TIME
AFTER ca 20 MINS.
λ max = 455 mμ
OPTICAL DENSITY
.200
.100
.400
.300
.200
.100
400
450
500
550
WAVELENGTH [mμ]

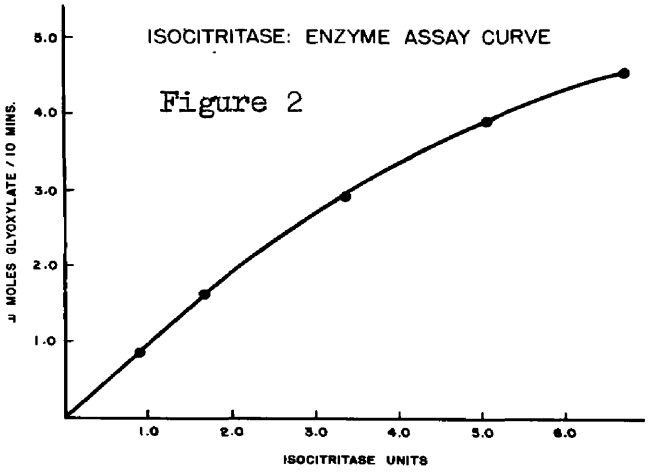


Figure 2

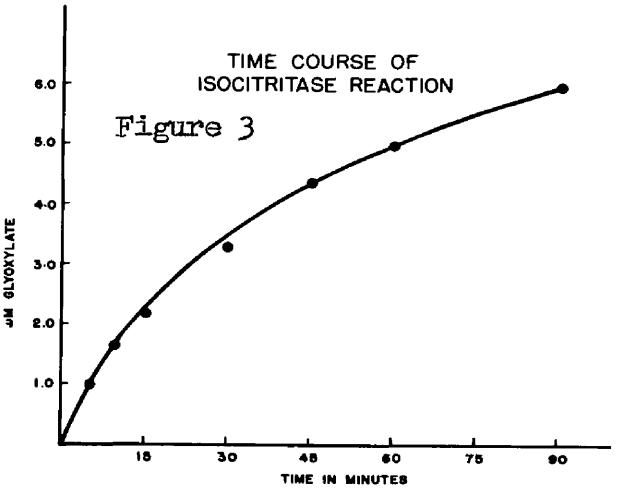


Figure 3

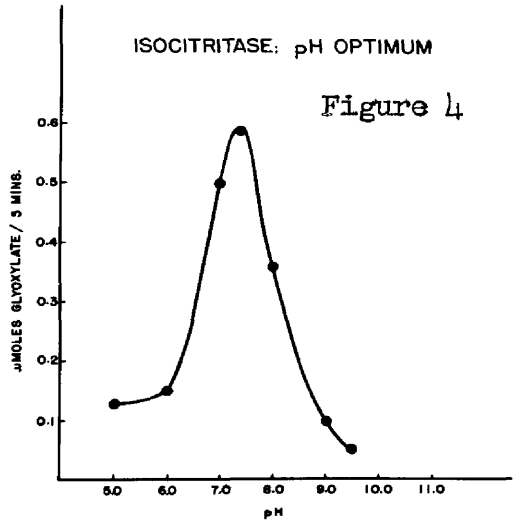


Figure 4

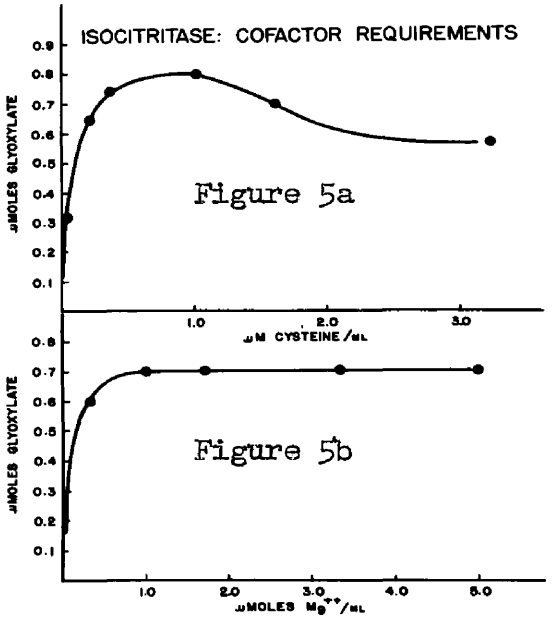


Figure 5a

Figure 5b

Figure 6

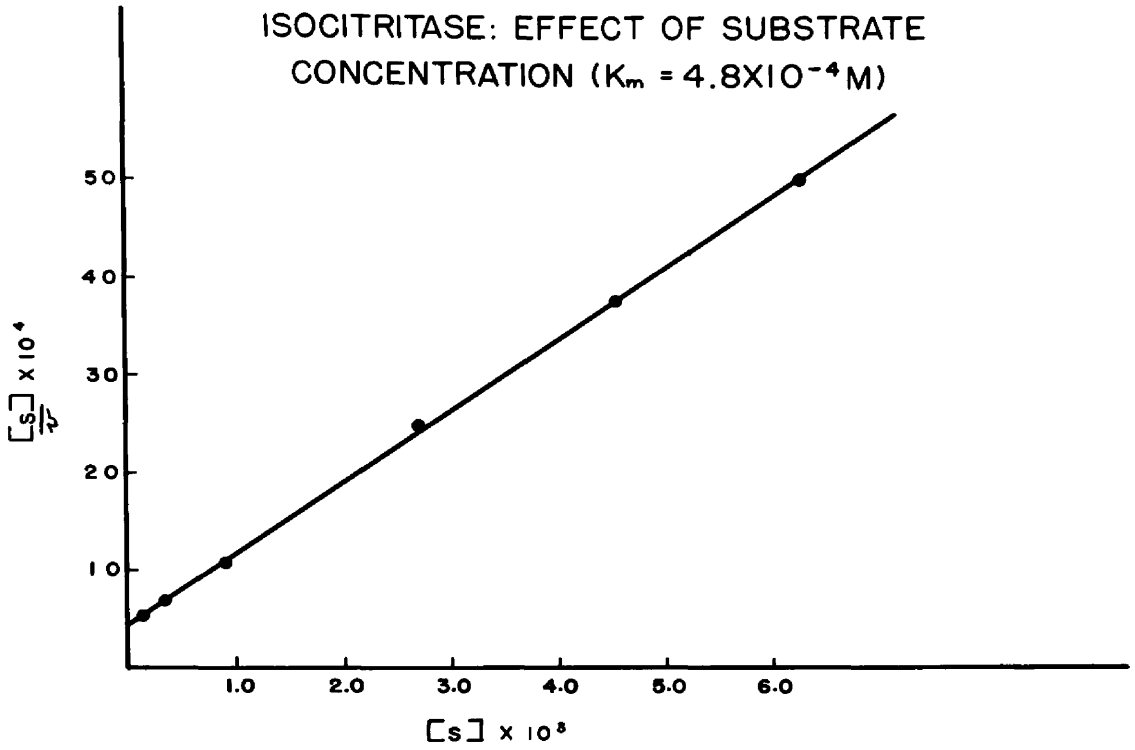
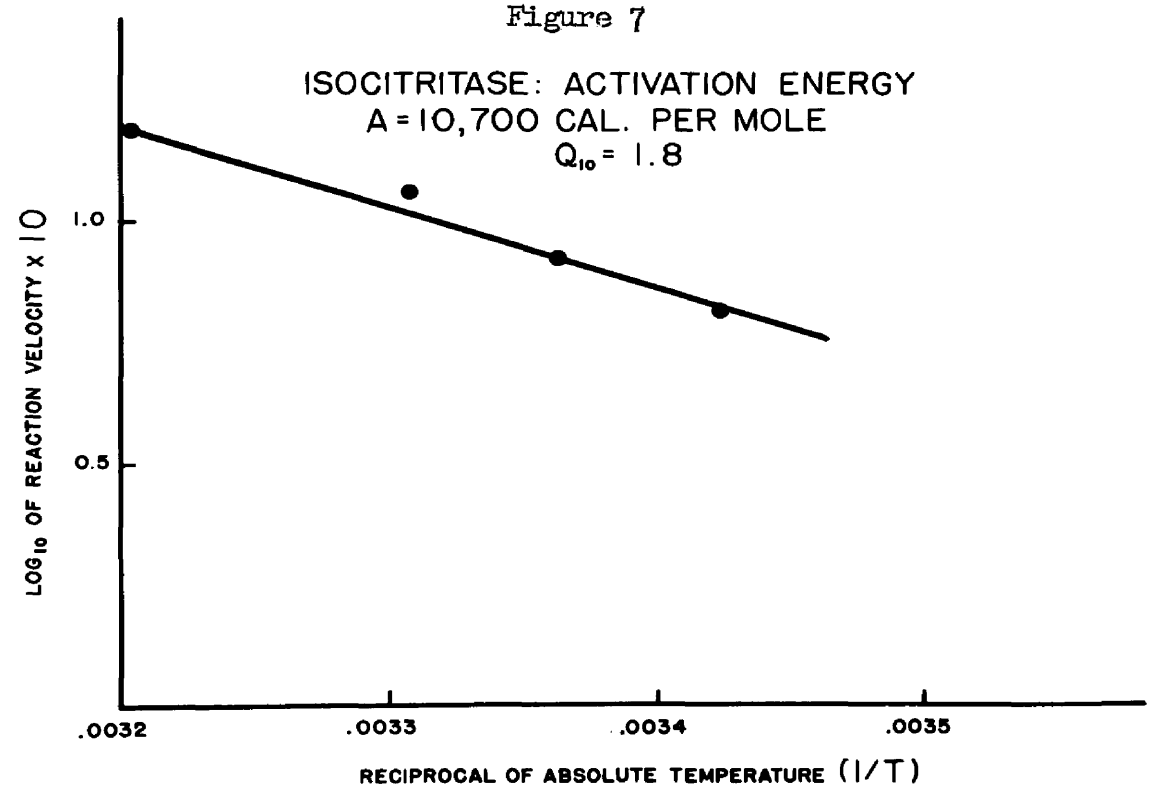


Figure 7



PART II

Glycine-Alanine Transaminase

1) Analytical Methods

Amino Acids: Glycine and alanine were determined by elution of their ninhydrin reaction products from paper chromatograms, followed by spectrophotometric measurements at 575 mu, according to the method of Kay et al. (1956). The chromatograms for quantitative work were run unidirectionally using Whatman No. 1 filter paper which had been previously washed. The wash was carried out using 1N acetic acid followed by three rinses in glass distilled water and one in propanol-ammonia-water (6:3:1). The latter was used as the solvent system for all quantitative work.

For the qualitative detection of amino acids involved in transamination reactions, one-dimensional paper chromatograms were run using butanol-ethanol-water (4:1:5), propanol-ammonia-water (6:3:1) and butanol-acetic acid-water (4:1:5) and the spots detected by spraying with 0.1 per cent ninhydrin in 95 per cent ethanol.

Pyruvic Acid: Pyruvic acid was determined colorimetrically according to the method of Straub as outlined by Green et al. (1945) with slight modification. To 1.0 ml of the sample containing 0.1 to 1.0 uM pyruvate, in a calibrated test tube was added 1.0 ml potassium hydroxide (100 gm per 60 ml water), followed by 0.5 ml of 2.0 per cent salicaldehyde in 95 per cent ethanol. After a ten minute incubation at 37°C, the solution was made up to 10 ml, cooled in an ice bath and

the color measured using a No. 42 filter in a Klett-Summerson colorimeter. As pointed out by Green et al. (1945) the color yield from a given amount of pyruvate varied somewhat from one determination to the next in a fashion not easily controlled. It was therefore necessary to include a pyruvate standard in all determinations and to calculate the amount in the sample therefrom. Klett readings were found to bear a linear relationship to pyruvate concentration up to ca. 1.0 uM in a 1.0 ml sample. The reaction was specific for pyruvate.

Glyoxylic and Pyruvic Acids: Glyoxylate and pyruvate were assayed spectrophotometrically in mixtures as their 2,4-dinitrophenylhydrazones using the same procedure described previously for determining glyoxylate alone. From the standard curves obtained for the DNPH derivatives of each acid at 490 and 540 mu, a simultaneous equation was derived which permitted the calculation of both their concentrations:

$$1.56x + 2.25y = A$$

$$1.50x + 1.47y = B$$

where

x = uM pyruvate per sample

y = uM glyoxylate per sample

A = optical density at 490 mu

B = optical density at 540 mu.

Glycine-Alanine Transaminase: Glycine-alanine transaminase was determined in a 1.0 ml assay mixture containing: 10 uM sodium glyoxylate monohydrate; 10 uM L-alanine; 40 uM phosphate buffer, pH 8.0;

5 uM magnesium chloride; and ca. 10 ug pyridoxal-5-phosphate. After a ten minute incubation at 37°C, the reaction was stopped by the addition of Straub Test reagents. The blank for the color measurement contained all additions except enzyme. Such a control is necessary to correct for a small amount of chemical transamination which occurs between glyoxylate and alanine in the strong alkali used in the test.

A unit of transaminase activity = that amount of enzyme catalyzing the production of 1.0 uM pyruvate in ten minutes in the above protocol.

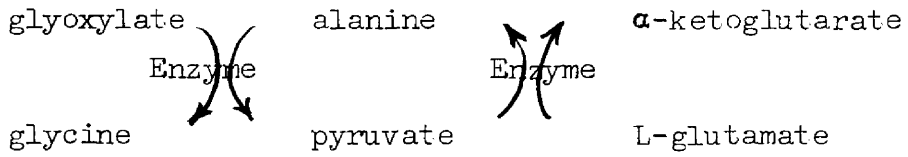
Specific activity = the number of units of activity per milligram protein.

Protein was determined as previously described.

2) Preliminary Observations

During the studies on isocitritase it was found that, when using crude preparations and long incubation periods (one to two hours), a-keto-glutarate and pyruvate were frequent products of side reactions involving glyoxylate. It seemed likely that a transamination between glyoxylate and endogenous amino acids was responsible. To verify this assumption glyoxylate was incubated with a number of amino acids (L-glutamate, L-glutamine, L-asparate, 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. Appropriate controls omitting one or the other presumed substrate were also incubated in each case. Transamination was detected by means of paper chromatography of the reaction mixtures followed by ninhydrin spray. An enzymatic transamination

occurred only in those reaction mixtures containing L-alanine. Evidently the α-ketoglutarate detected during the isocitritase runs was produced partially as a result of a direct chemical transamination between glyoxylate and endogenous glutamate and partially as a result of an enzymatic transamination mediated by alanine since an alanine-glutamate transaminase was also present:



Since it seemed possible that an alanine-glyoxylate transamination might constitute an important "pulling" reaction for the removal of glyoxylate produced by isocitritase, further studies were carried out on this enzyme.

3) Partial Purification

Step One: Preparation of crude extract. The plant material from four 14 hour cultures of Blastocladiella were harvested as described previously. After weighing, the mats were homogenized in glass distilled water (4 ml/gm) containing 10^{-4} M versene by grinding with glass beads (4 gm beads/gm plants) in an Omnimixer cooled in a salt ice bath (-5°C) at 16,000 rpm for three minutes. After decanting from the beads the homogenate was centrifuged at 22,000 x G for 30 minutes and the supernatant used for fractionation.

Because of the large amounts of endogenous amino acids in the crude extract, no direct estimation of the initial transaminase activity

could be made. The extract was therefore dialyzed for 20 hours against 0.2M phosphate buffer, pH 7.4, at 4°C, after which it was assayed for activity. Since dialysis always lowered the protein concentration through apparent denaturation of inactive protein, the initial specific activity was expressed as the number of micromoles of pyruvate produced by the dialyzed preparation per milligram protein in the original undialyzed preparation. This was possible since it was subsequently found that even prolonged dialysis of the enzyme in water or buffer did not affect the transaminase activity. A similar procedure was used for the protamine sulfate fraction.

Step Two: Protamine Sulfate Treatment A. 0.75 ml of a protamine sulfate solution (20 mgm/ml) was added slowly, with stirring for every 100 mgm of crude extract protein at 0°C. After stirring for an additional ten minutes the abundant precipitate which formed was removed by centrifugation at 30,000 x G for 15 minutes.

Step Three: First Acetone Fractionation. With the temperature of the extract maintained at -2°C to 0°C enough acetone (-10°C) was added rapidly dropwise to bring the concentration to 32 per cent. The solution was then cooled to -8°C and more acetone added to bring the concentration to 41 per cent. After allowing the temperature to rise to -6°C, the inactive precipitate which formed was removed by centrifugation at 6,000 x G for three minutes at -6°C.

The concentration of acetone in the supernatant was then brought to 47 per cent while maintaining the temperature at -10°C. After again

allowing the temperature to rise to -6° C, the precipitate was removed by centrifugation as before and discarded.

The active fraction was then obtained by bringing the acetone concentration to 57 per cent at -10°C. After allowing the temperature to rise to -6°C, the solution was centrifuged and the sediment resuspended in one-fifth the original volume of glass distilled water.

The slightly turbid, somewhat straw-colored solution thus obtained, was then dialyzed 20 hours against three changes of 100 volumes of distilled water containing 10^{-4} M versene at 1°C.

Step Four: Negative Adsorption with Calcium Phosphate Gel. Enough of a calcium phosphate gel solution (18.6 mgm/ml) was added to the dialyzed acetone fraction at 0°C to give a gel-protein ratio of 2 mgm gel/mgm protein. After allowing adsorption equilibrium to be reached by stirring for 15 minutes by hand the gel was removed by centrifugation and discarded.

The process was repeated once more using the same gel-protein ratio and the supernatant obtained by centrifugation used in the next step.

Step Five: Final Acetone Fractionation. In order to concentrate the transaminase and obtain some additional purification a second acetone precipitation was carried out.

While maintaining the temperature at -5°C to 0°C enough acetone (-10°C) was added to give a concentration of 62 per cent. The precipitate which formed was obtained by centrifugation and resuspended in

one-fourth volume glass distilled water. The resulting enzyme solution was then dialyzed for 20 hours against three changes of 500 volumes of distilled water.

The results of a typical transaminase purification are shown in Table VI.

4) Stoichiometry, Reversibility and Identification of Products.

Pyruvate was verified as the keto acid product of the transamination reaction between glyoxylate and alanine by means of paper chromatography of its 2,4-dinitrophenylhydrazone in butanol-ethanol-water according to the method of Cavallini (1950) and by the color obtained in the specific quantitative salicylaldehyde reaction of Straub (see Analytical Methods). Glycine was identified by means of paper chromatography in propanol ammonia, butanol-ethanol-water and butanol-acetic acid-water. The mobilities of all reactants are listed in Table VII.

Determinations of all reactants after one and two hour incubations of glyoxylate and alanine with enzyme as shown in Table VIII, permitted definition of the reaction as:

$$\text{Glyoxylate} + \text{L-Alanine} \rightleftharpoons \text{Glycine} + \text{Pyruvate}$$

When an attempt was made to demonstrate reversibility by incubating 10 uM pyruvate and 10 uM glycine in the usual reaction mixture, no disappearance of pyruvate could be detected nor was alanine produced. This, in spite of the fact that the "forward" reaction proceeded to only ca. one-third conversion (see Figure 9). It therefore seemed likely that pyruvate or glycine was inhibitory.

TABLE VI

RESULTS OF TRANSAMINASE PURIFICATION

Fraction	Protein Concentration (mgm/ml)	Specific Activity	Over-all* Recovery (per cent)	Purification Factor
1. Crude Extract	5.3	0.68	100	1
2. Protamine Sulfate	2.7	1.40	103	2
3. Acetone I	2.2	11.0	96	16
4. Gel Supernate B	0.7	29.0	100	40
5. Acetone II	0.9	55.0	60	80

Conditions: 10 uM sodium glyoxylate monohydrate; 10 uM L-alanine; 40 uM phosphate buffer, pH 8.0; 5 uM $MgCl_2$; 10 ug pyridoxal-5-phosphate. Enzyme, < 0.7 units. Total volume 1 ml. Temperature 37°C. Reaction stopped at 10 minutes by addition of Straub reagents.

*(Total units/total number of units in original extract) x 100

TABLE VII

RF'S OF TRANSAMINASE REACTANTS

Reactant	Solvent		
	Butanol-Ethanol	Propanol Ammonia	Butanol Acetic
Glyoxylate (2,4 DNPH)	0.40, 0.57		
Pyruvate (2,4 DNPH)	0.48, 0.61		
Glycine	0.36	0.41	0.15
Alanine	0.42	0.50	0.22

TABLE VIII

STOICHIOMETRY OF GLYOXYLATE-ALANINE TRANSAMINATION

Time	Glycine uM	Pyruvate uM	Alanine uM	Glyoxylate uM
1 hr	2.9	2.7*	2.5	2.8
2 hrs	3.5	3.6*	3.3	3.2

Conditions: As for transaminase assay except that incubation was for times indicated. Reaction stopped with 0.09 ml 2N HCl. Enzyme: Gel supernatant B.

*Determined by calculation from optical densities at 490 and 540 mu and Straub test (averaged).

Reversibility was subsequently demonstrated by means of the following experiments. Two parallel series of four reaction tubes each were set up according to the usual protocol and after initiation of the reaction a tube from each set was stopped at five minute intervals up to 20 minutes. In one set 20 uM glycine in 0.1 ml was added at ten minutes to the tubes to be stopped at 15 and 20 minutes. The results of this experiment are shown in Figure 10 where it may be seen that glycine brought about apparent reversal. That this was so, was confirmed in another experiment in which 2.0 uM pyruvate and 20 uM glycine were incubated for one hour in the usual reaction mixture. Under these circumstances there was a disappearance of 0.28 uM pyruvate with a corresponding appearance of alanine as demonstrated by qualitative paper chromatography.

5) Enzyme Properties

Stability: The enzyme may be stored for at least a week at $4^{\circ}C$ without loss of activity. Frozen (-18°) samples of the enzyme stored for two months showed no loss of activity. The enzyme is also stable to prolonged dialysis against various buffer solutions in distilled water.

Effect of pH: Highest activity is obtained at about pH 8.5. However, since as shown in Figure 11 considerable nonenzymatic transamination occurs in this pH range, all routine assays of the activity of the enzyme were carried out at pH 8.0.

Reaction Rate: As illustrated in Figure 8 the amount of pyruvate produced is directly proportional to the quantity of enzyme up to 0.7 units. All assays were carried out in this range since the method for the determination of pyruvate which was used, was suitable only in the range up to 1 uM of pyruvate.

A typical time course curve for the enzymatic transamination reaction is shown in Figure 9. The reaction was found to proceed to only ca. one-third conversion.

Activators and Inhibitors: Neither prolonged dialysis against distilled water, phosphate buffer or a 50 per cent saturated solution of ammonium sulfate caused the enzyme to exhibit any stimulation by the addition of pyridoxal-5-phosphate. The latter procedure did cause the irreversible loss of ca. 30 per cent of the original activity. Precipitation of the enzyme by dialysis against saturated ammonium sulfate for ten hours followed by dialysis for three days against 0.02M acetate buffer, pH 4.0 (Youatt, 1958) caused complete and irreversible loss of all activity which could not be restored even by prolonged incubation in the presence of pyridoxal phosphate and various metal ions (Fe^{++}, Mg^{++}, Cu^{++}) (Metzler, 1954).

In common with other pyridoxal phosphate containing enzymes, glycine-alanine transaminase was inhibited by both hydroxylamine and potassium cyanide (Colowick and Kaplan, 1953) (Table IX). However, inordinately high concentrations of the latter were required (10^{-2} M). Hydroxylamine inhibited completely at 10^{-3} M and ca. 40 per cent at 10^{-4} M. The inhibition of the latter concentration was completely overcome by

TABLE IX

INHIBITION OF TRANSAMINASE

Additions	uM Pyruvate	Per Cent 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 NH_2OH + 10^{-3} M pyridoxal phosphate	0.65	0

Conditions: As for transaminase assay except for additions as indicated.
Enzyme: Gel Supernatant B.

10^{-3} M pyridoxal phosphate. Isoniazid (Davison, 1956; Youatt, 1958) another reported inhibitor of vitamin B_6 enzymes did not inhibit even at 10^{-2} M.

Requirements for a metal ion have so far not been demonstrated for any transaminase. The glycine-alanine transaminase was found to be no exception. Dialysis against distilled water and various buffers containing versene failed to reveal the need for a metallic cofactor.

Substrate Specificity: In order to test the specificity of the partially purified preparations, incubations were carried out with a number of substrate pairs. The appearance of products was detected by means of qualitative paper chromatography with exceptions as noted in the Table X.

L-alanine could not be replaced by any other amino acid in systems containing glyoxylate. D-alanine was completely inactive. Though the original crude extract contained considerable glutamate-alanine transaminase activity it was completely lacking in the purified preparation.

An enzymatic transamination between pyruvate and asparagine, however, was detected. Further work will have to be done to determine whether this is due to the same or a different enzyme though structural considerations would seem to suggest that a separate enzyme is involved.

Effect of Light: Assays of transaminase activity were carried out in the light and in the dark under the same conditions used previously for light-dark assays of isocitritase. The results of this experiment are presented in Table XI. No stimulation by light was found to occur.

TABLE X

ENZYME SPECIFICITY OF GLYCINE-ALANINE TRANSAMINASE

Keto Acid	Amino Acid	Result
Pyruvate	Glycine	+
	L-isoleucine	-
	L-serine	-
	L-valine	-
	L-leucine	-
	L-lysine	-
	L-phenylalanine	-
	L-proline	-
	L-glutamine	-
	L-asparagine	+
	L-cysteine	-
	L-arginine	-
	L-tryptophan	-
Glyoxylate	L-alanine	+
	D-alanine	-
	L-phenylalanine	-
	L-cysteine	-
	L-valine	-
	L-tryptophan	-
	L-serine	-
	L-leucine	-
	L-lysine	-
	L-threonine	-
	L-aspartate	-
	L-glutamate	-
	L-isoleucine	-
	L-asparagine	-
Oxalacetate	L-alanine*	-
α-Ketobutyrate	L-alanine*	-
	L-glycine	-
α-Ketoglutarate	L-alanine*	-

Conditions: As for transaminase assay except that: incubation was for 2 hours; incubations of pyruvate contained 2 uM pyruvate and 20 uM of amino acid. All others contained 10 uM each of keto acid and amino acid. Enzyme: Acetone II.

*Activity determined by Straub test for pyruvate. All others by qualitative paper chromatography.

TABLE XI

IN VITRO EFFECT OF LIGHT ON TRANSAMINASE*

	Light	Dark
uM Pyruvate/10 minutes	0.76	0.75

Conditions: As for the transaminase assay, except that light tubes were subjected to light of intensity ca. 300 foot candles. Dark tubes were shielded with aluminum foil. Enzyme: Gel supernatant B.

*Average of duplicate determinations.

FIGURE LEGENDS

Figure 8. Transaminase: Enzyme Assay Curve. Conditions as for transaminase assay (see Table VI). Enzyme: Gel Supernatant B.

Figure 9. Time Course of Glycine-Alanine Transamination. Conditions as for transaminase assay except that incubation times were as indicated. Reaction stopped with 0.09 ml 2N HCl. Enzyme: Gel Supernatant B.

Figure 10. Reversibility of Glycine-Alanine Transamination. Two parallel series of four reaction tubes each were set up using the conditions described for the transaminase. 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 uM glycine in 0.1 ml was added to the 15 and 20 minute tubes. Enzyme: Gel Supernatent B.

Figure 11. Transaminase: Effect of Hydrogen Ion Concentration. Conditions as for transaminase assay except 40 uM tris buffer was used from pH 7.2 to pH 8.4 and 40 uM borate buffer from pH 8.4 to pH 10. Chemical controls were incubated for each pH and pyruvate determined using the pH 7.2 reaction mixture as control. Enzyme: Gel Supernatant B.

Figure 8

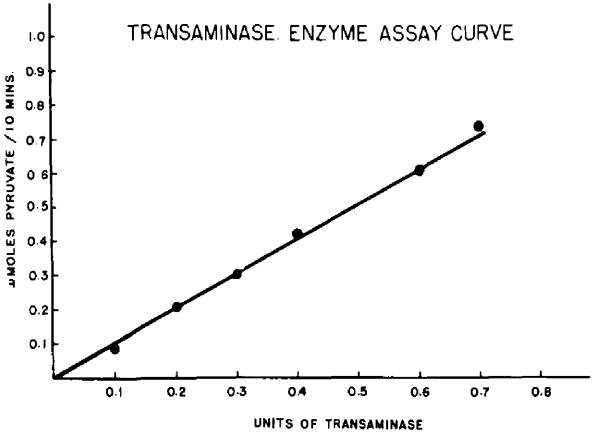


Figure 9

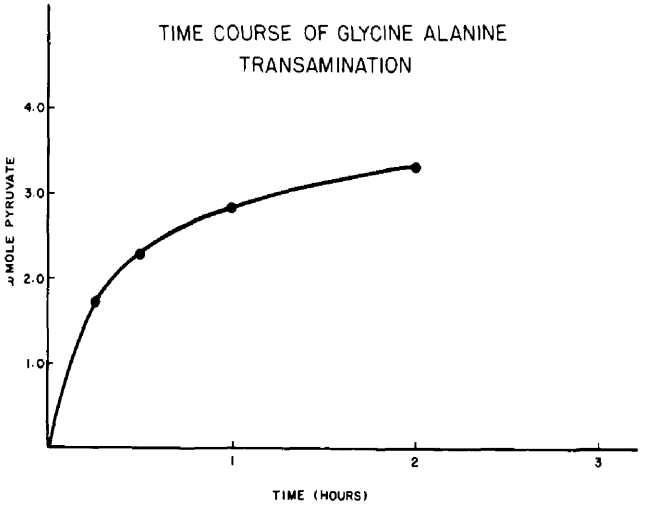


Figure 10

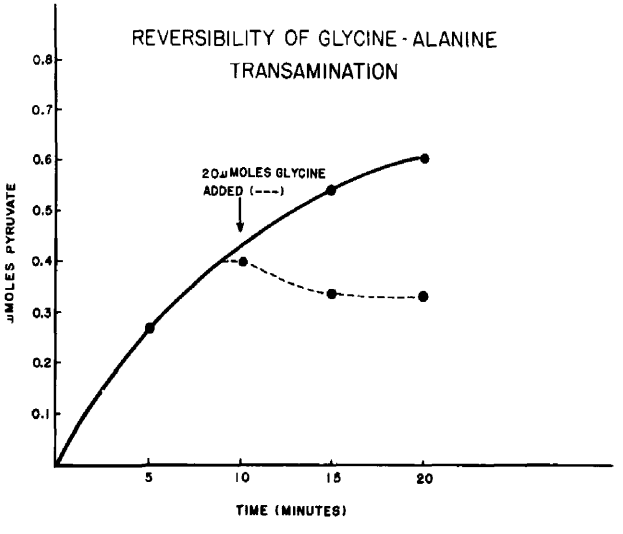
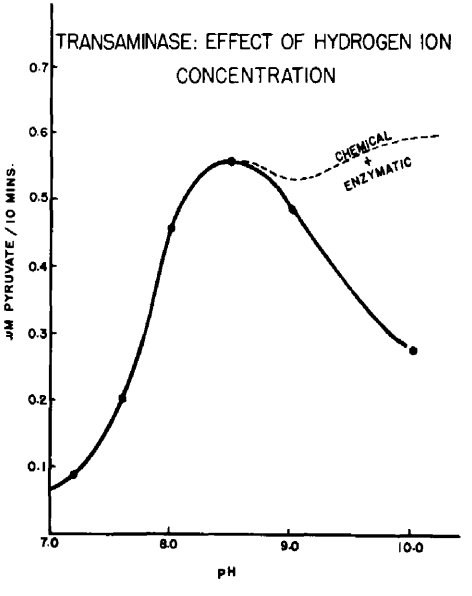


Figure 11



PART III

Studies on the Physiology of Development

1) Preparation of Zoospore Suspensions

Expressions of Swarmer Density: In order to obtain reproducible zoospore suspensions for inocula and other experiments involving swarmers it was necessary to have some rapid means of determining the density of suspensions. This was done turbidometrically using the Klett-Summerson colorimeter. Suspensions were read directly or diluted, depending on the degree of turbidity, using the No. 42 filter and an appropriate blank. The "swarmer density" of a suspension was defined as the corrected Klett reading.

Viable counts obtained by plating out dilutions of suspensions of known density permitted the calculation of a factor for converting swarmer density into numbers of viable cells:

One swarmer density unit = 4.8×10^{4} viable cells/ml.

The yield of zoospores from any one harvest was never great enough to permit dry weight determinations simultaneously with other studies. Therefore dry weight determinations were done on suspensions of known swarmer density and the following conversion factor obtained:

One swarmer density unit = 2.4×10^{-3} mgm dry weight per ml.

Preparation of Plates and Harvest of Zoospores: Large (12.5 cm) freshly poured petri plates containing ca. 40 ml PYG agar were inoculated with ca. 10^{5} viable zoospores in a total volume of 1.0 to 1.5 ml and the suspension spread evenly over the surface of the agar.

The plates were then incubated at 27°C. Under these conditions, germination of the mature plants was generally far advanced fourteen hours after inoculation. The plates were then flooded with 10-15 ml. of sterile distilled water and allowed to stand for about thirty minutes. After swirling the plates several times to suspend the spores, the suspension was transferred aseptically to a sterile container and the swarmer density determined. Frequently a second or third wash of the plates gave appreciably more swarmers. However, for use as inocula, only one wash was used to avoid contamination. By this procedure it was generally possible to obtain from 10^7 to 10^8 zoospores per plate.

Preparation of Suspensions for Physiological Studies: The following technique was developed to concentrate zoospores while keeping them intact and motile and was always strictly adhered to.

After harvesting and determination of density the suspension was centrifuged at 400 x G for exactly 1.5 minutes in 40 ml, conical tubes. After centrifugation, as much of the supernatant as possible was removed by means of a bacteriological pipette (care being taken not to disturb the loosely packed sediment). The zoospores were then resuspended in the residual medium and transferred to 12 ml centrifuge tubes and cooled to ca. 10°C. The concentrated suspension was then centrifuged at 1600 x G in a clinical centrifuge for exactly 30 seconds, the supernatant decanted and the sediment resuspended in the 0.01M phosphate buffer, pH 7.0.

The resuspended material was then washed by centrifuging as previously in a clinical centrifuge, the supernatant decanted and the process repeated.

Careful adherence to this procedure was critical. When followed carefully it generally resulted in zoospore suspensions exhibiting 80-100 per cent motility. Any deviation, however, generally lead to "rounding up" of the zoospores and loss of flagella. Repeated centrifugation in distilled water, hypertonic solutions (0.1M) or centrifugation at high relative centrifugal force generally lead to loss of motility, deformation and, on occasion, complete disintegration.

For the preparation of homogenates the swarmers were centrifuged at 1600 x G for three minutes in a calibrated 12 ml centrifuge tube in order to pack the cells. The pellet was then resuspended in five volumes phosphate buffer (0.1M, pH 7.4 containing 10^{-4} M versene + 10^{-3} M cysteine·HCl) and homogenized using a small (3 ml capacity) glass homogenizer or by means of grinding with a carborundum and glass mixture using a small mortar and pestle.

2) Cultivation of Synchronous, Single Generation Cultures of O.C. Plants

It was found that, using heavy zoospore suspensions, it was possible to obtain in a relatively short time (ca. 14 hrs) fairly large quantities (up to ca. 4 gm in 1.5 liters PYG with aeration) of plant material which was the result of a single generation of development. It seemed possible that a time-course enzymological analysis of such cultures might reveal clues to the functions of isocitritase and glycine-alanine transaminase in relation to ontogeny. It was necessary in preparation for these studies to establish a growth curve for single generation cultures of ordinary colorless plants and to determine their

protein content per milligram dry weight at various ages.

The curve was obtained by inoculating a series of four flasks equipped with aeration devices with suspension of zoospores of known density and incubating them for various periods of time up to 12 hours at 27°C in the light. Because there is very little increase in the weight of plant material during the early hours of growth it was necessary, in order to obtain sufficient material at all stages, to vary the inoculum accordingly by using decreasing amounts of the suspension as the incubation period became longer. Therefore, using a suspension containing 3.4×10^6 zoospores/ml, 150, 60, 15 and 10 ml were introduced into the 3, 6, 9 and 12 hour cultures respectively.

At the appointed times the cultures were examined microscopically (Figures 12 to 17) and then harvested by filtration, weighed, and aliquots used for the determination of dry weight and soluble protein. Protein determinations were carried out on 22,000 x G supernatants obtained by centrifugation of Omnimixer homogenates.

The growth curves (Figures 18 and 19) were obtained by adjusting the weights to a constant inoculum based on the three hour culture. Growth was found to be exponential throughout the growth period as indicated by a logarithmic plot of the dry weight data (Figure 18). Microscopic examination revealed that the plants were at all stages of quite uniform morphology (Figures 12 to 17) and size except that at twelve hours, when some plants were beginning to germinate, variations did appear (Figure 17). From these observations it was clear that such

cultures were the result of the very nearly synchronous growth of a single generation of ordinary colorless plants. The protein concentrations in the plants at various ages are presented in Table XIII.

3) Relationship Between Endogenous Respiratory Rate and Stage of Development of Ordinary Colorless Plants

Endogenous Respiration of Zoospores and Effect of Added Substrate and Inhibitors: Cantino and Hyatt (1953c) have already established the presence of most of the enzymes associated with the Krebs' cycle in mature ordinary colorless plants. Because of this and the close association of isocitritase and CO_2 fixation with the tricarboxylic acid cycle, it was of parenthetical interest, before proceeding with further studies on isocitritase, transaminase, and development to obtain some indication of the type of metabolism exhibited by zoospores.

Respiration studies were carried out using a conventional Warburg constant volume respirometer containing a total of 2.7 ml in the flasks. Oxygen uptake and carbon dioxide evolution measurements, were done simultaneously at 30°C using identical flasks with and without 0.2 ml ten per cent KOH respectively and containing 100 uM phosphate buffer pH 6.5. Correction for carbon dioxide retention by the buffer was made according to the method of Johnson as described by Umbreit et al. and the respiratory quotients calculated using the formula of Umbreit et al. (1957). A number of determinations were done on zoospore respiration. The endogenous Q_{O_2} of zoospores as an average of many experiments was found to be 102 ul O_2 per hour per mgm dry weight at pH 6.5 to 7.0 and temperature 30°C. The respiratory quotient was calculated to be 0.87.

The addition of the following intermediates did not affect the respiratory rate; glucose, succinate, acetate, malate, α-keto glutarate, pyruvate, glutamate and isocitrate.

Arsenite at a concentration of 4×10^{-4} M inhibited oxygen uptake 45 per cent (Table XII).

As would be expected malonate did not affect the oxygen uptake in the neutral range of pH but at pH 5.5 ($Q_{O_2} = 22$) malonate at a concentration of 2×10^{-3} M inhibited completely at 4×10^{-4} it inhibited 50 per cent. Succinate at ten times the concentration returned oxygen uptake to endogenous levels.

Thus presumptive evidence has been obtained for a functioning tricarboxylic acid cycle in zoospores. However, since the addition of various substrates failed to stimulate endogenous oxygen uptake further investigations, not within the scope of this work, will be necessary for confirmation.

Endogenous Respiration of Developing Plants: Since Brown and Cantino (1955) had found a very much lower Q_{O_2} for mature O.C. plants (ca. 9 ul O_2/mgm dry wt/hr) than reported here for zoospores. Q_{O_2} determinations were done at various stages of a single generation culture.

A series of 500 ml Erlenmeyer flasks containing 300 ml PYG and equipped with aeration devices were each inoculated with a heavy suspension of swarmers and incubated at 27°C. At four hour intervals up to the beginning of germination one of the flasks was removed and the plant material harvested by centrifugation in 250 ml centrifuge bottles. The material was then washed twice in distilled water by

TABLE XII

THE EFFECT OF INHIBITORS ON ZOOSPORE RESPIRATION

Additions	Concentration	O_2 Uptake	Per Cent Inhibition
None	-	56 u l/hour	-
Arsenite	4×10^{-4} M	31 u l/hour	44
None	-	46 u l/hour	-
Malonate	4×10^{-4} M	24 u l/hour	48
Malonate	4×10^{-4} M		
Succinate	4×10^{-3} M	37 u l/hour	20
Malonate	4×10^{-4} M		
Succinate	8×10^{-3} M	46 u l O_2/hour	None

Flask contained 100 uM glycyl glycine buffer pH 5.5, 0.4 ml swarmer suspension, 0.2 ml 10% KOH in center well and additions as indicated. Temperature 30°C.

centrifugation and after removing an aliquot for a dry weight determination resuspended in 0.01M phosphate buffer pH 6.5 for estimates of the rate of oxygen uptake.

As shown in Figure 20 the Q_{O_2} did decrease as the plants developed. However, even as they near maturity it is still appreciable and somewhat higher than values previously reported using multiple generation cultures which contained a good deal of biologically inactive material and dead plants.

4) Relationship of Isocitritase and Transaminase Activities to Development and Morphogenesis

Distribution of the Enzymes at Various Stages in the Life Cycle: Estimates were made of isocitritase and transaminase activities in extracts prepared from resistant sporangia, ordinary colorless plants and zoospores from ordinary colorless plants. Except in the case of zoospore isocitritase, all estimates were made on high speed supernatants prepared as described elsewhere. Because of the high levels of endogenous amino acids (particularly alanine (Lovett, 1959), it was not possible to assay the zoospores directly for isocitritase. The isocitritase activity of zoospore extracts was determined by assaying the fraction obtained by adding 0.65 volumes of saturated ammonium sulfate to an extract prepared using the carborundum-glass grinding mixture.

Representative data are presented in Table XIV. Specific activities for both enzymes are given for all stages. Activity in a dry weight basis are also given for all stages except R.S. where, because

TABLE XIII

SOLUBLE PROTEIN

Age (Hours)	Soluble Protein (mgm/gm)
0 (Zoospores)	371
3	393
6	350
9	380
12	312

TABLE XIV

DISTRIBUTION OF ISOCITRITASE AT VARIOUS STAGE OF THE LIFE CYCLE

Stage of Development	Isocitritase		Transaminase	
	Units per Gm Dry Wt	Units per Mgm Protein	Units per Gm Dry Wt	Units per Mgm Protein
Zoospores	82	0.23	271	0.79
Mature O.C.	55	0.28	193	0.58
R.S. (4 days)		0.50		0.99

Conditions: As for assays of respective enzymes described previously.

of the difficulty in obtaining complete breakage, accurate dry weight-activity relationships could not be obtained.

Both enzymes were detectable at all stages of the life cycle examined. Though it has not been possible to obtain sufficient quantities of R.S. zoospores for physiological studies the assumption has been that they do not differ from those of O.C. plants.

Relationship of Transaminase and Isocitritase Activities to the Development of O.C. Plants: The development of the enzymes during the growth of single generation cultures was traced by means of a series of experiments in which cultures of various ages, incubated in the light and the dark, were assayed for enzymatic activities. The methods for inoculating and harvesting cultures were as described in the growth curve experiment. Crude extracts were prepared using the Omnimixer as in the purification of isocitritase except that ten rather than 5 ml of 0.1M phosphate buffer pH 7.4 was used per gram wet weight.

Isocitritase was assayed directly as described earlier and transaminase was assayed after dialysis vs. 0.02M phosphate buffer pH 8.0 for 26 hours and the specific activity of the crude extract expressed as previously. Activities per gram dry weight and per plant were calculated from conversion factors obtained from the growth curve experiment.

It must be noted that there was considerable variation in the results obtained in runs done at different times. This is attributable to a lack of adequate means of strict temperature control. Consequently,

while time was used as the criterion of the development of cultures it was not always an accurate reflection of physiological maturity.

Since no consistent differences in either isocitritase or transaminase activities were found between cultures grown in the light and the dark the data for both conditions of growth were pooled in obtaining the plotted averages though data from light and dark are indicated as such in the graphs.

Figures 21, 22, 23 and 24 summarize the results. The number of isocitritase units per gram dry weight and per milligram protein decreases during the first three hours of growth, remains low until nine hours and then rises again to initial levels (Figure 22). When the data were plotted on a per plant basis (Figure 21) it was found that the number of units per plant remained relatively constant up to ca. three to six hours and then increased almost exponentially during the latter half of the generation time demonstrating that net synthesis of isocitritase does not occur until relatively late in development.

Glycine-alanine transaminase on the other hand though dropping somewhat in the number of activity units per gram dry weight of plant material and milligram protein as shown in Figure 23, is however, apparently synthesized from the very beginning of plant development. As shown in Figure 24 this synthesis is exponential. The relationship between enzyme synthesis and over-all synthesis of plant material is shown in Figure 25.

Relationship of Transaminase and Isocitritase Activities to the Development of R.S. Plants: The procedure and conditions used here

for the growth of semisynchronous cultures of resistant sporangia are those developed by Lovett (1959).

A twelve liter flask containing ten liters of PYG containing 0.75 gm sodium bicarbonate per liter was inoculated with 10 ml of a zoospore suspension (swarmer density = 20) and incubated with vigorous aeration at 25°C. Commencing at 24 hours and at 12 hour intervals thereafter samples large enough to yield ca. two grams of plant material were withdrawn by means of a siphon. After harvesting cell free extracts were prepared as described previously, except that a six rather than three minute grinding period was used because of the much thicker walls of R.S. as compared to O.C. Otherwise isocitritase and transaminase activities were assayed as previously.

The results of two such runs are shown in Figures 26 and 27. As may be seen in Figure 26 the specific activity of isocitritase remains fairly constant through the first 24 hours. However, beginning at 36 hours there is a sharp increase in activity with a peak being reached at 48 hours followed by a slow decline. The specific activity at 48 hours is higher than detected at any other stage of the life cycle (cf. Table XIV).

Transaminase activity was found to behave similarly as shown in Figure 27. Since resistant sporangia mature (i.e., are capable of germination) under these conditions at ca. 80 hours the level of activity of both enzymes reach their maxima at about three-fifths of the generation time.

FIGURE LEGENDS

Conditions for cultivation and harvest of cultures are given in detail in the text. Methods for enzymatic assays and preparation of extracts are described in detail elsewhere.

Figure 12. Zoospores from O.C. plants (500x). Note posterior flagella.

Figure 13. A germling three hours after initiation of growth in a near synchronous culture (500x).

Figure 14. A six hour old O.C. plant from a single-generation near-synchronous culture (500x).

Figure 15. A nine hour old O.C. plant from a near synchronous culture (500x).

Figure 16. A group of several nine hour O.C. plants illustrating the uniformity exhibited by plants in near synchronous cultures (240x)

Figure 17. Twelve hour O.C. plants from a near synchronous single-generation culture of (240x). Note that one plant has almost completed zoospore release.

Figure 18. Growth Curve of a Single Generation Culture of Blastocladiella.

Figure 19. Log Growth Curve of Individual Plant of Blastocladiella. The plotted data are the logarithms of the values obtained by dividing the dry weights obtained at the times indicated, by the corrected number of viable swarmers in the inoculum.

Figure 20. Relationship between Culture Age and Oxygen Uptake. Q_{O_2} = ul Oxygen consumed per milligram dry weight per hour.

Figure 21. Isocitritase: Relationship to Development of O.C. Plants. The amounts of enzyme per plant were calculated from specific activities using conversion factors obtained from the growth curve experiment.

Figure 22. Isocitritase: Relationship to O.C. Culture Development. Activities per gram dry weight were calculated from the specific activities on the basis of the protein per gram dry weight values determined in the growth curve experiment.

Figure 23. Transaminase: Relationship to O.C. Culture Development. Activities per gram dry weight were calculated by the same method used for isocitritase.

Figure 24. Transaminase: Relationship to Development of O.C. Plants. Transaminase activities per plant were calculated on the same basis as that used for isocitritase.

Figure 25. Enzyme Synthesis as a Function of Plant Growth. The plotted values were obtained from the log data used in Figures 19, 21 and 24.

Figure 26. Isocitritase: Relationship to R.S. Development.

Figure 27. Transaminase: Relationship to R.S. Culture Development.

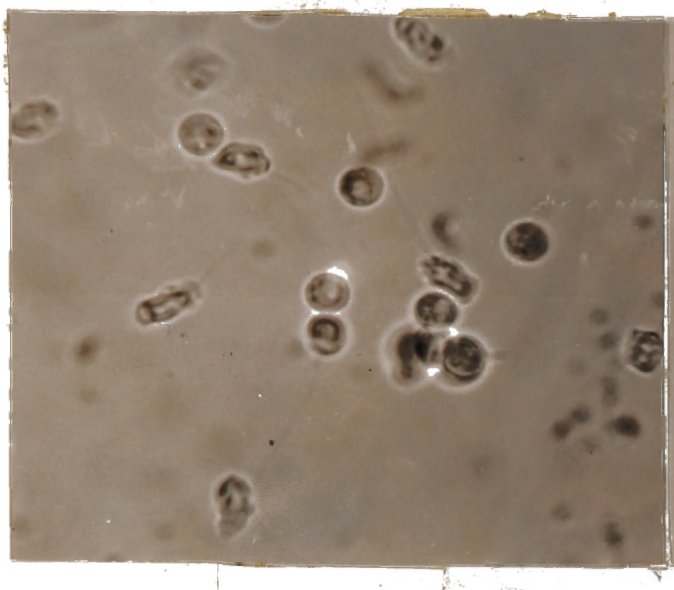


Figure 12

Figure 13

Figure 14

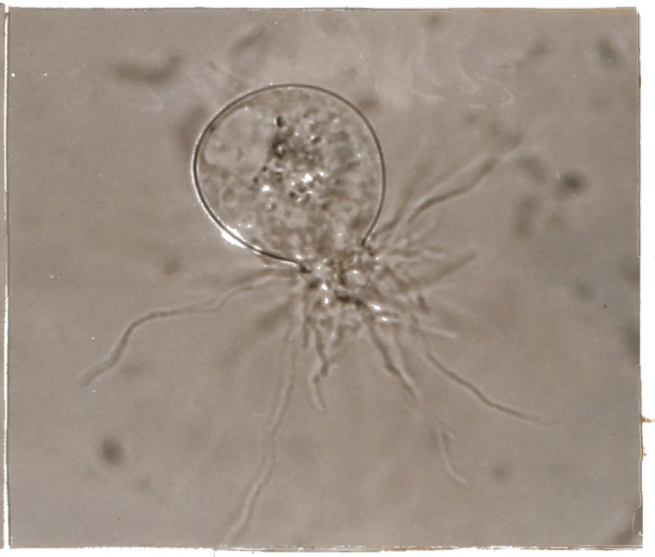


Figure 15

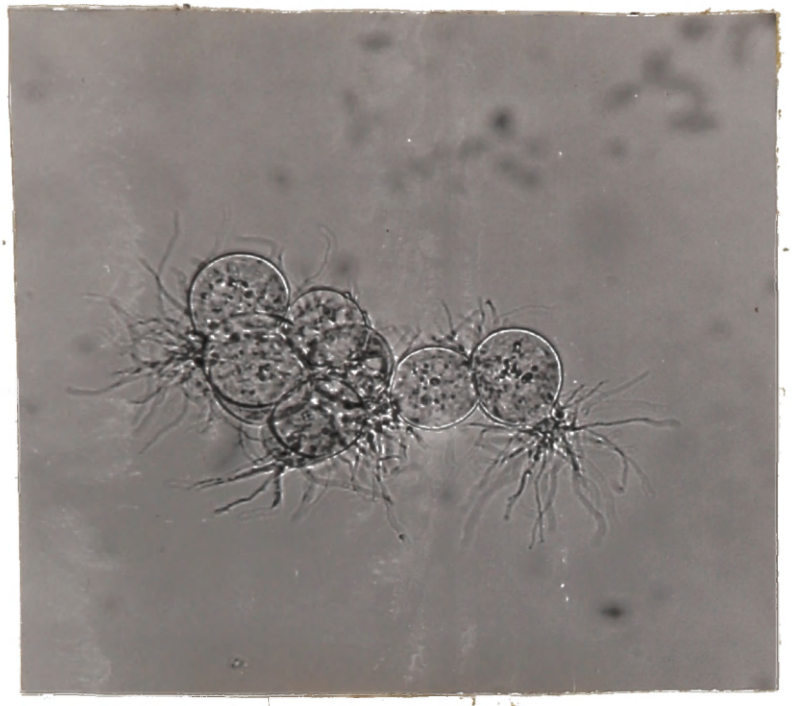


Figure 16

Figure 17

Figure 18

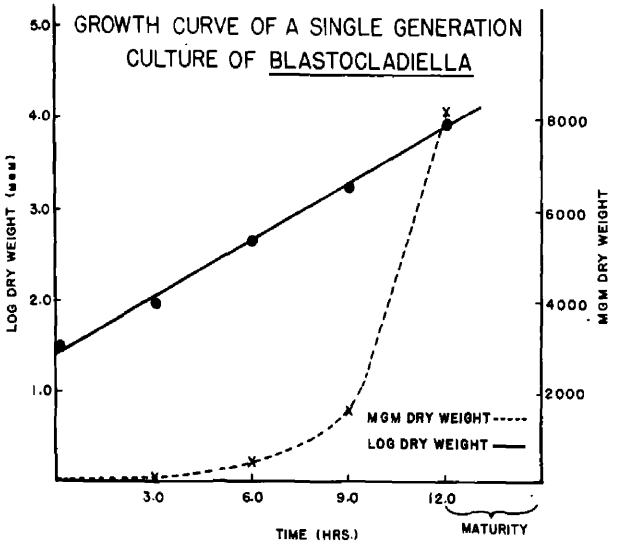


Figure 19

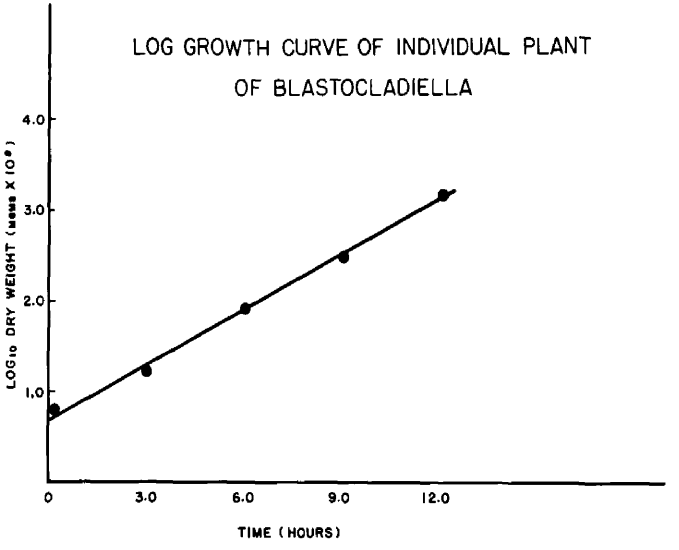


Figure 20

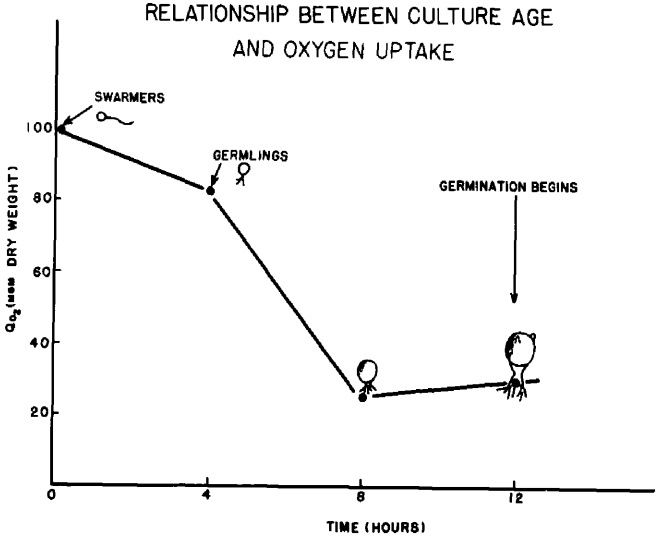


Figure 21

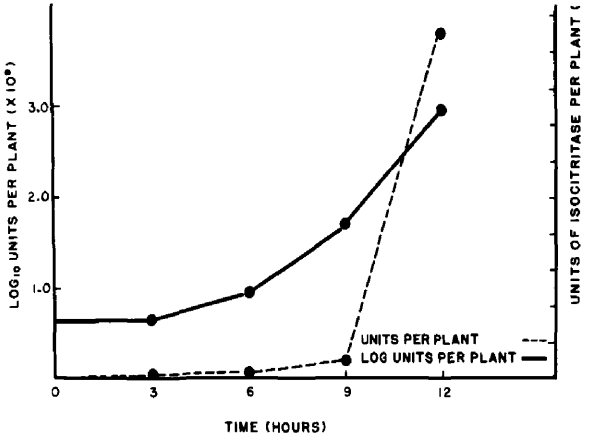


Figure 22

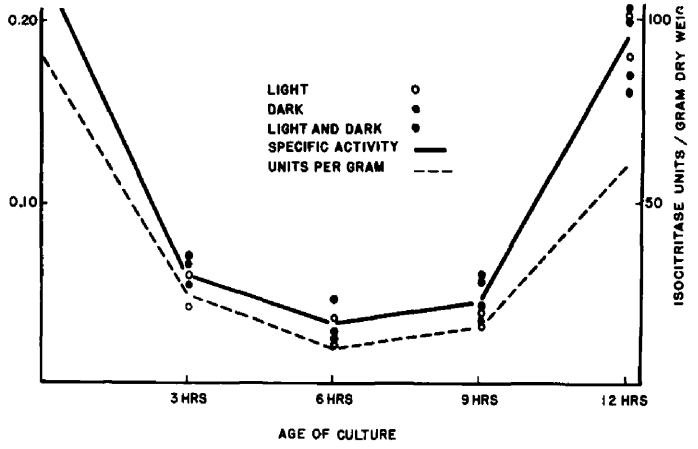


Figure 23

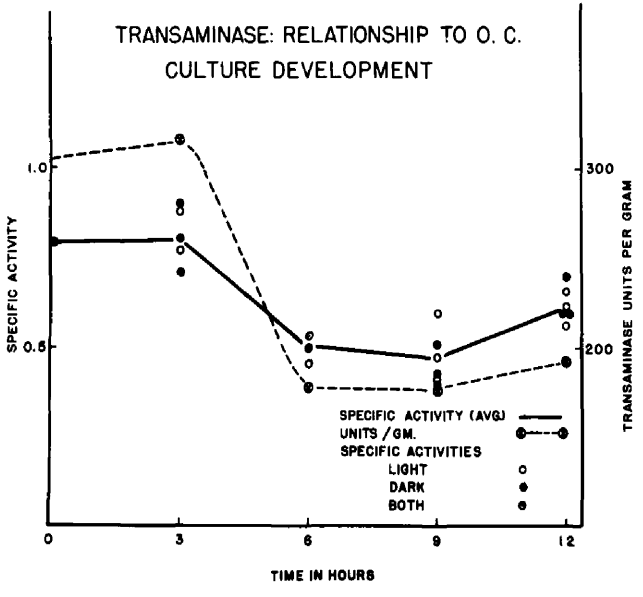


Figure 24

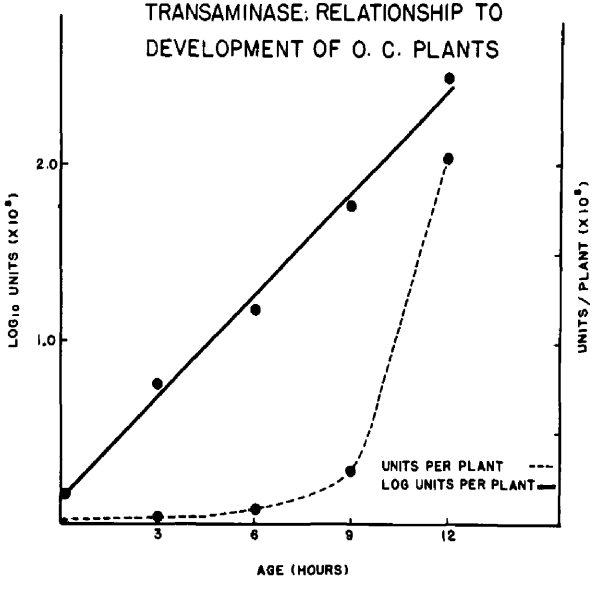


Figure 25

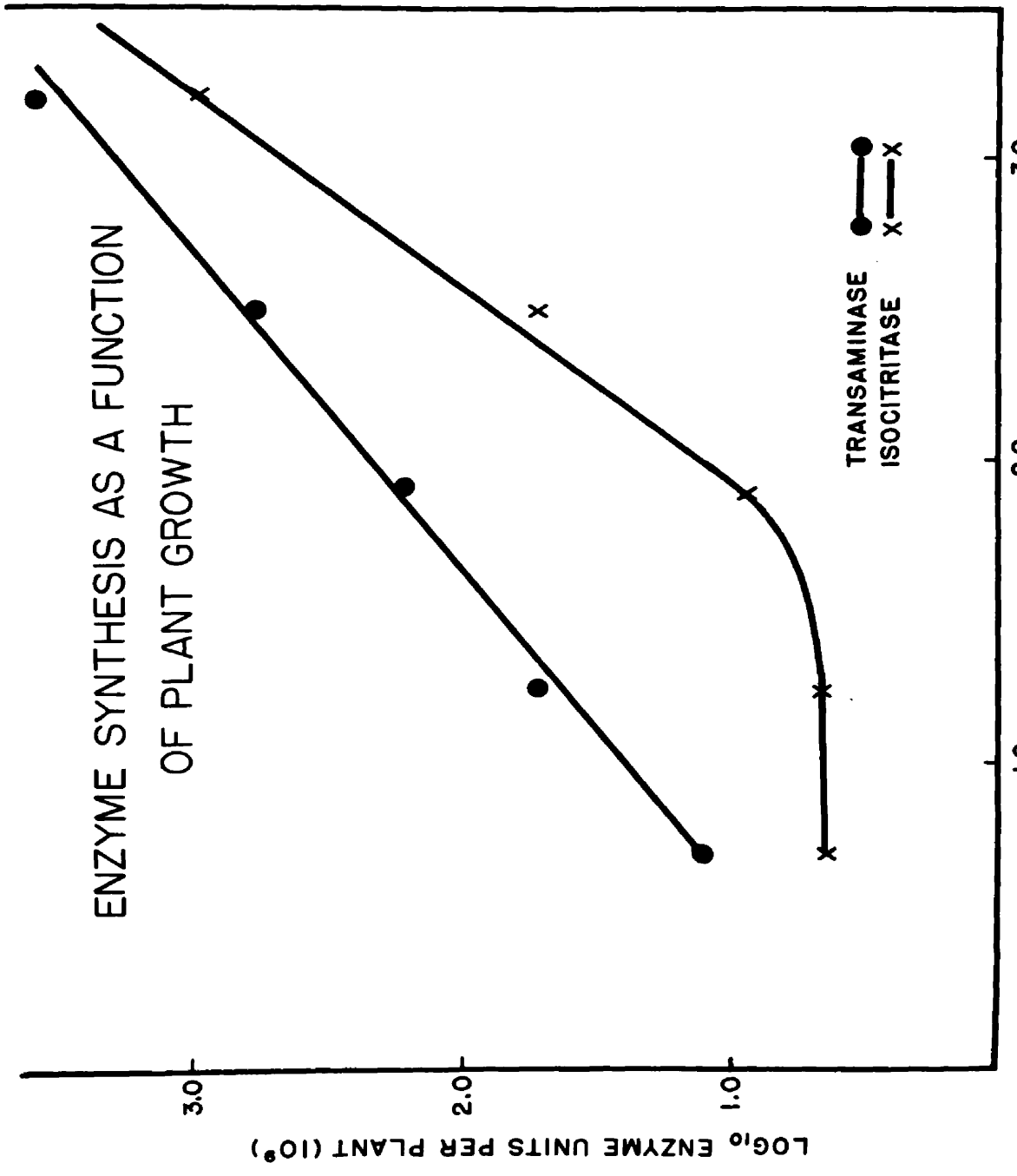

ENZYME SYNTHESIS AS A FUNCTION
OF PLANT GROWTH
TRANSAMINASE
ISOCITRITASE
LOG10 ENZYME UNITS PER PLANT (10⁹)
LOG10 WEIGHT PER PLANT (MGM X 10⁶)
3.0
2.0
1.0
1.0
2.0
3.0

Figure 26

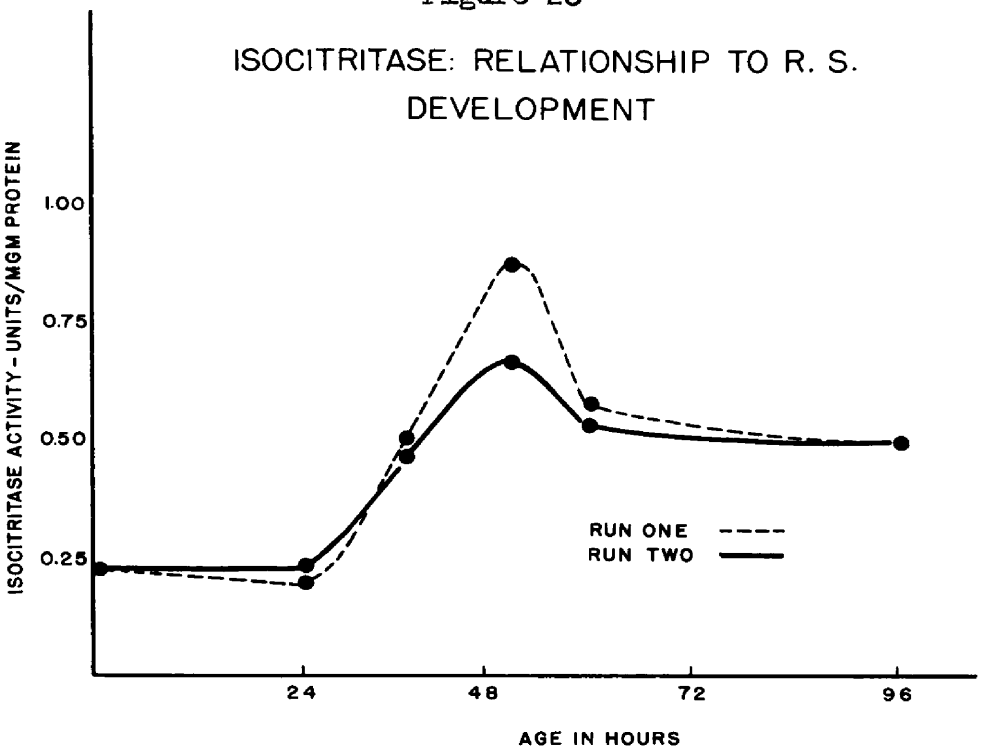
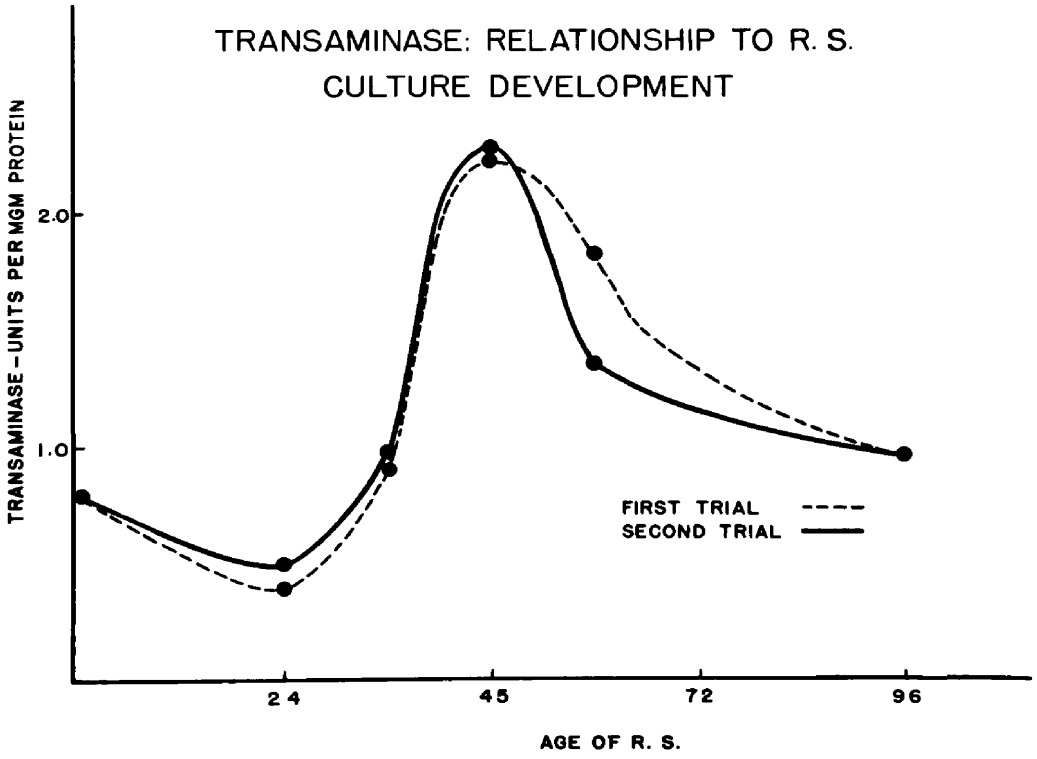


Figure 27



DISCUSSION AND CONCLUSIONS

The fundamental purpose of the work of which this thesis is a part, is to understand growth and differentiation in terms of the underlying biochemical mechanisms involved. In pursuit of this goal the physiology of Blastocladiella emersonii has been more thoroughly studied than that of any other aquatic fungus. Enzymological studies on this group of organisms 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 proceeding with a consideration of their possible roles in light stimulated growth and morphogenesis, a brief discussion of the properties of these enzymes seems appropriate.

The isocitritase of Blastocladiella has been found to resemble very closely the analogous enzymes from other sources for which similar levels of purification have been achieved. Thus, though it differs slightly in its pH optimum (pH 7.4 vs pH 8.2 for Pseudomonas) and ammonium sulfate solubility characteristics, the Michaelis constant (4.8×10^{-4}M) is almost identical to that of the enzyme from Pseudomonas aeruginosa (Smith and Gunsalus, 1957). The Michaelis constant of the yeast enzyme (Olson, 1959) has been determined under somewhat different conditions to be 1.2×10^{-3} M. Like the reaction catalyzed by the enzyme from other sources, the Blastocladiella isocitritase reaction is reversible and specific for the d-isomer. The isocitritases from Blastocladiella and Pseudomonas (Smith and Gunsalus, 1957) show very close similarity

in their saturation curves for both magnesium and cysteine, exhibiting maximum activities at 10^{-3} M concentrations for both cofactors. Worthy of mention is the fact, not discussed by Smith and Gunsalus, that the response curve for cysteine exhibits a maximum followed by decreasing activity with increasing cysteine concentration. This phenomenon is probably attributable to a chemical reaction between cysteine and glyoxylate. Brunel-Chapelle (1952) reported the occurrence of such a reaction at pH 9.0 and above. We have found that there is considerable disappearance of glyoxylate even at physiological hydrogen ion concentrations in the presence of a sufficiently large excess of cysteine. Olson (1959) has reported a similar observation in the course of studies on yeast isocitritase.

During our studies it was found that extracts of B. emersonii were capable of mediating the rapid conversion of glyoxylate to glycine by amino group transfer from alanine. Since this might have constituted a "pulling" reaction in the operation of the S.K.I. cycle and since, to our knowledge, there have been no previous reports of attempts to purify a glycine-alanine transaminase, further studies were carried out. By a series of acetone fractionations and gel treatments an eighty-fold purification of the enzyme was achieved. The purified enzyme was found to be quite stable and resembled other transaminases in exhibiting maximum activity in the alkaline pH range. Attempts to demonstrate stimulation of activity by pyridoxal phosphate were unsuccessful though it seems likely, in view of its almost universal role in enzymatic transaminations, that it is involved in the glyoxylate-alanine

transamination as well. The inhibition of the reaction by hydroxylamine and the reversal of the inhibition by pyridoxal phosphate would seem to indicate that this is so. We have concluded that the enzyme is probably very tightly bound in the haloenzyme though Meister (1955) has pointed out the theoretical possibility of enzymatic transaminations involving glyoxylate not requiring a cofactor.

In attempts to establish substrate specificity it was found that none of the amino acids tested were capable of replacing L-alanine in systems containing glyoxylate. D-alanine was completely inactive. However, an enzymatic transamination between pyruvate and asparagine was found to occur in our purified preparation. Whether this was due to the same enzyme or a different one, from that catalyzing the glyoxylate-alanine reaction is not at present known. Further study will be required to clarify this point.

It was expected that the equilibrium of the glyoxylate-alanine transamination would be far in the direction of glycine production (Metzler et al., 1954). However, in time course runs it was found that conversion to glycine and pyruvate never exceeded ca. one-third conversion while the reverse reaction yielding alanine was only barely 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 to believe that the equilibrium constant of the enzymatic reaction should be different than chemical transaminations involving glyoxylate.

Our primary interest in the study of isocitritase was based on indirect evidence that it was involved in the proposed S.K.I. cycle mechanism for CO_2 fixation as the "pulling" reaction tending to remove isocitrate from the site of the reductive carboxylation of α-ketoglutarate. It was thought that glyoxylate and some of the succinate produced were then integrated into the biosynthetic machinery which yields increased growth under conditions of illumination but, in the presence of high bicarbonate concentrations, a resistant sporangium. Moreover it now seems possible, on the basis of the high specific activity of the transaminase as compared to isocitritase and the probable equilibrium of the glyoxylate-glycine conversion towards glycine production, that transamination constitutes at least one of the important reactions responsible for the further metabolism of glyoxylate.

Therefore, a number of experiments were carried out in an attempt 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. Therefore, we first tested the purified enzymes for a stimulation of their reactions by white light and established unequivocally that neither is the specific locus of the light effect.

A method was then developed for the cultivation of O.C. cultures which, on the basis of their morphological uniformity and the logarithmic nature of their growth curves, were considered to be very nearly synchronous for most of the generation time. Support for this contention is found in the recent results of Turian and Cantino (1960) where,

using similar cultures, nuclear division was found to be ca. 80 per cent synchronous for at least the first six hours of growth. In addition a new technique was developed that permitted for the first time, both for B. emersonii and aquatic fungi generally, physiological studies of zoospores using both motile, intact cells and cell free preparations. Meanwhile Lovett (1959) also established the conditions for the growth of synchronous cultures of resistant sporangial plants. Thus, where previous physiological studies aimed towards solving developmental problems were limited to the use of heterogeneous preparations of mature ordinary colorless or resistant sporangial plants, it has now become possible to study biochemical changes as they occur from the germination of the tiny zoospore to the development of a mature plant along either of the alternative developmental pathways.

Armed with these new techniques, we then attempted to follow the behavior of isocitritase and glycine-alanine transaminase during the development of synchronized, single-generation cultures of ordinary colorless plants in both the light and the dark. By so doing it was thought that the changes taking place during growth and differentiation, and the effect of light thereon, might be reflected in corresponding changes in the two enzymes which would give clues to their function in phenomena associated with growth.

The results of a number of these experiments failed to reveal any effect of light on the activity levels of either glycine-alanine transaminase or isocitritase. The specific activity of isocitritase was high in the zoospores, dropped sharply as development began, remained

low until ca. six hours, and then rose again to initial levels. Transaminase activity, on the other hand, changed relatively little during plant growth; it dropped only ca. one-third from starting levels in the zoospores to those in mature plants.

A more meaningful picture for the purpose of interpretation, evolves by considering the synthesis of the two enzymes as reflected in the amounts of enzyme per plant. Thus we found that glycine-alanine transaminase was synthesized exponentially (Figure 24) throughout the generation time as a linear function of plant growth (Figure 25), while, isocitritase synthesis exhibited a definite lag (Figures 21 and 25) extending to between the third and sixth hours after germination of the zoospores. While it is very difficult to arrive at any conclusions regarding glycine-alanine transaminase, the lag in isocitritase synthesis might offer possible clues to its role in development.

The significance of this lack of isocitritase synthesis may 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 the control of enzyme synthesis. A great deal of work has been done on the phenomenon of adaptive enzyme formation in microorganisms as a possible mechanism for the control of enzyme synthesis. One of the significant findings of these researches has been that there is a remarkable correlation between the need for particular enzyme functions and the synthesis of the enzymes involved. Thus, for example, it has been found that facultatively anaerobic bacteria (Englesberg and Levy, 1955) and yeast (Slonimski, 1953) when

cultivated under anaerobic conditions synthesize the Krebs' cycle enzymes only at a very low rate, but in the presence of oxygen a rapid synthesis of the whole array of respiratory enzymes is brought about.

Thus we might conclude that the lack of synthesis of isocitritase during the early hours of development of an O.C. plant, reflects a situation in which the reaction it catalyzes is not required, and that isocitritase is not operating until relatively late in the generation time. It would therefore follow that CO_2 fixation could not be taking place via the operation of a S.K.I. cycle involving isocitritase until late in development. Although no studies have yet been carried out to indicate carbon dioxide fixation is so limited, recent evidence clearly establishes that the effect of light on development is not. Thus, Turian and Cantino (1960) have recently found by cytological studies and chemical analyses that DNA synthesis and nuclear division take place more rapidly in the light than in the dark throughout O.C. ontogeny!

On the basis of the preceding discussion and the evidence that both nucleic acid synthesis and the isocitritase-dependent S.K.I. cycle are stimulated by light, we have evolved the following as a working hypothesis: light must be having its effect not directly on CO_2 fixation but on the metabolic path or paths leading to nucleic acid synthesis and, ultimately, growth at a point beyond the S.K.I. cycle. During the early portion of the generation time it would seem that increased nucleic acid synthesis and other reactions associated with increased growth are taking place at the expense of a readily available source of metabolites which, at least in part, may be alternatively

supplied by isocitritase or its products. Beginning at three to six hours this readily available source of precursors becomes depleted or insufficient, necessitating either the initiation of, or a shift to, an alternative mechanism of supply. Since this period corresponds to the decrease in endogenous respiratory activity (Figure 20), it is possible that the supply deficiency is due to a decrease in the production of substances associated with a functioning Krebs' cycle. In any case, in response to the requirement for an alternative supply source isocitritase synthesis 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 that is produced is at least in part transaminated with alanine to yield glycine which might, in turn, be used in producing thymine (Elwyn and Sprinson, 1954) and purines (Goldthwait et al., 1956) for nucleic acid synthesis. Other, as yet, unknown pathways for the utilization of glyoxylate, as well as glycine and succinate may also be in operation.

In a further attempt to elucidate the roles of isocitritase and transaminase in development, synchronous cultures of resistant sporangial plants were also studied. The results that were obtained 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.

The specific activities of both glycine-alanine transaminase and isocitritase were found to increase sharply, beginning at about the twenty-fourth hour, reaching maximum levels at forty-eight hours which

were three to four 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 (Lovett, 1959). All of these events occur at approximately three-fifths of the generation time, the period beyond which R.S. morphogenesis becomes an irreversible process!

These results, though admittedly of a correlative nature, give significant support to our contention that isocitritase is a key component of the bicarbonate trigger mechanism. Moreover, we now have reason to believe that glycine-alanine transaminase is also involved in this chain of events.

In attempting to integrate these findings into the original hypothesis of morphogenesis, the following picture emerges: bicarbonate acts to stop and eventually reverses the forward progress of the Krebs' cycle by inhibiting the two successive oxidative decarboxylations mediated by α-ketoglutarate oxidase and isocitric dehydrogenase. As this happens most of the enzymes of the tricarboxylic acid cycle disappear or are rendered inoperative and there is a disturbance of the critical balance of reactions which constitute the metabolic apparatus of the cell. As a result there is a de novo synthesis of some enzymes and increased synthesis of others to establish a new equilibrium satisfactory to the needs of the cell. At ca. thirty-six hours this remodelling process nears completion and the organism becomes irreversibly committed to R.S. formation as isocitritase, glycine-alanine transaminase and other enzymes involved in morphogenesis reach maximum levels. Isocitric

dehydrogenase, the only Krebs' cycle enzyme which remains active, begins to mediate with greater and greater efficiency the reductive carboxylation of α-ketoglutarate, while isocitritase pulls the reaction by removing the isocitrate that is produced. At least part of the glyoxylate that is formed is converted to glycine and, if the S.K.I. cycle is involved, some of the succinate is recycled by way of a TPN-specific reaction to α-ketoglutarate to accept more CO_2 and the remainder is further metabolized. Somehow these reactions help supply building blocks for the synthesis of the characteristic structural components of the resistant sporangium. Between forty-eight and sixty hours the synthesis of gamma-carotene, melanin, lipid, chitin and other associated substances nears completion, the need for intermediates supplied by the reductive carboxylation of α-ketoglutarate disappear and, as a result, the levels of isocitritase and transaminase decrease.

It would be premature to discuss in 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 by Cantino and Horenstein (1959) that glyoxylate and succinate <u>together</u>, direct products of the isocitritase cleavage, will replace the light effect, and our discovery of a direct metabolic route from glyoxylate to glycine are strongly suggestive of likely routes of metabolism. Thus Kikuchi <u>et al</u>. (1958) have reported an enzyme catalyzed reaction yielding delta-aminolevulinic acid from succinate and glycine which, though the conditions were not clearly defined, was stimulated by light. Moreover, studies with radioactive

tracers have incriminated delta-aminole ulinic acid as an intermediate in the conversion of glycine to thymine and purines (Shemin, 1956) as well as carotene synthesis (MacKinney et al., 1955); and this brings us to the recent observation (Turian and Cantino, 1960) that thymine will substitute for light in DNA synthesis! From these findings we cannot help but regard as an attractive one the possibility that delta-aminole ulinic acid is the link connecting glyoxylate, glycine and succinic acid to light stimulated growth and, in R.S. morphogenesis, at least carotene synthesis.

The relationship between light and growth even in chlorophyll-containing organisms is poorly understood dispite the fascinating and fundamental biochemical studies being carried out by workers all over the world. The biochemistry of morphogenesis is such a young science that only a few workers working with fewer organisms are engaged in its study. It is no wonder then, that our knowledge is yet so fragmentary and our interpretations so speculative. Nevertheless, such interpretations have the value that they will lead to further work which someday will solve the puzzle of morphogenesis, such as that involved in the biology of Blastocladiella emersonii.

SUMMARY

1) The enzyme isocitritase, which had been postulated to occur in Blastocladiella, has been purified from extracts of this organism ca. fifty-fold and some of its properties studied.

2) A second enzyme, glycine-alanine transaminase, which has not been previously described, was purified ca. eighty-fold and its properties similarly examined.

3) New methods were developed which permitted, for the first time among the aquatic fungi, enzymological studies at all stages of the growth of Blastocladiella, from the zoospores to the mature ordinary colorless plant.

4) The syntheses of isocitritase and glycine-alanine transaminase, as reflected in activity measurements, were followed during the development of O.C. and R.S. plants. An attempt was then made to integrate the results obtained into an interpretation of the function of these enzymes in morphogenesis and lumisynthesis.

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APPENDIX

Abbreviations used:

DNA - Deoxyribonucleic acid

TPN - Triphosphopyridine nucleotide

DPN - Diphosphopyridine nucleotide

TPNH - Reduced TPN

DPNH - Reduced DPN

S.K.I. - Succinate-ketoglutarate-isocitrate

O.C. - Ordinary colorless

R.S. - Resistant sporangia(1)