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Tricarboxylic acid cycle enzymes and bicarbonate fixation in morphogenesis of Blastocladiella emersonii.

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TRICARBOXYLIC ACID CYCLE ENZYMES AND BICARBONATE FIXATION IN MORPHOGENESIS OF *BLASTOCLADIELLA EMERSONII*.

by

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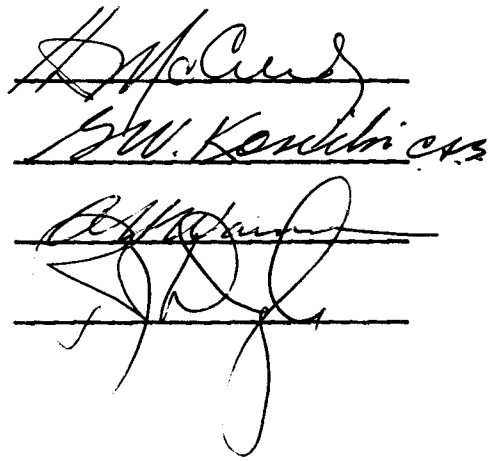

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ABSTRACT

The characteristics of mass synchronous cultures of ordinary colourless (OC) and resistant sporangial (RS) plants of Blastocladiella emersonii were studied. During their exponential growth, OC plants consumed little glucose and produced no lactic acid. Similarly, RS plants did not utilize glucose or produce lactic acid while growing exponentially for the first 24 hours. During the next 24 hours, glucose was consumed with the concomitant production of lactic acid which was then reutilized. Lactic acid gradually accumulated again at maturity.

Enzyme studies on cell-free extracts from spores, mature OC and mature RS plants demonstrated the presence of all tricarboxylic acid (TCA) cycle enzymes, except α-keto-glutaric dehydrogenase. Included was the detection of AMP stimulated, NAD-isocitric dehydrogenase and citrate condensing enzyme not previously found in Blastocladiella. TCA cycle enzyme activity was higher in RS than OC plants at all stages examined.

An attempt was made to correlate enzyme syntheses with development through assays performed at various stages during the synchronous development of OC and RS plants. In OC plants, the activities of all enzymes measured increased during growth. Increasing levels of these enzymes were also found during development of RS plants with maximum activities at 36 hours, the point of no return in ontogeny.

Also, preliminary studies utilizing short periods of C^{14}-bicarbonate fixation in young RS plants were made. During the first 4 minute incubation, 4% of the total C^{14}-bicarbonate in the medium was distributed in a number of amino acids, TCA cycle intermediates and other compounds. Aspartic acid incorporated the most label.

These results are discussed in light of previous results, and in particular, Cantino's hypothesis concerning the relationship between bicarbonate induction and TCA cycle enzymes in the morphogenesis of B. emersonii.

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I also wish to thank my brother, Dr. B. H. Khouw, for his help with the glass-blowing work needed for the design of the culture systems used in this study.

Finally, I wish to dedicate this thesis to my wife, Eugenia, who has helped in so many ways.

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INTRODUCTION

The zoospores of the water mold, Blastocladiella emersonii, are capable of developing into either of two types of plants. In the presence of bicarbonate they produce resistant sporangial (RS) plants while in its absence, they form ordinary colourless (OC) plants. Results from numerous studies to determine the mode of action of bicarbonate have led Cantino (1966) to hypothesize that bicarbonate induces "lesions" in the TCA cycle, and thereby causes the accumulation of TCA-intermediates which are then shunted to other reactions necessary for the genesis of RS plants.

Most of the work on TCA cycle enzymes was carried out with heterogenous multigeneration cultures, and in many instances, the results and conclusions derived therefrom have not been verified from studies using synchronous cultures. It seemed necessary therefore to re-examine the TCA cycle enzymes in B. emersonii in synchronous cultures. It was hoped that the results of such a study would contribute further to an understanding of the bicarbonate effect.

In the work reported here, the activities of the TCA cycle enzymes were followed during the synchronous development of both OC and RS plants. With the exception of α-ketoglutaric dehydrogenase, the activities of all cycle enzymes were detected in all stages of development

of Blastocladiella with highest levels of activity in RS plants, particularly at 36 hours. In an attempt to detect the site(s) of bicarbonate fixation, short exposures to C^{14}-bicarbonate were carried out. These data indicate that incorporation of bicarbonate was greatest in aspartic acid, whereas other amino acids and the TCA cycle intermediates incorporated little radioactivity.

CHAPTER I

LITERATURE REVIEW

Morphogenesis and Tricarboxylic Acid Cycle in *Blastocladiella emersonii*

The motile, uniflagellated spores of *Blastocladiella emersonii* (Cantino and Hyatt, 1953a), are capable of developing into two types of plants. Cantino (1951) found that in the presence of 10^{-2} M sodium bicarbonate in a peptone medium, only dark brown resistant sporangial (RS) plants are produced, while in the absence of bicarbonate only ordinary colourless (OC) plants are formed.

Both types of plants consist of an upper globose sporangium, and a basal stalk subtended by a rhizoid system. In RS plants, the sporangium has a thick chitinous, melanin impregnated wall (Cantino and Horenstein, 1955; Cantino, Lovett and Horenstein, 1957) which encloses protoplasm containing large quantities of lipoidal particles and gamma carotene (Cantino and Horenstein, 1956). On the other hand, the sporangia of OC plants do not contain melanin or gamma carotene, have thin chitinous walls and, at maturity, bear several discharged papillae through which the zoospores are finally liberated. A diagram of the life cycle of *B. emersonii* is presented in Figure 1a.

In attempting to determine the mode of action of bicarbonate, Cantino (1951) found that the ability of bicarbonate to induce a high proportion of RS plants in a

Figure 1a --- Life Cycle of B. emersonii.

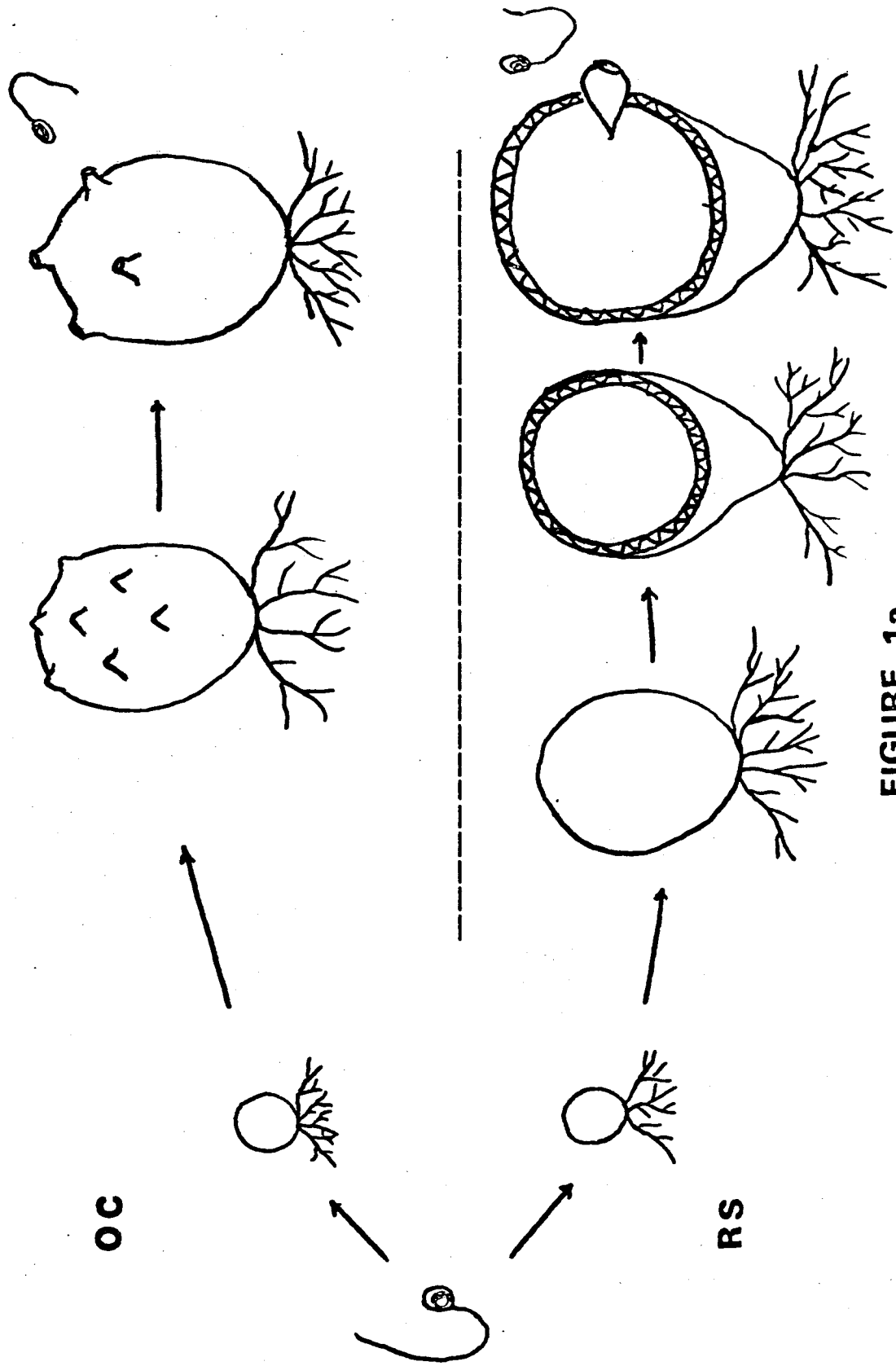

FIGURE 1a

buffered medium containing a casein hydrolysate requires the simultaneous presence of either an intermediate, such as α-ketoglutarate or citrate, or an inhibitor such as arsenite or semicarbazide. In the absence of bicarbonate, none of these compounds is active. It was then postulated that bicarbonate probably induces a decrease in the rates of α-ketoglutarate oxidation and isocitrate decarboxylation within the TCA cycle. As a result, there is an accumulation of intermediates between α-ketoglutarate and citrate or its C_2 precursors, and subsequent activation of various shunt reactions leading to the production of RS plants.

In later studies, Cantino (1952) found that the bicarbonate effect is related to critical periods in the development of both OC and RS plants. Thus beyond three-fifths of the maturation time of either plant type, addition or removal of bicarbonate does not influence the subsequent course of development. A change in permeability was considered to be partially responsible.

Cantino and Hyatt (1953b) isolated a mutant of B. emersonii, BEM, which was unable to form RS plants. Subsequent studies carried out by Cantino and Hyatt (1953c) on this mutant as compared to the wild type showed that extracts of mature wild type OC plants exhibited most of the TCA cycle enzymes, including aconitase, NADP-isocitric dehydrogenase, α-ketoglutaric dehydrogenase, succinic dehydrogenase, fumarase, malic dehydrogenase and cytochrome oxidase. On the other hand, extracts of the mutant 'lacked'

two enzymes, aconitase and α-ketoglutaric dehydrogenase, both of which, according to the hypothesis, should be present to permit RS formation. Thus these results were considered to support the hypothesis.

In attempting to study the causative relationship between the action of bicarbonate and the new biosynthetic pathways leading to the characteristic cell constituents of RS plants, it was suggested that bicarbonate fixation may be coupled to melanin production. First, Cantino (1953) found that melanin formation in RS plants could be uncoupled in vivo from the rest of the morphogenetic process by means of incorporating phenylthiourea into the growth medium. In subsequent in vitro studies, Cantino and Horenstein (1955) found that melanin formation in RS plants is associated with a cell wall bound, and phenylthiourea sensitive polyphenoloxidase system, not present in OC plants. It was also claimed that the system was capable of mediating electron transport between substrate and oxygen or NADP. α-ketoglutarate seemed to function in this case as an essential factor for activity because of its reductive carboxylation and concomitant oxidation of NADPH. Thus, it appeared that an important link between the site of bicarbonate activity and a morphological change, namely melanin formation, characteristic of RS plants had been found.

In these same studies Cantino and Horenstein reported the presence of NADP-isocitric dehydrogenase and succinic

dehydrogenase in extracts of RS plants, but not α-keto-glutaric dehydrogenase or cytochrome oxidase. The absence of the latter two enzymes in RS plants appears to support the hypothesis that bicarbonate is interferring with the decarboxylation of α-ketoglutarate and therefore disrupting the TCA cycle.

Further studies on the TCA cycle enzymes were reported by Brown and Cantino (1955). They found that the aerobic respiration of mature OC plants is not stimulated above the endogenous level ($Q_{O_2} = 9$) by either glucose or malate. However, in OC plants malate is oxidized to lactate, oxaloacetate and traces of pyruvate. Based on these observations, Cantino (1955) suggested that the TCA cycle in B. emersonii is probably a weakly functioning system whose main role is not to supply energy but to serve as a biosynthetic centre for the production of RS plants.

With the discovery that light stimulated growth of B. emersonii (Cantino and Horenstein, 1956), an additional role for bicarbonate was revealed. Cultures of OC plants produce greater yields of plant material in the light than in the dark. Moreover, bicarbonate is still required but at lower concentration (10^{-4} M) than needed for morphogenesis in the absence of light. In a series of studies with labelled and unlabelled bicarbonate, it was found that illumination induced a large increase in CO_2 fixation by intact plants over dark levels and the amount of C^{14}-α-ketoglutarate decreased, whereas C^{14}-succinate

increased within the plants. In addition, citrate, fumarate, malate, lactate and perhaps oxalate also became labelled. Glucose was consumed more rapidly in the light while depressing bicarbonate fixation.

It was also found that extracts of OC plants mediated a reduction of NADP which was inhibited by light, whereas the oxidation of NADPH in the presence of α-ketoglutarate and bicarbonate was stimulated. Moreover, when NADPH and light were present, succinate induced some CO_2 fixation. When these extracts were incubated in a solution containing α-ketoglutarate, C^{14}-bicarbonate and NADPH, C^{14}-α-ketoglutarate, together with C^{14}-isocitrate and C^{14}-succinate were identified. These studies on the effect of light were considered to have supplied direct corroborative evidence for the role of α-ketoglutarate as the site of CO_2 fixation in B. emersonii.

Subsequent studies by Cantino and Horenstein (1957) showed that light induces a marked increase in the size, volume, and in certain constituents of OC plants. Moreover, the maturation time of these plants is increased considerably under illumination, and the delay of maturity is proportional to light intensity and exogenous CO_2, both of which are replaceable by an equimolar mixture of succinate and glyoxylate (Cantino and Horenstein, 1959).

The early studies of differentiation in B. emersonii suffered from two significant shortcomings. Firstly, they were based on comparison between cultures presumed to

consist of mature OC and RS plants, and thus the results rather than the course of morphogenesis were studied. Secondly, the cultures employed were the result of several generations of growth from the initial inoculum, and as such, contained indeterminate and varying amounts of dead and immature plants. However, these shortcomings were overcome when techniques were developed for obtaining large quantities of viable zoozpores and for growing synchronous, single generation cultures of both OC (McCurdy and Cantino, 1960) and RS plants (Lovett and Cantino, 1960).

Using synchronous cultures, McCurdy and Cantino (1960) studied the role of isocitritase and glycine-alanine transaminase during the development of B. emersonii. They found that the synthesis of these enzymes occurs during growth of both OC and RS plants. In OC plants transaminase synthesis appears to be a linear function of plant growth throughout the growth period, whereas isocitritase shows an initial lag. In RS plants, however, the activities of these two enzymes parallel each other during development, reaching maximum levels at about three-fifths of the maturation time, the point of no return in ontogeny. It was suggested that the sequence of reactions mediated by these enzymes constitutes an important shunt mechanism for removing isocitrate produced by the reductive carboxylation of α-ketoglutarate.

In studying the role of glucose-6-phospate dehydrogenase during the synchronous development of RS

plants, Lovett and Cantino (1960) found that enzyme synthesis occurs throughout growth and at high levels of activity. Since this enzyme generates NADPH, it was suggested that the high glucose-6-phosphate dehydrogenase levels in RS plants might constitute a source of NADPH required for the reductive carboxylation of α-ketoglutarate.

Studies of the aerobic respiration of spores, OC and RS plants were also made. McCurdy and Cantino (1960) found that high endogenous respiration of spores (Q_{O_2} = 100) is inhibited by arsenite and malonate; they concluded that the TCA cycle was probably responsible for the high endogenous respiration of spores. While the effect of these inhibitors was not studied in OC plants, they did observe a progressively decreasing Q_{O_2} during growth of these plants. In similar studies, Cantino and Lovett (1960) reported that the Q_{O_2} of RS plants decreases during growth. When they compared the Q_{O_2} levels of OC and RS plants on the basis of total maturation time, they found that at any equivalent stage of development, the Q_{O_2} of RS plants was only a fraction of that of OC plants. It was concluded that bicarbonate depressed aerobic metabolism in B. emersonii, and that this was consistent with the hypothesis that bicarbonate disrupts the TCA cycle in RS plants.

In subsequent studies on the key enzymes of the postulated bicarbonate trigger mechanism, Lovett and Cantino (1961) reported that during growth of RS plants, the activity of NADP-isocitric dehydrogenase increases

several fold, whereas that of α-ketoglutaric dehydrogenase decreases. However, this pattern is completely reversed if bicarbonate is removed before 36 hours --- the point of no return in ontogeny. These authors concluded that bicarbonate induces isocitric dehydrogenase synthesis and α-ketoglutaric dehydrogenase depletion during morphogenesis. The latter was said to explain (Cantino, 1966) the decrease in Q_{O_2} noted in the presence of bicarbonate.

On the basis of various observations there appears to exist a close correlation between the activity of various TCA cycle enzymes and the involvement of bicarbonate in morphogenesis in Blastocladiella. It is nevertheless apparent that the assumptions and conclusions of the postulated bicarbonate trigger mechanism require a critical re-examination of the TCA cycle activity using synchronous cultures. It is the purpose of this thesis to carry out such a re-examination.

CHAPTER II

EXPERIMENTAL WORK

SECTION I

Materials and Methods

Organism and media --- Blastocladiella emersonii obtained from Dr. E. C. Cantino, Michigan State University, East Lansing, Michigan, U.S.A., was used. Stock cultures were maintained at 22-24 C on Difco PYG agar containing per litre: peptone, 1.25 g; yeast extract, 1.25 g; glucose, 3.0 g and agar 20 g. For liquid cultivation of OC and RS plants, liquid PYG medium and liquid PYG supplemented with 8.9×10^{-3} M $NaHCO_3$ were used, respectively.

Chemicals --- Unless otherwise specified, all chemicals were of reagent grade. Adenosine 5'-monophosphate (AMP), nicotinamide adenine dinucleotide (NAD), reduced NAD (NADH), nicotinamide adenine dinucleotidephosphate (NADP), reduced NADP (NADPH), coenzyme A (CoA), 2,6-dichlorophenol-indophenol (DPIP), phenazine methosulfate, bovine serum albumin, DL-isocitric lactone, L-malic acid, α-ketoglutaric acid, oxaloacetic acid and malic dehydrogenase (MDH) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.. Glucose oxidase reagent was obatined from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.. $NaHC^{14}O_3$ was purchased from Nuclear Research Chemicals, Orlando, Florida, U.S.A.. DL-isocitric acid was prepared by hydrolysing its lactone with KOH and neutralizing with HCl

before use. All other chemicals were dissolved in water and neutralized.

Culture procedures --- Heavy spore suspensions and synchronous cultures of OC plants were prepared and grown as described by McCurdy and Cantino (1960). Synchronous cultures of RS plants were prepared as described by Lovett and Cantino (1961). The culture vessels used were 12 litre, 2-necked round bottom flasks equipped with an inoculation funnel, a siphon for removing samples and an aeration tube. Filtered air (3 litres per minute) was admitted through two fritted glass dispersion tubes and agitation was maintained by means of magnetic stirrer using a teflon coated magnetic bar. OC cultures were initiated with 2-5 x 10^8 spores, and RS cultures with about 10^7 spores. All cultures were incubated in a water bath at 25±0.2 C under room illumination. Cultures were harvested by filtration on Whatman #1 paper, washed with 1-2 litres distilled water, and sucked 'dry' for 5 minutes. The plant materials, when not used immediately, were stored at -20 C until needed. Dry weights were obtained by heating under vacuum at 75 C to constant values.

Analysis of culture media --- Ten millilitres of spent media from OC and RS cultures were collected at appropriate intervals for pH, glucose and lactic acid determinations. pH was monitored with a Beckman Expandomatic pH meter. Glucose was assayed with glucostat reagent (Worthington). Lactic acid was determined according to the method of Baker

and Summerson (1941), using lithium lactate as standard.

Cell-free extract preparations --- Except as noted, 0.5 g wet weight of freshly harvested or frozen cells were suspended in 5 ml cold 0.1M potassium phosphate buffer containing 10^{-4} M ethylenediaminetetraacetic acid (EDTA) at pH 7.4 (henceforth referred to as phosphate buffer) and disrupted by ultrasonic treatment at full power (20 kc/sec Brownwill sonic oscillator) for 3 minutes in 30 second pulses. The sonicated extract was centrifuged at 500 x g for 10 minutes, and the resulting supernatant fluid centrifuged at 25,000 x g for 30 minutes. The pellet was suspended in 1.5 ml of the cold phosphate buffer, and the supernatant fraction used for enzyme assays. For the assay of NADP-isocitric dehydrogenase, the 25,000 x g supernatant fluid was dialysed for 6 hours against 200 volumes of 0.02 M phosphate buffer, pH 7.4. For preparation of the citrate condensing enzyme, 0.4 M KCl in 20% ethanol was used in place of the phosphate buffer. All operations were carried out at 0-4 C. The protein content of extracts was determined by the method of Lowry et al. (1951).

Enzyme assays --- Except where otherwise noted, the assays used were those described in Colowick and Kaplan (1955). All spectrophotometric assays were carried out at 25 C using 3 ml quartz cuvettes (1 cm light path) in a Beckman spectrophotometer, model DU, equipped with a thermospacer and Gilford automatic absorbance meter. A molar extinction coefficient of 6.22×10^3 at 340 mμ was

used for both pyridine nucleotides. One unit of activity is defined as the activity of enzyme mediating the conversion of 1 μmole NADH or NADPH per minute at 25 C. Enzyme activities were recorded as specific activity or enzyme units per plant. The latter was calculated as described by Lovett and Cantino (1960).

NADH and NADPH oxidases were assayed by the decrease in absorbancy at 340 mμ at 25 C in reaction mixtures containing Tris-(hydroxymethyl)amino methane HCl (Tris-HCl) buffer, pH 7.4, 300 μmoles; and substrate, 0.2 μmoles.

The citrate condensing enzyme (citrate-oxaloacetate lyase, CoA acetylating, E.C.4.1.3.7) was assayed as described by Srere and Kosicki (1961) by measuring the acetyl CoA dependent increase in absorbance at 340 mμ. The reaction mixture contained Tris-HCl buffer, pH 8.1, 300 μmoles; malate, 30 μmoles; NAD, 0.81 μmoles; acetyl CoA, 0.42 μmoles; MDH, 20 units; and 25,000 x g supernatant fluid containing 20-300 μg protein.

Aconitase (citrate isocitrate hydrolyase, E.C.4.2.1.3) was assayed by following the increase in absorbancy at 240 mμ due to the formation of cis-aconitate with potassium citrate as the substrate. The reaction mixture contained Tris-HCl buffer, pH 7.4, 250 μmoles; citrate, 20 μmoles; and 25,000 x g supernatant fluid containing 30-250 μg protein. Using a molar extinction of 3.3×10^3 for aconitate, one unit of activity is defined as 1 μmole of aconitate formed per 5 minutes at 25 C.

NAD-Isocitric dehydrogenase (L_s-isocitrate:NAD oxido-reductase, E.C.1.1.1.41) was assayed by the increase in absorbancy at 340 mμ as described by Sanwal et al. (1963). The reaction mixture contained Tris-HCl buffer, pH 7.4, 250 μmoles; $MgCl_2$, 10 μmoles; NAD, 1.25 μmoles; AMP, 1.0 μmole; D,L-isocitrate, 10 μmoles; and 25,000 x g supernatant fluid containing 200-900 μg protein.

NADP-Isocitric dehydrogenase (L_s-isocitrate:NADP oxido-reductase, E.C.1.1.1.42) was assayed by the increase in absorbancy at 340 mμ. The reaction mixture contained Tris-HCl buffer, pH 7.4, 300 μmoles; $MgCl_2$, 10 μmoles; NADP, 0.2 μmoles; D,L-isocitrate, 10 μmoles; and dialysed 25,000 x g supernatant fluid containing 100-500 μg protein.

Attempts to assay α-ketoglutaric dehydrogenase were carried out using several methods. They included: (1) spectrophotometric methods for the reduction of NAD (Von Tigerstrom and Cambell, 1966), DPIP (Sanadi, 1952), $K_3Fe(CN)_6$ (McFadden et al., 1967) or cytochrome c (Cantino and Hyatt, 1953b); (2) the manometric determination of CO_2 evolution (Hogen and Kornberg, 1961); and (3) the chemical determination of succinyl hydroxymate for succinyl CoA formation. The hydroxymate was determined as described by Lipman and Tuttle (1945). Cell-free extracts from 500 and 25,000 x g supernatant fractions as well as the 25,000 x g particulate fractions from freshly harvested or frozen plants were used.

Succinic dehydrogenase (succinate:oxidoreductase,

E.C.1.3.99.1) was assayed spectrophotometrically as described by Ells (1959). The reaction mixture contained Tris-HCl buffer, pH 7.4, 200 µmoles; KCN, 10 µmoles; DPIP, 0.83 µmoles; phenazine methosulfate, 0.5 µmoles; succinate, 50 µmoles; and the 25,000 x g particulate suspension containing 100-900 µg protein. The decrease in absorbancy at 600 mµ due to DPIP was recorded at one minute intervals for 5 minutes. Using a molar extinction coefficient of 16.1×10^3 for DPIP, one unit activity is defined as the reduction of 1 µmole DPIP per minute at 25 C.

Fumarase (L-malate hydrolyase, E.C.4.2.1.2) was assayed as the increase in absorbancy at 240 mµ in a reaction mixture containing Tris-HCl buffer, pH 7.4, 300 µmoles; malate, 15 µmoles; and 25,000 x g supernatant fractions containing 40-200 µg protein. One unit activity is defined as 1 µmole fumarate formed per minute at 25 C, using a molar extinction coefficient of 2.4×10^3.

Malic dehydrogenase (L-malate:NAD oxidoreductase, E.C. 1.1.1.37) was assayed by measuring the oxidation of NADH in the presence of oxaloacetate. The reaction mixture contained Tris-HCl buffer, pH 7.4, 350 µmoles; NADH, 0.2 µmoles; oxaloacetate, 0.8 µmoles; and 25,000 x g supernatant fluid, 5-50 µg protein.

Label studies --- One million RS plants (36 hour old) grown synchronously in PYG-$NaHCO_3$ at 25 C as described above, were harvested and collected as usual. The plants were suspended in 100 ml fresh PYG-$NaHCO_3$ containing 100 µc

$NaHC^{14}O_3$ (1 µmole) in 500 ml conical flask having a sample outlet at the bottom. At 4, 10, 15 and 20 minute intervals, 10-30 ml withdrawals were made and the plants rapidly collected on filter paper by suction (less than 20 seconds), and transferred to 40 ml hot 80% ethanol. The suspensions were subsequently acidified with HCl to liberate unfixed $NaHC^{14}O_3$, and after standing for 30 minutes with constant stirring, the suspensions were sonicated for 3 minutes (20 kc/sec Brownwill sonic oscillator), centrifuged at 1000 x g for 15 minutes, and the supernatant fluid collected. The sediments were each washed twice with 10 ml hot 80% ethanol and the washings combined with the original ethanolic extract. The ethanol extract was evaporated in vacuo at 40 C to 2-3 ml and any insoluble material removed by centrifugation. Counts of radioactivity were made, on suitable aliquants (0.3 ml) of the concentrated extracts, using a thin-window gas-flow plancet counter, model FC-72A, (Baird Atomic, Inc., Valley Stream, N.Y., U.S.A.). All counts were corrected for background.

Aliquants (50 µl and 100 µl) of the concentrated extract from the 4 minute sample were chromatographed in two dimensions using phenol;water (80:20, v/v) and butanol: propionic acid:water (2:1:1.5, v/v) as solvents. C^{14}-spots were located autoradiographically by developing 14 x 17" Kodak x-ray sheets (no screen type) which had been in contact with the chromatograms in the dark for 14 days. Identification of the C^{14}-compounds were made by, (1)

staining the chromatograms with 2% ninhydrin and 0.2% bromcresol purple solutions and by, (2) co-chromatography with the standard compounds.

SECTION II

Results

Growth of OC and RS plants --- Mass synchronous cultures of OC plants incubated under the conditions previously described were found to have a maturation time of 15-16 hours. Figure 1 shows a typical growth curve of a mass synchronous OC culture. Such a culture system was characterized by a uniform size of OC plants at all stages of growth, and an exponential increase in dry weight throughout the entire growth period. A slight initial decrease of exogenous glucose was always detected during the first five hours of growth, but the concentration remained virtually constant thereafter. Neither the appearance of lactate nor a change in pH of the medium was observed during the 15-hour growth period. On the other hand, mass synchronous cultures of RS plants showed an entirely different pattern of growth. Figure 2 shows a typical RS growth curve. Initially, the growth of RS plants was similar to that of OC plants. There was an initial phase of exponential increase in dry weight lasting about 36 hours, followed by 48-60 hours of little change in weights. During the first 24 hours of growth of RS plants, the glucose concentration remained constant, but during the next 24 hours, there was a sharp drop to 46% of the original concentration, followed by a period of little change. Lactate production increased markedly after a 24 hour lag

Figure 1 --- Growth Curve of a Synchronous Culture of OC Plants in PYG at 25±0.2 C.

Figure 2 --- Growth Curve of a Synchronous Culture of RS Plants in PYG containing Bicarbonate at 25±0.2 C.

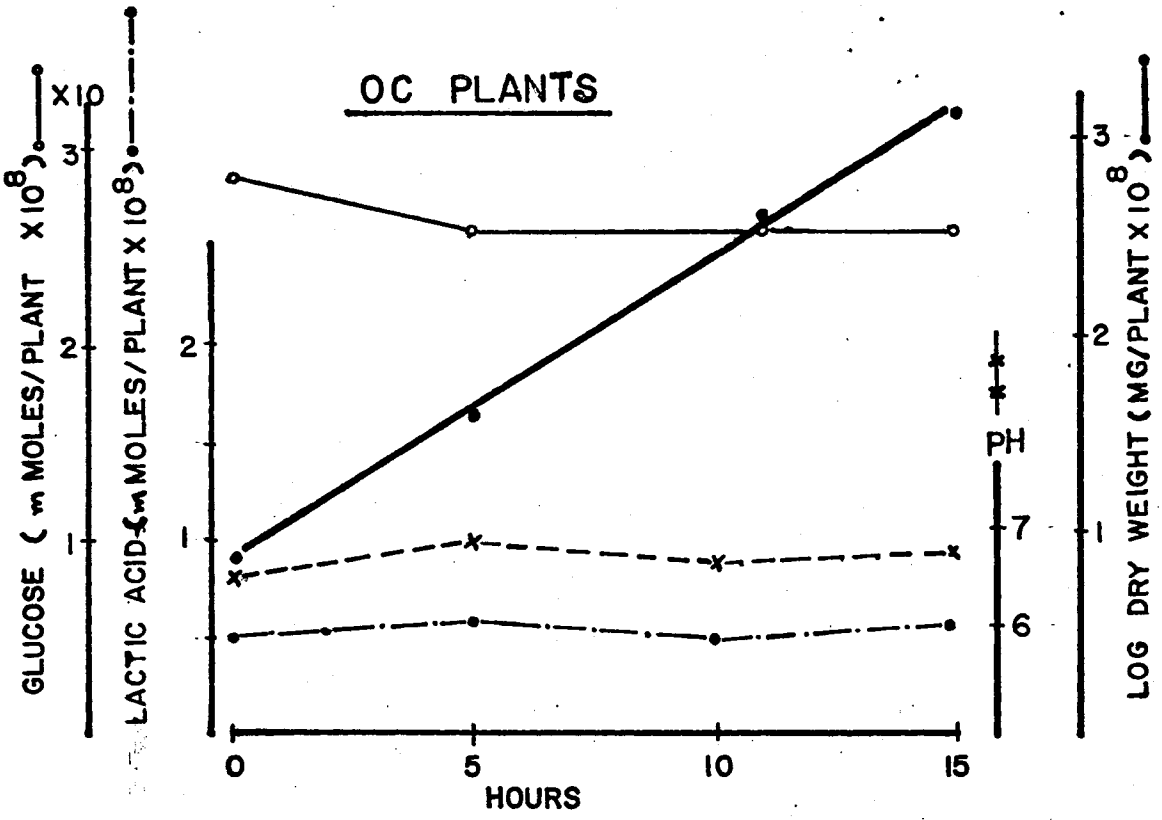

FIGURE 1

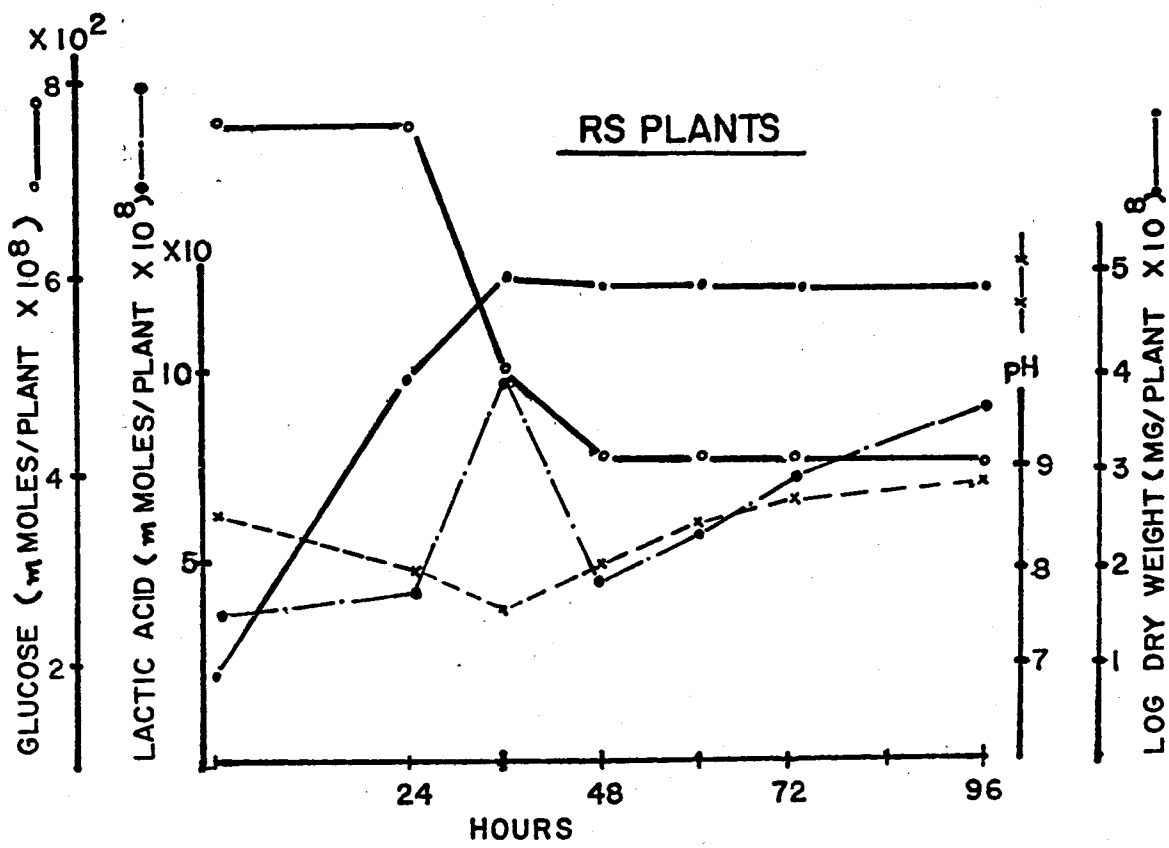

FIGURE 2

period, fell abruptly by 48 hours, and then increased gradually until 96 hours. The pH of the medium decreased steadily to a minimum of pH 7.5 at 36 hours, and then gradually returned to the initial value of pH 8.8 by the end of growth of RS plants.

TCA cycle enzymes and development --- As an initial step in tracing the TCA cycle enzyme activities in B. emersonii, attempts were made to establish their distribution in various cell-free fractions obtained by sonication and centrifugation. The results show that most of the TCA cycle enzymes were present in the 25,000 x g supernatant fraction, except succinic dehydrogenase and NADH oxidase, which were found mainly in the 25,000 x g particulate fraction. Since most of the assays of dehydrogenases involved the reduction of NAD or NADP, it was necessary to test for the presence of NADH and NADPH oxidases in the extracts. Neither oxidase could be demonstrated in the 25,000 x g supernatant fractions. Some NADH oxidase activity was detectable, however, in the 25,000 x g particulate fractions.

After having established a suitable assay system, studies were made on the activities of the TCA cycle enzymes in spores, mature OC and mature RS plants. The results are summarized in Table I.

With the exception of α-ketoglutaric dehydrogenase, all TCA cycle enzymes were present in cell-free extracts of B. emersonii. Despite numerous attempts to assay

TABLE I --- Activities of TCA cycle enzymes in B. emersonii.

Enzymes	Spore		Mature OC		Mature RS	
	Units mg protein	Units plant x10^6	Units mg protein	Units plant x10^6	Units mg protein	Units plant x10^6
Citrate Condensing Enzyme	0.075	0.004	0.063	0.325	0.272	6.500
Aconitase	0.135	0.005	0.030	0.145	0.040	8.000
NAD-Isocitric Dehydrogenase	0.034	0.002	0.064	0.332	0.021	4.480
NADP-Isocitric Dehydrogenase	0.030	0.001	0.083	0.350	0.031	6.560
α-Ketoglutaric Dehydrogenase	-	-	-	-	-	-
Succinic Dehydrogenase	0.190	0.002	0.121	0.159	0.019	2.700
Fumarase	0.245	0.010	0.199	0.682	0.062	6.830
Malic Dehydrogenase	1.900	0.080	1.630	5.620	1.100	90.250

α-ketoglutaric dehydrogenase in different preparations by various methods, this enzyme could not be demonstarted. Two enzymes, citrate condensing enzyme and NAD-isocitric dehydrogenase, which have not been previously reported as present in B. emersonii, were also detected. In the case of citrate condensing enzyme, the activity was easily demonstrated and was always high. Most of the AMP-stimulated NAD-isocitric dehydrogenase activity (> 80%) was located in the 25,000 x g particulate fraction.

The activities of the enzymes mediating the conversion of C_4-dicarboxylic acids were high at all stages compared to those enzymes mediating the conversion of tricarboxylic acids. The activities of all enzymes, expressed in units per plant, were higher in RS plants than in either spores or OC plants.

Since these results contradicted the report by Cantino (1965) that, with the exception of NADP-isocitric dehydrogenase, the TCA cycle enzymes are weak or absent in RS plants, and examination of the TCA cycle enzyme activity profile during development of both OC and RS plants was undertaken. The results are presented in Figures 3 to 16. Since the protein content of Blastocladiella varies with growth, these data are expressed in enzyme units per plant.

In both OC and RS plants, the levels of activity of all the enzymes increased during exponential growth (see later in discussion). However, in RS plants, these levels of activity increased to a maximum at 36 hours and decreased

CITRATE CONDENSING ENZYME

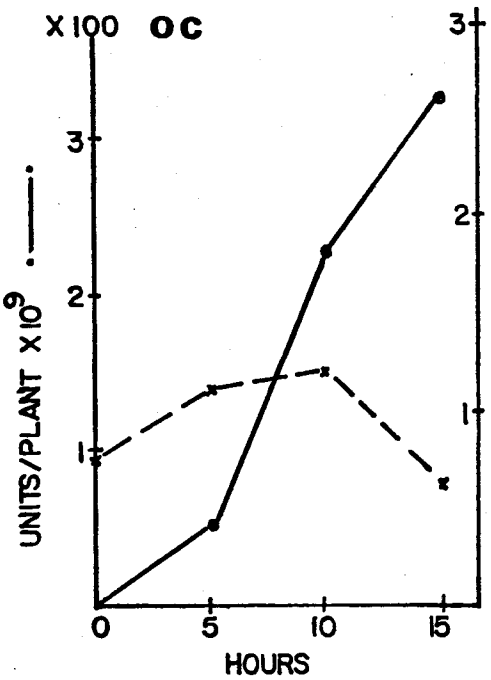

FIGURE 3

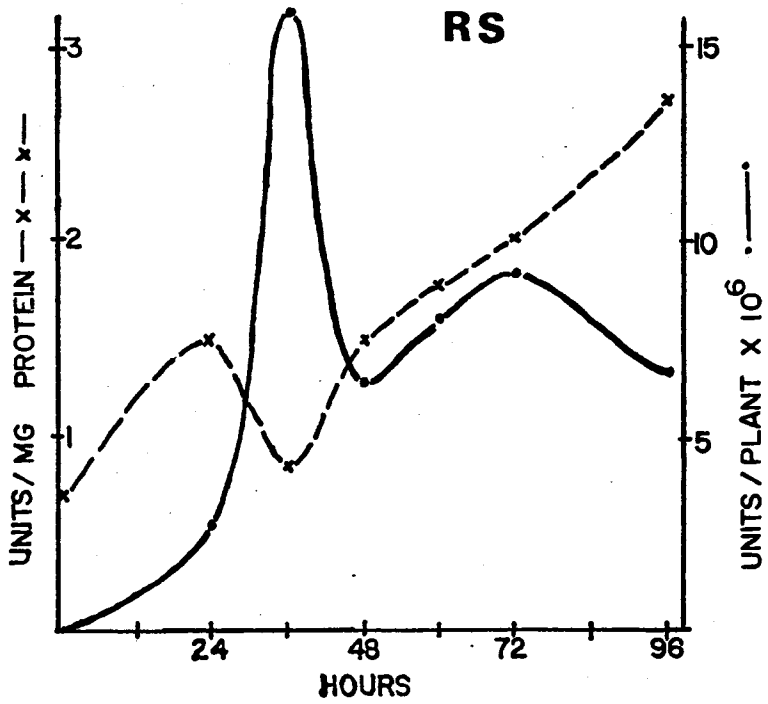

FIGURE 4

ACONITASE

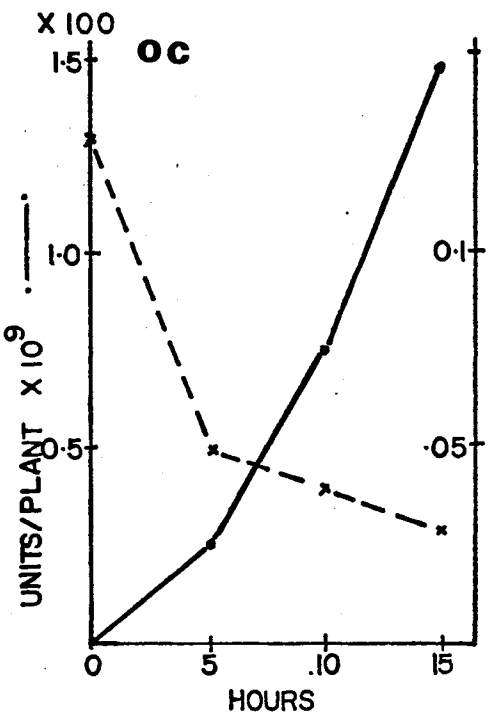

FIGURE 5

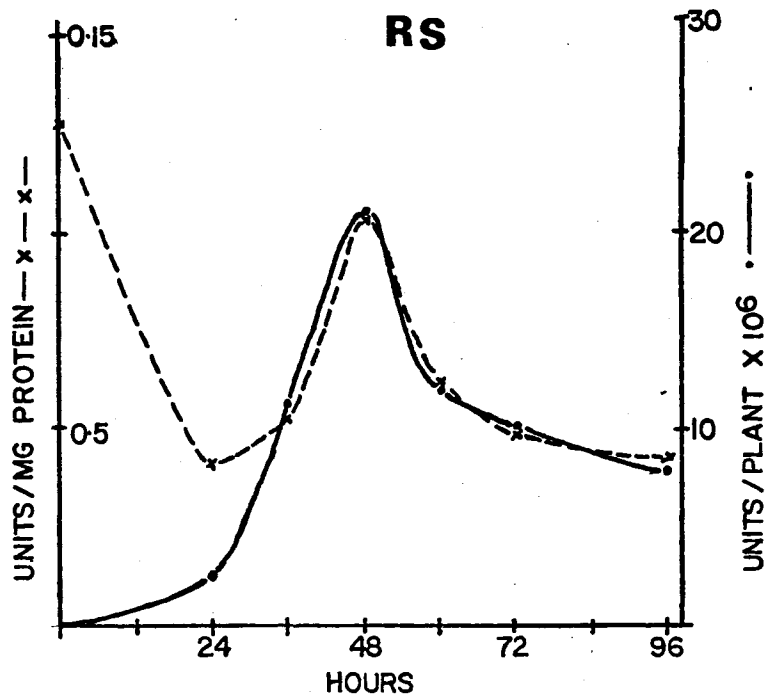

FIGURE 6

NAD-ISOCITRIC DEHYDROGENASE

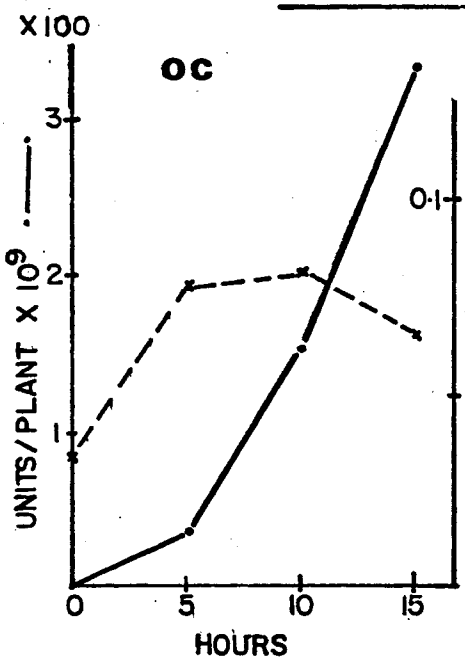

FIGURE 7

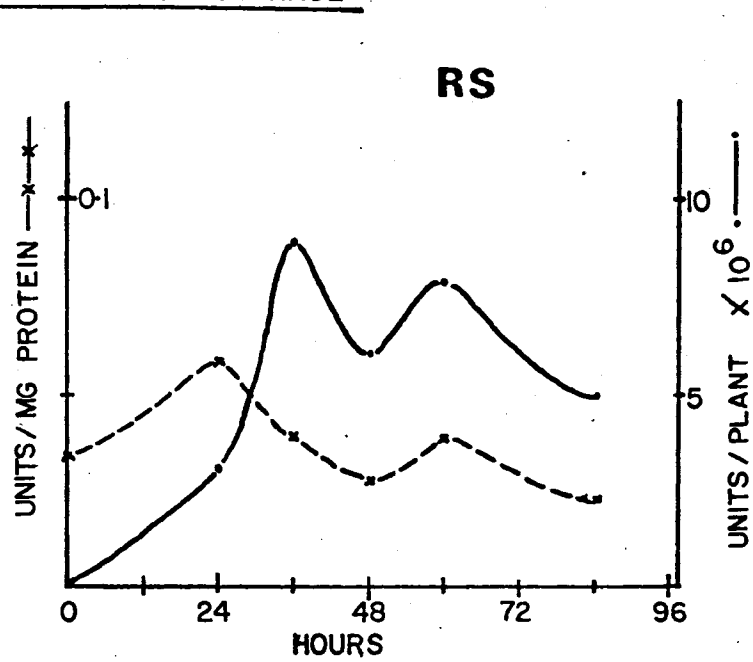

FIGURE 8

NADP- ISOCITRIC DEHYDROGENASE

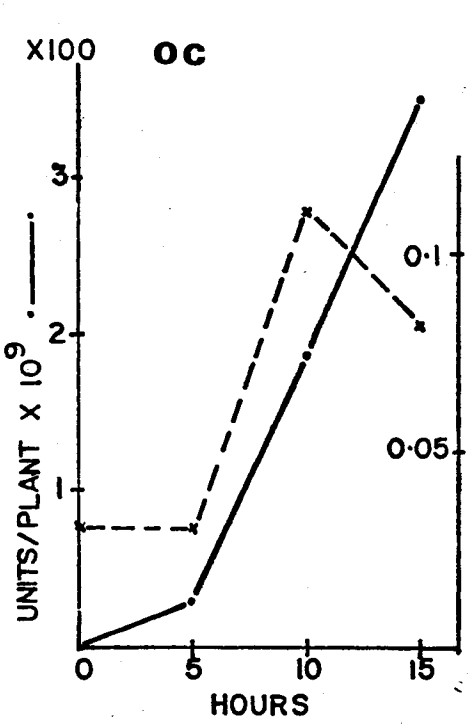

FIGURE 9

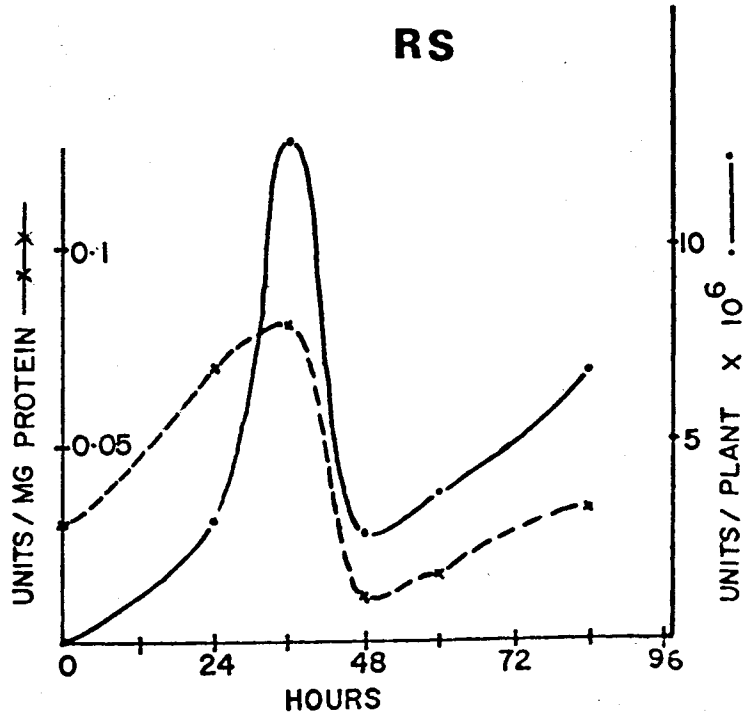

FIGURE 10

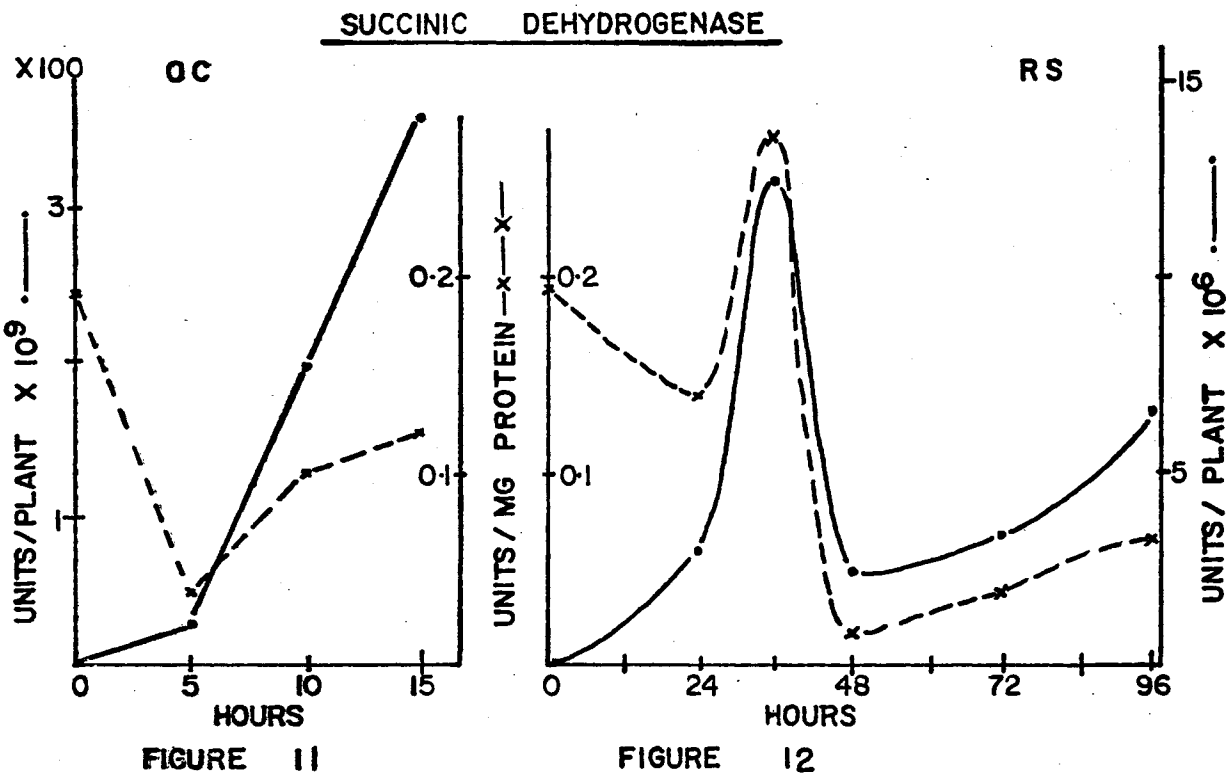

FIGURE 11

FIGURE 12

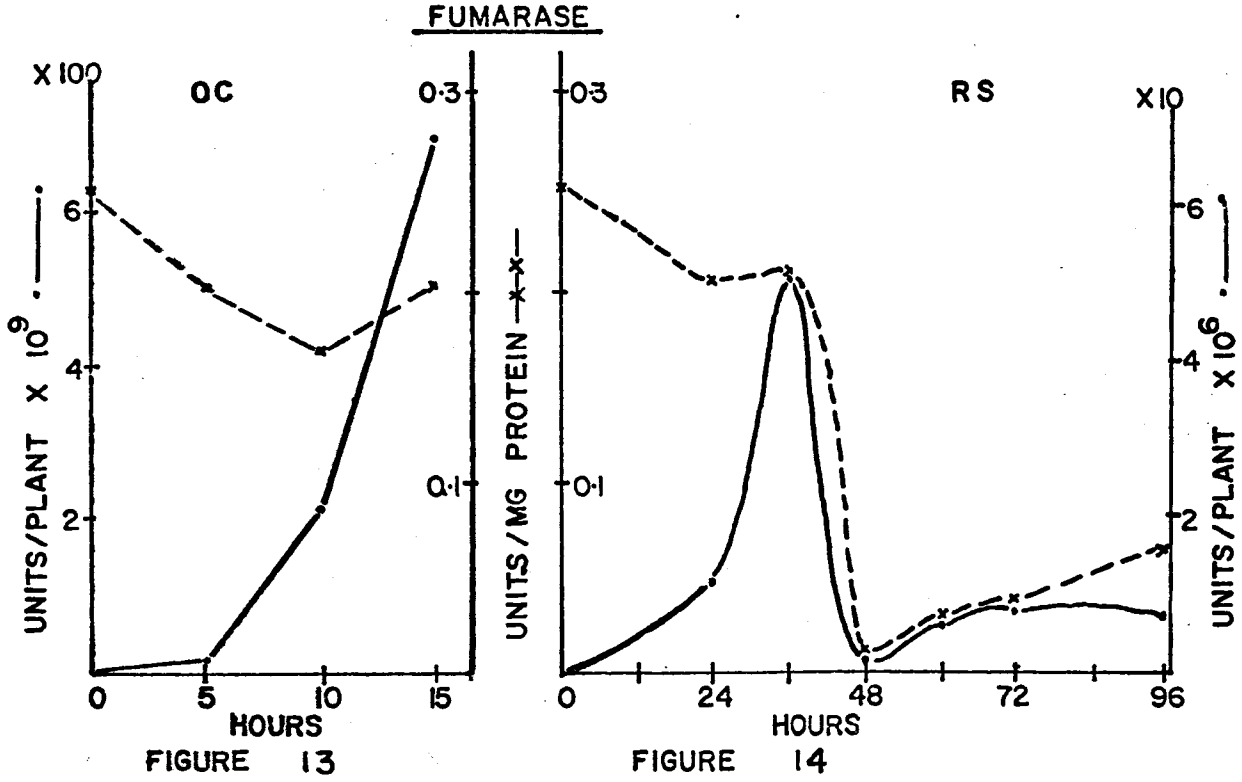

FIGURE 13

FIGURE 14

MALIC DEHYDROGENASE

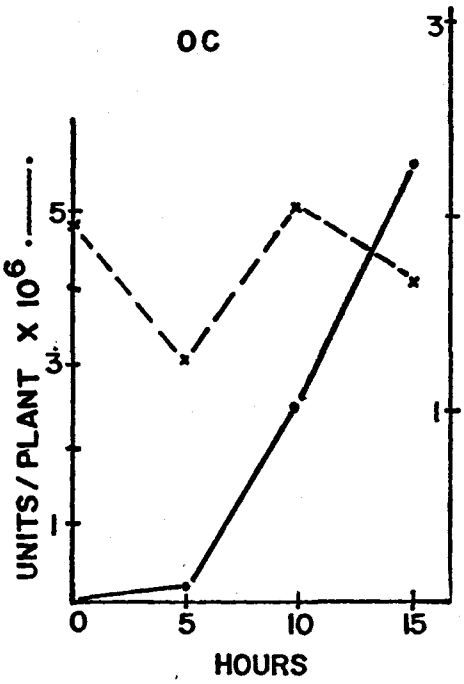

FIGURE 15

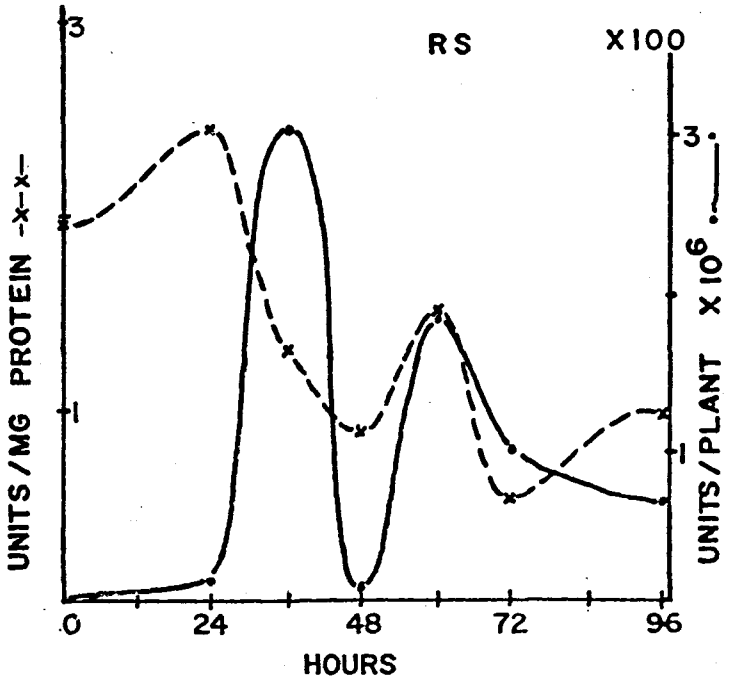

FIGURE 16

at 48 hours, which for aconitase, was the period of maximum activity. For some enzymes, particularly the dehydrogenases, a second maximum at about 60-72 hours was also noted. The levels of activity at the point of no return of RS plants (36 hours) were exceptionally high, three times higher than at any other RS stage, and 30-100 times higher than OC plants. Furthermore, the final levels of activity in RS plants were about the same as those at 24 hours and were even higher than in mature OC plants.

Labelling studies --- In order to more fully understand the site of CO_2 fixation and the bicarbonate trigger mechanism, a C^{14}-bicarbonate fixation study was carried out with the RS plants. Figure 17 shows the pattern of C^{14}-incorporation in the ethanol extracts of 36 hour RS plants. Considerable clumping of plants occurred at this stage, and the first sample taken was 4 minutes after introducing the plants into the C^{14}-bicarbonate containing medium. During this 4-minute period, 4% of the initial C^{14}-bicarbonate was incorporated. Initially, there was a very rapid uptake of C^{14}-label into the ethanol soluble fraction, but decreased after 10 minutes. Based on chromatographic and autoradiographic analyses (Figure 18), the greatest amount of label appeared in aspartic acid with lesser amounts in glycine and malic acid. Other labelled compounds which contained a significant amount of label were identified to be, in order of decreasing activity: α-ketoglutaric acid, lactic acid, serine, arginine, glutamic acid, alanine, isocitric

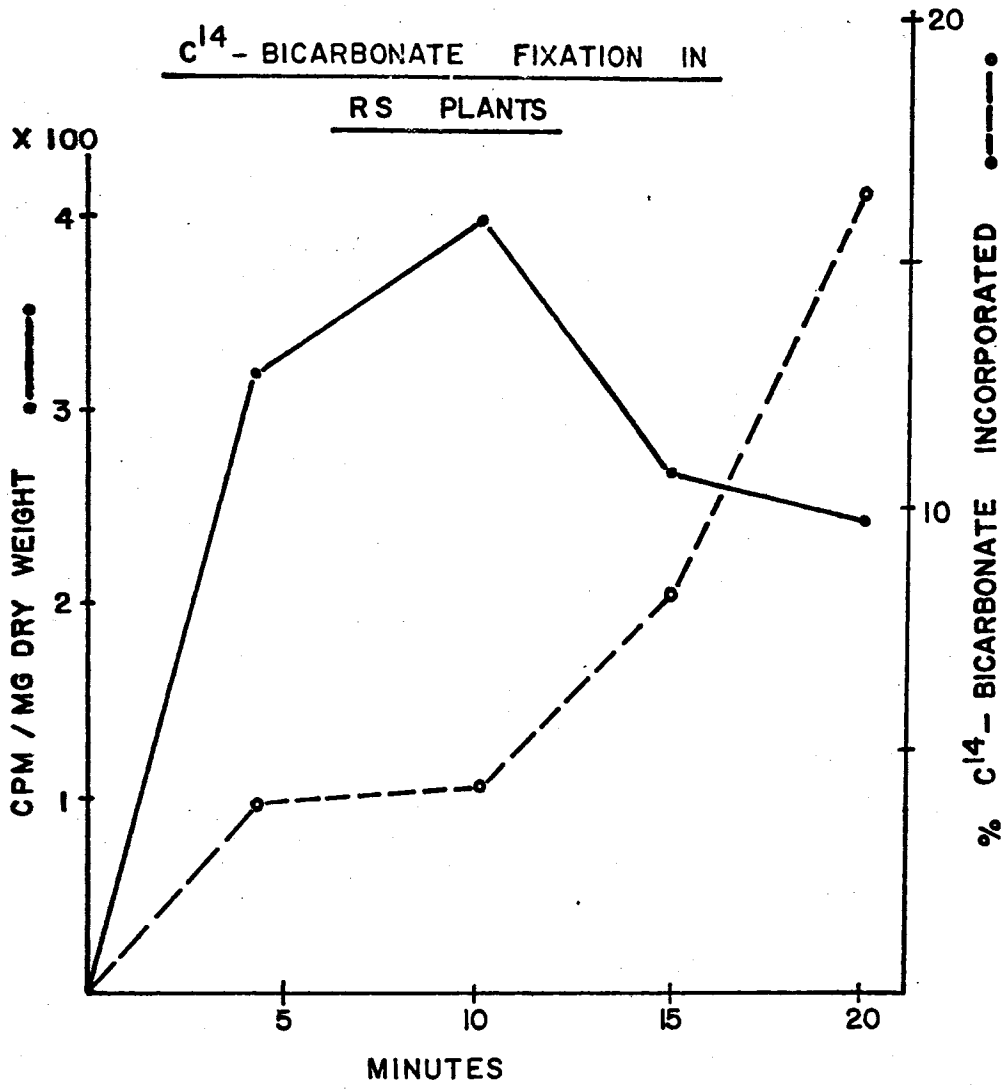

FIGURE 17

The time course of C^{14}-bicarbonate fixation in alcohol soluble fraction of 36 hour RS plants in PYG-bicarbonate medium containing 100 µc $NaHC^{14}O_3$

Figure 18. Tracing of the autoradiogram.

C^{14}-compounds:

1 = aspartic acid
2 = serine
3 = glutamic acid
4 = glycine
5 = arginine
6 = alanine
7 = isocitric or citric acid
8 = α-ketoglutaric acid
9 = malic acid
10 = lactic acid
11 = succinic or pyruvic acid
12 & 13 = not identified

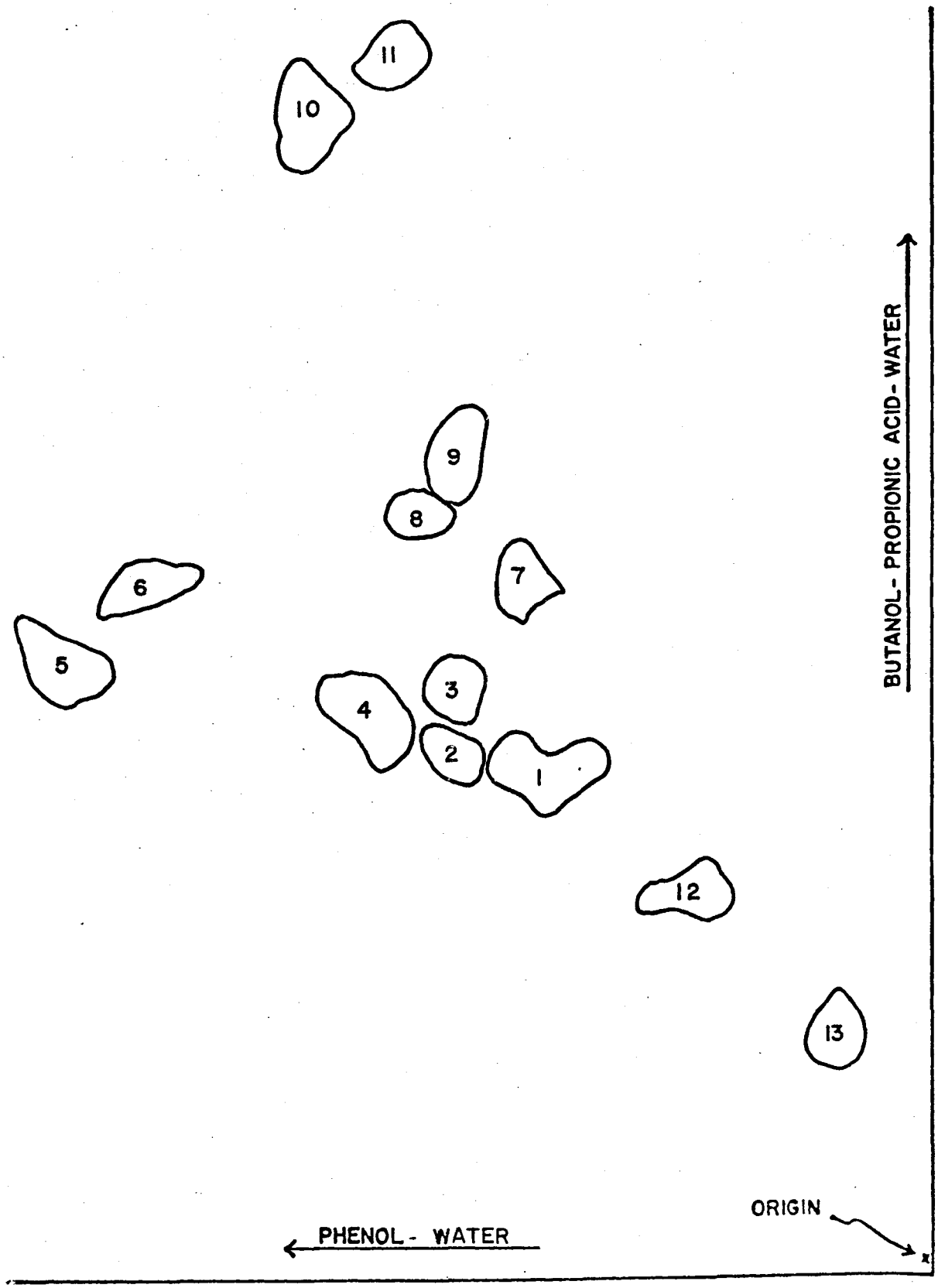

FIGURE 18

or citric acid, succinic or pyruvic acid and two unidentified compounds, numbered 12 and 13.

CHAPTER III

DISCUSSION AND CONCLUSIONS

The main purpose of the work reported here was to re-examine the behavior of the TCA cycle enzymes during the development of B. emersonii in synchronous cultures. It was hoped that such a study might offer clues to the biochemical mechanisms underlying morphogenesis in this mold. In some respects, our results contrast with previous work and the assumptions derived therefrom (Cantino, 1961 and 1966). Before considering the results of the enzymological studies, it is appropriate to consider the characteristics of the synchronous cultures used in this work particularly as they affect to some degree the interpretation of the enzymological studies.

Growth of OC and RS plants --- In mass synchronous cultures, OC plants consume very little glucose during their entire growth period and liberate no lactic acid into the medium, as a consequence of which the pH remains virtually constant (Figure 1). It seems that the metabolism of OC plants is mainly oxidative. A similar conclusion was also reached by Griffin (1965). Many of the early reports (Cantino, 1951; 1959; 1961; 1965 and 1966) described the metabolism of OC plants as homofermentative. This may indeed be the case in multigeneration cultures with poor aeration. However, in the highly aerated, single-

generation cultures studied here, lactic acid was not produced nor was appreciable glucose utilized. Moreover, McCurdy (personal communication), and Cantino and Goldstein (1961) also failed to detect any appreciable pH changes in aerated, single-generation cultures. The evidence suggests therefore that Blastocladiella is capable of both oxidative and fermentative metabolism.

During the early period of exponential growth, RS plants, like OC plants, do not utilize glucose. Beginning at 24 hours and continuing for the next 24 hours, they consume 43% of the total glucose, a value which correlates well with the transient production and subsequent decrease in concentration of lactic acid (Figure 2). It is not known what proportion of the glucose utilized appears in lactic acid or in the Blastocladiella polysaccharide which is synthesized during this period (Goldstein and Cantino, 1961). There is also a decrease of pH in the medium during the period of early acid production, but the explanation of the subsequent rise in pH during the later phase of development when lactic acid is again produced is not apparent. These results are similar to those of Cantino and Lovett (1960) except that they did not report any early transient accumulation of lactic acid.

Cantino and coworkers have generally compared OC and RS plants on the assumption that their respective maturation times and fractions thereof (expressed as % generation time) reflect the achievement of analogous and

therefore comparable stages of growth and development. On this basis, many differences between OC and RS plants have been noted. Examples are differences in Q_{O_2} (Cantino and Lovett, 1960), polysaccharide synthesis (Cantino and Goldstein, 1961), growth rate (Cantino, 1966) and numerous enzyme systems (Cantino, 1961 and 1966). It is doubtful whether such comparisons are valid. For if one examines the overall pattern of growth, it is obvious that the exponential growth rate of OC and RS plants are similar. The significant distinction between the two is that while exponential growth in OC plants ends in 15 hours with the subsequent release of zoospores, that of RS plants continues for some 12 additional hours, followed by 70 hours of continued development in the absence of growth. It seems, therefore, that the exponential phase is the only period during which the two types of plants can be validly compared. The failure of either plant to aerobically metabolize glucose during exponential growth further suggests that there are similarities in energy metabolism during the growth phase. We have therefore chosen not to use the method of analysis employed by Cantino and co-workers.

TCA cycle enzymes --- From previous enzymatic studies on the TCA cycle enzymes in multigeneration cultures of OC and RS plants, Cantino (1961 and 1966) found that most of the TCA cycle enzymes, including cytochrome oxidase, are present in OC but not in RS plants, except NADP-isocitric dehydrogenase, which remains fully functional in both.

Moreover, the Q_{O_2} of RS is much lower than that of OC plants. These results were considered as evidence in support of the hypothesis that bicarbonate first reversed the flow then induced multiple enzymatic lesions in the TCA cycle. Our results on mature plants contrast markedly with these findings. All of the TCA cycle enzymes were found to be present in spores, mature OC and RS plants, except α-ketoglutaric dehydrogenase. Unable to detect citrate condensing enzyme, Cantino (1965) considered the pathway controlling the entry of C_2 units into the TCA cycle to be a very weak system. Our discovery of high citrate condensing enzyme activity indicates that there is no road-block to the entry of C_2 units into the TCA cycle. NAD-isocitric dehydrogenase is also of considerable interest, since the enzyme is usually associated with an energy generating function, and is intimately bound to mitochondria (Niederpreum, 1965). In Blastocladiella, the major portion of this activity is in the soluble fraction. Whether this observation is an artifact of the preparation or reflects the true state of affairs remains uncertain.

Our failure to detect α-ketoglutaric dehydrogenase, one of the key enzyme of the bicarbonate trigger mechanism, is puzzling. The presence of this enzyme has been reported by Cantino and co-workers many times in various contexts, yet, we were unable to confirm its presence in B. emersonii. It is worth noting that in recent studies of this enzyme in spores and RS plants, Lovett and Cantino (1961) used a

direct chemical method for determining the disappearance of α-ketoglutarate from a reaction mixture containing CoA, NAD, a buffer and a high concentration of extract. However no attempt was made to confirm the accumulation of the expected product of the reaction. In view of the high endogenous content of amino acids and the presence of transaminases catalyzing the conversion of α-ketoglutarate to glutamate in Blastocladiella (McCurdy and Cantino, 1960; and McCurdy, personal communication), the disappearance of α-ketoglutarate reported by Lovett and Cantino cannot be attributed unequivocally to the action of this dehydrogenase. Thus the function of α-ketoglutaric dehydrogenase in development must be regarded as ambiguous.

TCA cycle enzymes and development --- Since our results obtained using mature plants do not support the claim that RS plants are devoid of the TCA cycle enzymes except of those presumed to be involved in the bicarbonate mechanism, it is of particular interest to examine the correlation of enzyme activities with the ontogeny of OC and RS plants.

The results (Figure 3 to 16) show that in OC plants all the enzymes increase during growth when expressed on a per plant basis. A similar picture is obtained with RS during exponential growth although higher levels are achieved at the end of the longer exponential growth period in RS than in OC plants. The most striking feature of RS plants is the dramatic rise in all TCA cycle enzymes which occurs at 24 and which builds to maximum levels at 36 hours

corresponding to a cessation of growth and the period of maximum glucose utilization. Several other enzymes also attain maximal activity at this stage, namely isocitritase and glycine-alanine transaminase (McCurdy and Cantino, 1960), as well as glucose-6-phosphate dehydrogenase and glucosamine synthetase (Lovett and Cantino, 1960). Maximum bicarbonate fixation also occurs at this stage of development (Cantino, 1967). It appears that the 36 hour is a critical period in RS development.

It is obvious on the basis of our enzymatic data that bicarbonate does not lead to a loss in the TCA cycle enzymes. On the contrary, its presence is correlated with an increase in activities. It is not the unique property of NADP-isocitric dehydrogenase alone that it continues to be detectable during the genesis of RS plants as suggested by Cantino (1966), but it is the property of the entire complement of the TCA cycle enzymes, with the exception of α-ketoglutaric dehydrogenase. Thus our data do not seem to offer any support to that part of the hypothesis which assumes a CO_2 induced loss of enzymes in RS plants. However, the results of our enzyme studies do not have any immediate bearing on the notion that NADP-isocitric dehydrogenase mediates a CO_2 linked reductive carboxylation of α-ketoglutarate, and that this is the reaction in bicarbonate induced morphogenesis. Neither do our preliminary labelling studies support this assumption.

Labelling studies --- In the labelling studies, the C^{14}-bicarbonate incorporated into the ethanol soluble fraction is found to be distributed in a number of amino acids, TCA cycle intermediates and two unidentified compounds (Figure 18), with only small amounts of label in α-ketoglutarate and isocitrate. The most rapidly labelled compounds resulting from 4 minutes exposure to C^{14}-bicarbonate is aspartic acid. Since it may be rapidly formed from oxaloacetic acid (OAA) via transamination in extracts of Blastocladiella, it seems likely that OAA may be the initial product of bicarbonate fixation. It is possible that phosphoenolpyruvate (PEP) or pyruvate is the site of CO_2 fixation. There are numerous enzyme systems capable of mediating PEP or pyruvate linked CO_2 fixation (Wood and Utter, 1965), and any one of these enzymes may be involved. However none of these enzymes has been demonstrated in Blastocladiella. Previously Cantino (1965) discounted the possible involvement of these enzymes, because he was unable to obtain any OAA from his fixation studies. Neither was OAA obtained in our studies; however, considering its acid lability and its rapid amination noted above, its detection might not have been expected.

Although it is possible that a CO_2 fixation reaction mediated by PEP or pyruvate can produce OAA and result in the increase of the TCA cycle intermediates and enzymes, it is not clear how our results can be related to the induction of RS by bicarbonate. However, it may be

constructive to attempt some speculation as a basis for further work.

During their periods of exponential growth, OC and RS plants have many similarities. Morphologically they are almost identical (Cantino, 1966); they do not utilize glucose or produce lactic acid (Figure 1 and 2) and presumably use similar substrates for growth; they have similar Q_{O_2} (McCurdy and Cantino, 1960; Cantino and Lovett, 1960) and show the same rate of polysaccharide synthesis (Cantino and Goldstein, 1961). However, at the end of exponential growth (about 15 hours), OC plants produce numerous papillae through which zoospores are liberated. On the other hand, plants growing in the presence of 10^{-2} M bicarbonate, continue their exponential growth for another 10 hours, and only if bicarbonate is present for another 12-15 hours are RS plants produced. Although plants grown in the presence of bicarbonate for 24 hours are headed towards formation of the RS type, the process remains fully reversible until 12 hours later. It appears that the initial effect of bicarbonate is one of delaying the maturation of the plants or diverting their development from cell wall changes (papillae) and cleavage of spores characteristic of OC plants. If so, this would be similar to the effect of a lower concentration of bicarbonate in the presence of light which delays cleavage, increases maturation time and allows additional DNA replication (Cantino, 1959 and 1966). As a result of the prolonged

exponential phase and the subsequent requirement for the synthesis of constituents characteristic of RS, the demand of RS plants for energy and biosynthetic intermediates is increased. Perhaps as a result of depletion of other substrates in the medium, glucose is utilized to meet the demand after 24 hours with accompanying increase in TCA cycle activity and increased enzyme synthesis. Although the exact function of bicarbonate at this early stage is not known, if indeed its fixation produces OAA, the additional supply of C_2-receptor compounds would provide further stimulus to TCA cycle activity.

At first these enzymatic changes are reversible and subject, as is differentiation, to modulation by bicarbonate (Cantino, 1967). Beyond the point of no return, as a result of the products derived from OAA and therefore bicarbonate, the cells become irreversibly committed to RS formation. It may be that some fundamental change in informational macromolecules occurs which is triggered by products derived from OAA. In this connection Cantino's observation (Cantino, 1967) that bicarbonate induced, reversible changes in RNA occur just before the point of no return, which become irreversible thereafter, is suggestive; however, this phenomenon requires a more definitive examination. Deering's observation (see discussion in Cantino, 1967) that U.V. treatment of zoospores permits development only as far as the point of no return may also be relevant. It suggests that some

critical step, presumably involving DNA, RNA or both and occurring at the point of no return is blocked by early treatment with U.V.

It is apparent from the work reported here that considerably more work must be done on the analysis of events up to and including the point of no return. What is required is more careful characterization of plants growing in the presence and absence of bicarbonate in order to detect the earliest stage in exponential growth at which there appear significant differences between the two types of plants in structure, composition, and metabolism. The finding that glucose is not utilized during early exponential growth suggests that the study of metabolism in Blastocladiella shoud be predicated on the probability that an amino acid (perhaps, glutamate ---personal communication, David Sonneborn, U. of Wisconsin) is the initial carbon and energy source.

The results of the experiments with C^{14} indicate that it would be useful to examine Blastocladiella for the presence and behaviour of enzymes catalyzing CO_2-fixation via pyruvate or phosphoenolpyruvate. However, additional and more definitive labelling studies will have to be carried out at all stages of ontogeny to determine whether in fact the site of bicarbonate fixation is the same at all stages and to determine in what way bicarbonate fixation and products derived therefrom may be related to both the reversible and irreversible events involved in RS formation.

In respect to the latter it is obvious that a great deal more attention must be focused upon the reported changes in nucleic acid composition occurring beyond the point of no return (Cantino, 1961a; Murphy and Lovett, 1966) as probably the most fundamental response to bicarbonate in the induction of RS plants.

CHAPTER IV

SUMMARY

During exponential growth, OC plant in mass synchronous cultures consumed little glucose and produced no lactic acid. Similarly, RS plants did not utilize glucose or produce lactic acid during the first 24 hours of growth exponentially. During the next 24 hours, glucose was consumed but the lactic acid produced was rapidly reutilized. The acid was produced again at maturity.

Cell-free extracts of spores, mature OC and mature RS plants exhibited the activities of all the TCA cycle enzymes, except α-ketoglutaric dehydrogenase. Included were the activities of AMP-stimulated NAD-isocitric dehydrogenase and citrate condensing enzyme. TCA cycle enzyme activities were higher in RS than in OC plants or spores.

The levels of activity of TCA cycle enzymes increased during exponential growth of synchronously developing OC and RS plants. In RS plants, these enzyme activities reached a maximum at 36 hours, the point of no return in ontogeny.

In 36-hour RS plants, C^{14}-bicarbonate was rapidly incorporated into a number of amino acids, TCA cycle intermediates and two other unidentified compounds. Aspartic acid incorporated the most label.

An attempt to speculate about the bicarbonate induced morphogenesis in B. emersonii was made.

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