THE RELATION BETWEEN BIOCHEMICAL AND MORPHOLOGICAL DIFFERENTIATION IN *BLASTOCLADIELLA EMERSONII*

By

James S. Lovett

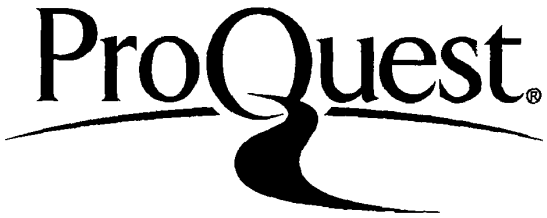
A THESIS

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

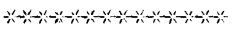
Department of Botany and Plant Pathology

1959



ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. E. C. Cantino for his enthusiastic support and able guidance during the course of this study.



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Approved Edward C. Cantino

ABSTRACT

The enzyme glucosamine synthetase (glutamine-F-6-P transamidase) was purified ca. 19-fold from extracts of the aquatic Phycomycete Blastocladiella emersonii, by centrifugation, protamine sulfate, fractionation, and adsorption on tricalcium phosphate gel. The pH optimum, the time course, and relation between enzyme concentration and reaction rate were established for the partially purified synthetase. The reaction catalyzed by the enzyme isolated from Blastocladiella was found to be the same as that of the enzyme of Neurospora crassa; i.e., d-fructose-6-phosphate + l-glutamine ⟶ d-glucosamine-6-phosphate + l-glutamic acid.

In preliminary studies concerning the biochemical basis of differentiation in Blastocladiella, the free amino acid pools were extracted and compared between the two alternative mature forms of the organism, the zoosporangial, and resistant sporangial plants. The latter were found to contain only 50% as much soluble amino acid nitrogen as the former, and there were, as well, certain qualitative differences between the two types. In addition the activity of glucosamine synthetase in zoospores, mature zoosporangial plants, and mature resistant sporangial plants was established.

The unexplained differences among the various forms of the organism made a more refined experimental approach to the problem of differentiation a necessity. Therefore, a procedure was developed for growing well synchronized, large scale cultures of resistant sporangial plants

of Blastocladiella throughout the complete generation period in a glucose-peptone-yeast medium containing bicarbonate. The increases in size and dry weight of individual plants were determined, and a photomicrographic record of their developmental morphology obtained, during the growth of cultures under such conditions.

In studies of the genesis of the resistant sporangium, the activity of the synthetase, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase enzymes was determined during development in synchronous culture. Utilizing the same culturing techniques, the time sequence of chitin, lipid, melanin, and nitrogen synthesis was established. The free nitrogen pools in developing resistant sporangial plants were shown to undergo both quantitative and qualitative changes during differentiation.

Using the approach of comparative biochemistry the significance of the changes in the cellular components as they related to the structure and function of the developing resistant sporangial plant was discussed. An attempt was made to integrate the physiological and morphological processes involved in the initiation and differentiation of the individual resistant sporangium of Blastocladiella.

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INTRODUCTION

The problem of cause and effect relationships underlying the differentiation of morphologically complex structures has attracted the attention of man from the time of Aristotle. Until the proposal of the cell theories of Schleiden and Schwann, and the work of Pasteur in the mid 1800's, however, little real progress was made toward understanding development. In fact, it required the discoveries of these men and others to finally lay to rest the speculations of the *naturphilosophen* concerning "preformation," "spontaneous generation," etc., which had seriously hindered any empirical approach to the problem. Although tremendous strides have been made in embryology since that time, the inherently-complex, multicellular systems utilized have impeded the development of a completely satisfactory explanation of the processes involved in growth.

Little enough is known concerning the regulation of development in a single cell. When all the complexities and interrelationships among the units in a multicellular structure are added together, the distinction between the properties due to the individual, and those due to the population becomes most difficult. It would appear axiomatic that an understanding of the physiological and morphological capabilities of a single cell should precede attempts to elucidate the interactions among cells. Such approaches have been, and are being increasingly utilized, particularly the study of isolated plant and animal cells or tissues in pure culture. A second and somewhat simpler experimental

approach has involved the study of organisms which contain one, or at most a few cells at maturity. The algae and fungi have shown themselves to be most suitable for such studies. A case in point is the elegant work of Hämmerling (1953) concerning nuclear-cytoplasmic relationships in the morphological development of the unicellular, uninucleate alga, Acetabularia. Many members of the lower fungi also appear to provide excellent material for experimental morphology. They can be grown under controlled experimental conditions and display a distinct but relatively simple morphology. Generally speaking, however, with a few important exceptions the potentialities of these organisms have not been exploited. The greatest percentage of work in the mycological area has been confined to investigations of the environmental conditions necessary for growth and reproduction (Hawker, 1957).

If attention is turned to studies of morphogenesis at the cellular and physiological level, very few examples can be found for the fungi. Notable among these is the work of Nickerson and his colleagues (1956, and references therein) on the biochemical mechanisms of cell division in yeasts. Another important contribution has come from the studies of Wright and Anderson (1959) on biochemical differentiation in the slime mold Dictyostelium discoidium. Probably the most thorough approach, from the standpoint of a broadly biological study closely coordinated with a biochemical investigation of differentiation, has been that of Cantino and co-workers (1951-1959) with the aquatic Phycomycete, Blastocladiella emersonii. In addition to the intrinsic knowledge gained for the particular organism, each of the studies mentioned has

helped to provide information necessary for an understanding of developmental phenomena common to the cells of many, or perhaps all, organisms.

LITERATURE REVIEW

Morphogenetic Studies of Blastocladiella emersonii

The simple aquatic fungus, Blastocladiella emersonii, was isolated in pure culture and its life history and development described by Cantino (1951), and Cantino and Hyatt (1953b). After an initial period of motility the small, uniflagellate spores of this fungus settle down, retract their flagella, and germinate in a bipolar fashion. The lower portion of the growing plant develops into a single-celled, branching, rhizoidal system. The upper portion, also consisting of a single multinucleate cell, develops into either a colorless, papillate, zoosporangium with a thin, chitinous wall (referred to as an ordinary colorless or O.C. plant), or, a brown, melanin-pigmented, thick-walled, pitted resistant sporangium (R.S.) containing conspicuous lipid globules. When the mature plants of either type are placed under suitable conditions, they discharge motile spores (swarmers) and thus complete the life cycle with no conventional intervening sexual stage.

In contrast to the closely related genus, Blastocladia, which required a carbon dioxide atmosphere of almost 100% to form R.S. (Emerson and Cantino, 1948), Blastocladiella spontaneously produced such structures under the crowded conditions in second generation colonies. When the conditions necessary for R.S. formation were studied, it was found that either calcium carbonate or sodium bicarbonate induced the development of R.S. It was further established that on casein hydrolysate medium, where bicarbonate alone would not suffice, both

α-ketoglutarate and citrate increased the percentage of R.S. formed, and that arsenite and semi-carbazide (both inhibitors for the decarboxylation of α-ketoglutarate) could replace the organic acid requirement (Cantino, 1951).

Having acquired the ability to control the pathway of development, a nearly ideal system was available for studying the metabolic interactions involved in the ontogeny of the organism.

The bicarbonate stimulus was required for only three-fifths of the generation time of the R.S. plants; conversely, it could not induce R.S. differentiation in O.C. plants after three-fifths of their generation time had elapsed (Cantino, 1952). These results, together with those from permeability studies, led to the interpretation that the effect of the bicarbonate was to block decarboxylation reactions in the tricarboxylic acid cycle, and that the resulting pile-up of intermediates caused a shift in the metabolic patterns toward R.S. development, i.e., thicker wall, increased pigment, lipid synthesis, etc. It was further suggested that these changes led to a gradually increasing impermeability to both internal and external bicarbonate ions. At approximately three-fifths of the generation time the system was assumed to become autocatalytic due to the increased retention of metabolically produced bicarbonate; removal of the external stimulus after this stage could no longer reverse the process (Cantino, 1952).

A comparative survey of citric acid cycle enzymes in the wild-type O.C. plants of Blastocladiella, and in an orange mutant (B.E.M.) derived therefrom, provided additional information used to support the theory

that bicarbonate helped to slow down and, with the assistance of other pulling reactions, reverse the Krebs cycle at the α-ketoglutarate stage (Cantino, 1953; Cantino and Hyatt 1953b, 1953c). Most of the enzymes normally associated with that oxidative system were found to be present in O.C. homogenates, including: aconitase, a TPN[1] specific isocitric dehydrogenase, a DPN or TPN coupled α-ketoglutarate oxidase system, succinoxidase, fumarase, a DPN specific malic dehydrogenase, and cytochrome oxidase. The B.E.M. mutant, however, which could not form R.S. plants, even in the presence of bicarbonate, lacked both aconitase and α-ketoglutarate oxidase activity. The failure of B.E.M. to respond to bicarbonate was therefore interpreted as being due to the absence of those two enzymes intimately associated with the two successive decarboxylations presumed to be the sites of action.

To strengthen their theory, Cantino, et al., sought to connect, in a cause and effect manner, what had been discovered concerning the bicarbonate "trigger mechanism" with certain of the morphological manifestations of the R.S. plant. When thalli were grown on bicarbonate medium containing thiourea (an inhibitor of the copper-containing polyphenol oxidases), R.S. plants were formed which were devoid of melanin, but normal in all other respects (Cantino, 1953). This demonstrated that a process concomitant with development could be uncoupled from it without otherwise disrupting its normal progress. It was also established in vitro that a wall-bound polyphenol oxidase found in R.S.

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[1]Refer to Appendix I for the abbreviations used in this thesis.

and presumably involved in melanogenesis, could not be detected in O.C. plants, thus indicating its appearance, de novo, as a result of the bicarbonate induction (Cantino and Horenstein, 1955). This thiourea-sensitive, cyanide-insensitive system not only mediated electron transport between substrate (e.g., tyrosine and catechol) and oxygen, but also between substrate and TPN, but not DPN. Alpha-ketoglutarate alone, or that compound plus bicarbonate, always stimulated the reaction. It was suggested that the effect was due to coupling between the reductive carboxylation of α-ketoglutarate, yielding oxidized TPN, and a "quinone oxidase" reaction in the polyphenol oxidase system (i.e., catalyzing the oxidation of o-quinone to hydroxy-o-quinone) regenerating reduced TPN.

The report that R.S. plants contained an active isocitric dehydrogenase, but a feeble succinic dehydrogenase and no α-ketoglutarate oxidase or cytochrome oxidase activity, demonstrated that there had been, as proposed, important shifts in the citric acid cycle enzymes of the R.S. (Cantino and Horenstein, 1955). A bicarbonate-induced increase in the α-ketoglutarate content of O.C. plants was also consistent with the proposed action of that ion, i.e., inhibition of the keto-acid decarboxylation (Cantino, 1956).

The presence of γ-carotene in the B.E.M. mutant (Cantino and Hyatt, 1953b) and in the R.S. of the wild type (Cantino and Horenstein, 1956) provided an interesting positive correlation between the presence of the pigment and lesions in the Krebs cycle. Presumably, however, the synthesis of γ-carotene could not have resulted directly from the reversal

of the α-ketoglutarate decarboxylation since this enzyme was absent from B.E.M.

The important discovery of light stimulated growth and light-stimulated carbon dioxide fixation in Blastocladiella helped to further clarify the role of bicarbonate in R.S. formation (Cantino and Horenstein, 1956). Tracer studies with uniformly labeled glucose-C^{14} and bicarbonate-C^{14} were undertaken with preformed O.C. plants incubated in the light and in the dark. The most significant results were: (a) glucose-C^{14} gave rise to labeled glutamic and aspartic acids long before any detectable radioactivity appeared in the citric acid cycle compounds; (b) Carbon-14 labeled bicarbonate was rapidly incorporated in the Krebs cycle compounds and, in particular, light caused a marked increase in labeled succinate and a decrease in the labeled α-ketoglutarate, as compared to dark controls; and (c) a compound tentatively identified as oxalate acquired a significant quantity of C^{14}. In vitro experiments with R.S. homogenates exhibited a slight light inhibition of the isocitric dehydrogenase activity and, conversely, a stimulation by light of its reversal in the presence of α-ketoglutarate, bicarbonate, and reduced TPN. When the latter was carried out using bicarbonate-C^{14}, the fixed Carbon-14 was found in α-ketoglutarate, oxalate, isocitrate, and a slight amount in succinate. When the same extracts were incubated with succinate, bicarbonate-C^{14}, and reduced TPN, fixation of carbon-14 also occurred. It was presumed, but not established unequivocably, that the succinate was carboxylated to produce α-ketoglutarate. In the light, reduced DPN yielded only one-fifth the

fixation obtained with reduced TPN in this system, and in the dark no fixation occurred with the former nucleotide.

Two important conclusions were drawn from the above observations. First, the citric acid cycle seemed an unlikely path leading to the synthesis of glutamate and aspartate. Second, light in some way stimulated the reductive carboxylation of α-ketoglutarate and the subsequent cleavage of the isocitrate so formed to yield succinate and a C_2 compound such as glyoxalate. The succinate was also presumed to undergo a light stimulated reductive carboxylation, via an undetermined TPN dependent system, to produce α-ketoglutarate and complete the cycle. This theory has recently been strengthened by the isolation from Blastocladiella (R.S. and O.C.) of the enzyme, isocitritase, which cleaves isocitrate to produce equimolar quantities of succinate and glyoxalate (McCurdy, 1959). The demonstration in vivo that succinate and glyoxalate could substitute for the effect of light and bicarbonate served to further support the interpretation given above (Cantino and Horenstein, 1959). It was also established that the glyoxalate was rapidly converted to glycine by transamination with alanine, thereby providing a pulling reaction for the whole S.K.I. (succinate-ketoglutarate-isocitrate) cycle (McCurdy, 1959). A possible connection, via the use of glycine for thymine biosynthesis, has recently been proposed between the S.K.I. cycle and light stimulation of nucleic acid synthesis and nuclear reproduction in Blastocladiella (Turian and Cantino, 1960).

The significance of the preceding observations with respect to the formation of R.S. was as follows: (1) it helped to verify the scheme

proposed for R.S. induction by providing a pathway which could operate in the absence of most of the citric acid cycle enzymes; (2) it indicated additional points where intermediates could accumulate due to the bicarbonate trigger effect and, in so doing, influence other reaction sequences; and (3) it strongly suggested that light stimulated growth and carbon dioxide fixation, and R.S. induction, were actually two different expressions of the potentiality of a single enzyme system in response to different environmental conditions. The ability of light to reduce the concentration of bicarbonate necessary to induce the formation of R.S. was taken as a confirmation of this interpretation (Cantino, 1957).

The identification of chitin as the primary cell-wall constituent in Blastocladiella was established by glucosamine and acetate analyses following acid hydrolysis of purified wall material (Cantino, Lovett, and Horenstein, 1957). Homogenates of R.S. and O.C. plants contained comparable glucosamine-acetylating activity in systems containing acetate, coenzyme-A, and ATP (or acetylmethionine and ATP). In all cases the wall-bound activity with the non-phosphorylated glucosamine was low. In contrast to acetylation, the chitinase activity of O.C. supernatants with finely divided, purified, Blastocladiella chitin proved to be 3 to 7 times that of the R.S.

In addition to the change in chitin nitrogen, analyses of the total acid soluble and water soluble nitrogen fractions of both R.S. and O.C. plants had indicated a considerable intracellular redistribution of the nitrogenous constituents between the two plant forms (Cantino, Lovett,

and Horenstein, 1957). With the exception of these analyses, and a preliminary report concerning differences in the electrophoretically separable, soluble proteins in the two plant types, little work had been done on the nitrogen metabolism of Blastocladiella. However, it appeared almost certain that the changes which occurred during differentiation involved fundamental transformations in nitrogen metabolism. The research reported herein was initiated to determine, in so far as possible, if this were so. It seemed likely that an understanding of such changes would help considerably in the ultimate formulation of a coherent explanation of growth and differentiation in Blastocladiella at the cellular and organismal levels.

Glucosamine Synthetase and the Biosynthesis of Chitin

Although the presence of chitin in the cell walls of fungi, and the fact that it contained nitrogen, was established as early as 1811 by Braconnot, and by Lassaigne in 1843 (Tracey, 1955) respectively, its biosynthesis received little attention until recent years. Most of the work reported during the intervening period was concerned with its identification and isolation from various sources. Two factors are perhaps most responsible for the contemporary interest in the synthetic reactions of hexosamine compounds. The first is the relatively recent availability of the biochemical techniques for studying enzymatic reactions with microquantities of biochemical compounds. The second is the ubiquitous presence of hexosamines in the mucopolysaccharides which are being intensively studied in connection with the biochemistry of blood, and arthritic disorders.

Whatever the subsequent fate of the hexosamines, most of the biosynthetic sequences utilizing these compounds appear to require the synthesis of glucosamine phosphates as a first step. Harpur and Quastel (1949) demonstrated the phosphorylation of d-glucosamine (GA) by brain extracts in the presence of ATP. Although the products were not isolated, the Michaelis constants, and the competition observed between d-glucosamine, d-glucose, and d-fructose in mixtures suggested that all three compounds were phosphorylated by the same enxyme. N-acetylglucosamine (AG) was unaffected by the enzyme and acted as a competitive inhibitor for all three compounds. A similar synthesis was reported with Bakers-yeast preparations by Grant and Long (1952). These authors also found evidence for competition between GA and glucose for the same enzyme. The ability of hexokinase to phosphorylate GA was established by Brown (1951) utilizing a crystalline yeast enzyme. Analyses of the reaction product (isolated by barium fractionation), indicated that it was a monophosphate possessing reducing properties and a free amino group. The position of the phosphate ester at carbon-6 was ascertained by comparative sodium metaperiodate oxidations with GA and d-glucose-6-phosphate (G-6-P). The structure was established to be that of d-glucosamine-6-phosphate (GA-6-P).

Leloir and Cardini (1953) reported that partially purified preparations from Neurospora crassa catalyzed the synthesis of GA-6-P from fructose-6-phosphate (F-6-P), or G-6-P, and glutamine. The enzyme, which had been purified ca. 8-fold by acetone fractionation, had a specific activity of 0.3 μM GA/mg. protein/hr., and temperature and pH

optima of 30° C. and 6.4 to 6.8, respectively. Both the hexose phosphates and glutamine exhibited a stabilizing effect on the rather labile enzyme. No co-factor requirement could be demonstrated for the reaction, nor could any of a number of structurally related compounds substitute for either of the hexoses, or replace glutamine as the amino donor. Blumenthal, et al. (1955), with more highly purified preparations demonstrated that F-6-P was the primary substrate for the Neurospora enzyme (glutamine-F-6-P transamidase, c.f. Comb and Roseman, 1958). Crude extracts from a Penicillium species had the same requirements.

Pogell and Gryder (1956, 1957a,b) partially purified an enzyme (aminotransferase) from rat liver which utilized G-6-P and glutamine for GA-6-P synthesis. The approximately 2 to 3-fold purification attained did not eliminate enzyme activity with F-6-P as substrate, but merely reduced it as compared to that with G-6-P. The two hexose phosphates were equally active in the crude homogenates. The system had a pH optimum of 7.4 and, as with the Neurospora reaction, no co-factor requirement could be found. This enzyme also resembled the Neurospora one in that the very labile enzyme could be stabilized by the addition of hexose phosphates. Pogell and Gryder considered the greater stability obtained with G-6-P, as compared with F-6-P, an indication of its role as a more immediate precursor for the reaction. Neither the Neurospora, nor the rat liver enzyme was subjected to more than a partial purification and, therefore, no critical studies have been made to elucidate either the mechanism or the equilibrium of the

reaction. The paucity of definitive information concerning these GA-6-P synthesizing systems may well be a reflection of the instability of the enzyme proteins and the consequent difficulties inherent in their purification. The same problem arose in the purification of the Blastocladiella enzyme.

The conversion of GA to F-6-P and ammonia has been studied extensively in Escherichia coli (Soodak, 1955; Faulkner and Quastel, 1956; Wolfe, et al., 1956a,b,c, 1957, 1959; Roseman, 1956; Comb and Roseman, 1956, 1958), in Aerobactor cloacae (Imanaga, et al., 1957a,b), and in brain extracts (Faulkner and Quastel, 1956). It was established that a common pathway in the three organisms occurred through the phosphorylation of GA with ATP to produce GA-6-P, and subsequent deamination of that compound to yield F-6-P and ammonia. The ubiquitous distribution of hexokinase, and its demonstrated ability to phosphorylate GA make it legitimate to expect the presence of the aforementioned system in many organisms.

Leloir and Cardini (1956) reported that preparations from hog kidney catalyzed GA-6-P formation from F-6-P and ammonia. The reversible reaction required N-acetylglucosamine-6-phosphate (AG-6-P) as a co-factor and displayed optimal activity at pH 8.4. An equilibrium constant of ca. 0.12 to 0.18 was given for the reaction ($F\text{-}6\text{-}P + NH_3 \rightleftharpoons GA\text{-}6\text{-}P$), i.e., it had a strong tendency toward the production of F-6-P and ammonia. Comb and Roseman (1956) showed that the E. coli deaminase was also reversible in partially purified preparations. The same

workers (1958) made a comparative study with purified deaminases from hog kidney and E. coli.

The results indicated that the reaction mechanism was the same for both enzymes and that the AG-6-P stimulated, but was not required for, the reaction. The direct participation of AG-6-P in the conversion was ruled out by the use of isotopically labeled compounds. Although F-6-P and ammonia formation was greatly favored, the reversibility was confirmed for both deaminases. It was, however, demonstrated that a rapid synthesis of AG-6-P from F-6-P and ammonia could be obtained with both enzymes if the reaction was coupled with a purified GA-6-P acetylase (Davidson, Blumenthal, and Roseman, 1957) and acetyl-coenzyme-A.

In summary, GA-6-P has been shown to be synthesized by at least four different enzyme systems: (1) from GA and ATP in a kinase reaction; (2) by the glutamine-F-6-P transamidase system; (3) by the aminotransferase reaction with G-6-P and glutamine; and (4) by a reversal of the deaminase systems. A second name, glucosamine synthetase, suggested by Roseman (personal communication) for reaction (2) will be used in this paper for the sake of convenience.

Although the known biosynthetic pathways that ramify from GA-6-P are rapidly enlarging, only those pertaining to chitin synthesis will be summarized here. The acetylation of GA-6-P with acetyl-coenzyme-A to form AG-6-P has been demonstrated in Bakers-yeast (Brown, 1955), Neurospora (Leloir and Cardini, 1953; Davidson, Blumenthal, and Roseman, 1956,1957), Penicillium, Streptococcus, rabbit muscle, and human liver (Davidson, Blumenthal, and Roseman, 1957). The AG-6-P has been shown

to undergo a phosphoacetylglucosamine mutase reaction (AG-6-P $\rightleftharpoons$ AG-1-P) in Neurospora (Leloir and Cardini, 1953; Reissig, 1956), and in hog kidney (Leloir and Cardini, 1956). Either glucose-1,6-diphosphate (G-1,6-DP) or N-acetylglucosamine-1,6-diphosphate could serve as the co-factor for the conversion. Reissig partially separated the enzyme from the phosphoglucomutase present in Neurospora extracts and found a ratio of 86% AG-6-P:14% AG-1-P at equilibrium. He also observed the formation of AG-1,6-DP when the enzyme was incubated with AG-1-P and G-1,6-DP.

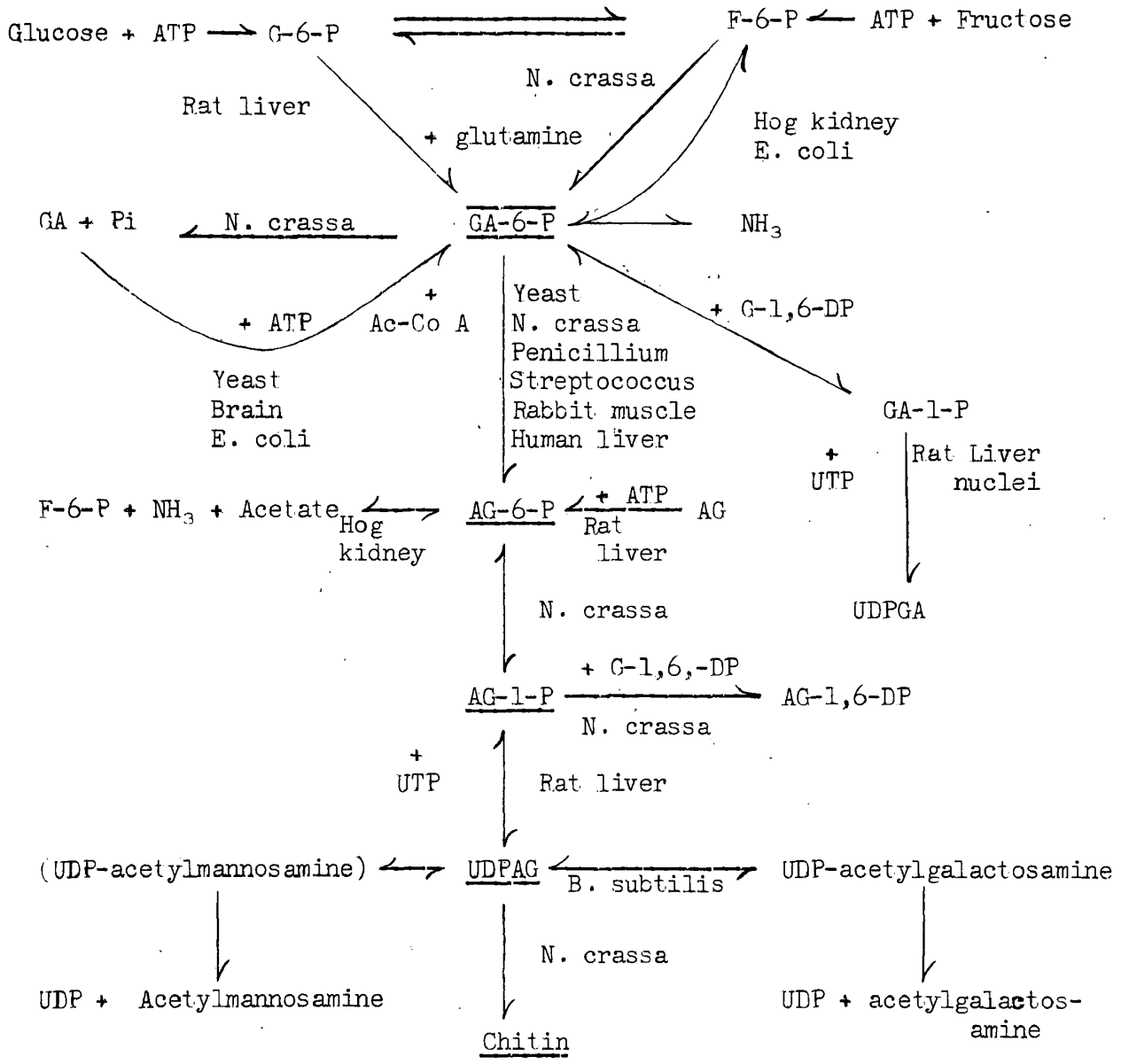
Maley and Lardy (1956) established the ability of rat liver preparations to form uridinediphosphate-N-acetylglucosamine (UDPAG) from AG-1-P and UTP. UDPAG was first isolated from Bakers-yeast by Cabib, Leloir, and Cardini, 1953) and has also been found in rat liver (Smith and Mills, 1953) and mung bean (Solms and Hassid, 1957). Of particular interest was the demonstration by Glaser and Brown (1957a,b) that a particulate fraction from Neurospora could incorporate the AG moiety of UDPAG into chitin-like compounds. The reaction was accelerated by the presence of high molecular weight chitodextrins.

Although the reactions described in the preceding paragraphs have been studied in a variety of organisms and tissues all but one have been found in the mycelia of Neurospora. Perhaps it can be assumed, therefore, that the whole biosynthetic sequence leading to chitin, as outlined, occurs in Neurospora and, by analogy, in other fungi producing chitinous polysaccharides.

Some of the hexosamine compounds and their interactions are indicated schematically in Figure 1 for the convenience of the reader.

Figure 1

Sugar-Phosphate and Hexosamine-Phosphate Interconversions



MATERIALS AND METHODS

Culturing and Harvesting Procedures

To obtain reproducible experimental results it was necessary to standardize, in so far as possible, the culturing and harvesting techniques used in this study. For this reason the procedures utilized have been described in considerable detail.

Stock Cultures.--All cultures of Blastocladiella were grown on a basic medium (PYG) containing: 1.25 gm. Difco yeast extract, 1.25 gm. Difco peptone, and 3.0 gm. glucose per liter of water (Cantino and Horenstein, 1955). To this basic medium either Brom-cresol purple (BCP), sodium bicarbonate plus BCP, or 2% Difco agar was added as described below.

Stock cultures were maintained as colonies of R.S. plants on Petri plates of PYG agar. Swarmer inocula were obtained by placing blocks of agar bearing R.S. in a small volume of sterile water (Barner and Cantino, 1952). After swarmer discharge (5 to 15 hours depending upon the history of the culture), plates or flasks were inoculated with the suspensions using standard bacteriological techniques.

Cultures for Zoospore Harvests and Large Scale Inocula.--Ten or 15 cm. diameter Petri dishes of PYG agar were inoculated with heavy swarmer suspensions and incubated at 20 or 24°C. for 20 or 16 hours, respectively; these conditions produced mature first generation plants ready to discharge. The plates were then flooded with approximately 10 ml. of

sterile water and allowed to stand at room temperature for 15 to 60 minutes for extensive spore discharge to occur. The resulting suspensions were then used to inoculate liquid cultures (see below) and stock plates, or they were harvested according to the method of McCurdy (1959) for analytical studies.

Standardization of Zoospore Inocula.--A standard curve for the concentration of swarmer suspensions was established by determining the absorption of a dilution series in a Klett-Summerson photoelectric colorimeter at 420 mμ, corrected for the absorption of the suspending medium, and by total viable counts on these same suspensions using PYG agar. The slope of the curve obtained was 2.14×10^{-5}, or the number of swarmers per milliliter = corrected absorption/2.14×10^{-5}. The numbers of viable swarmers used for inoculating flasks were routinely determined as described above.

Liquid Cultures.--For preparation and purification of enzymes, cultures of O.C. plants were grown in either 3- or 4-liter Erlenmeyer flasks containing 1.5 or 3.4 liters, respectively, of PYG medium and 10^{-5}% BCP. These flasks were equipped with an aeration tube, an inoculation tube, and a side-arm test-tube containing 1 N potassium hydroxide for neutralizing cultures during growth (Cantino and Horenstein, 1955). Initially the O.C. plants were grown with aeration for 3 to 4 days at room temperature, i.e., for several generations. At the time of harvest 5 ml. of the flocculent suspension of plants in each culture was used to inoculate a new flask. For the major portion of the enzyme

purification work, however, mature, healthy, first generation plants were obtained by inoculating 1.5 liter flasks with dense swarmer suspensions and aerating the cultures vigorously over lights (ca. 300 F.C.) at room temperature (24°C.) for 13 to 15 hours. By this method nearly synchronous, first generation cultures were obtained (McCurdy, 1959) since the total population was placed in the flask as swarmers, i.e., all at the same stage of development.

For producing R.S. plants flasks were set up in essentially the same manner as for O.C. cultures except that the alkali tube was removed, and sodium bicarbonate of different concentrations was added to the PYG broth. However, large scale synchronized cultures for studying R.S. ontogeny were grown in 12-liter, flat-bottom flasks containing 10 liters of PYG broth, indicator, and 8.9×10^{-3} M sodium bicarbonate before autoclaving. These flasks were also equipped with a glass siphon tube connected by Tygon tubing to a suction apparatus for aseptically removing samples of the culture at various time intervals.

All R.S. cultures except those intended for a 12 hour harvest, were inoculated with ca. 1.37×10^6 swarmers per liter of medium. This was the optimal population density for normal growth and adequate yield for the experimental procedures employed. Twelve hour cultures were inoculated with ca. 3.86×10^7 swarmers per liter of medium, to ensure a yield of sufficient plant material during this short growth period.

Resistant sporangial cultures were routinely grown with only normal laboratory illumination. The cultures for the early ontogeny experiments with individual 1.5-liter flasks were grown with aeration at room

temperature. The subsequent 1.5-liter R.S. cultures for the growth studies, and the large synchronized cultures were grown with aeration in a water bath at 22 to 24°C.

Harvesting Procedures.--All liquid cultures were harvested by vacuum filteration in a Büchner funnel using a porous filter paper. To ensure rapid filtration the contents of the flask were collected without being sucked down completely; the plant material was then freed of all media with numerous small washes of distilled water (total volume = 4 liters per 1.5 liters of medium harvested). After the final wash, the plant material was sucked down tightly and the suction maintained for 4 minutes to remove all excess water from the mat.

Dry Weight Determinations.--The wet mat, which separated easily from the filter disk, was weighed immediately after filtration and a portion of this material (0.5 gm. or more if available) dried to constant weight at 95°C.

Zoospore dry weights were obtained as follows: the density of a swarmer suspension (collected and washed as previously described) was determined by reading an aliquot in the Klett-Summerson colorimeter. The suspension containing a known number of spores was then transferred quantitatively to a previously tared bottle and dried to constant weight at 95°C.

Homogenization Procedure.--Plant material was regularly homogenized with a mixture of buffer, or water, and glass beads in a Servall Omnimixer operated at about 12- to 15,000 r.p.m. The temperature was

maintained between 0 and 4°C. by the use of an ice bath. The thin-walled O.C. plants required about 3 minutes for complete homogenization (i.e., until 95% or better of the plants were broken), while R.S. plants required from 3 to 7 minutes depending upon their stage of development.

The glass beads (200 μ in diameter) were washed before use by sequential treatment with 1 N sodium hydroxide, 1 N hydrochloric acid, and 10^{-1} M EDTA, pH 8.0, followed by thorough rinsing with glass distilled water.

Analytical Methods

General Chromatography.--Paper chromatography was carried out by the descending technique for both one- and two-dimensional work using Whatman # 1 filter paper. For two-dimensional resolution of amino acid mixtures, the extract was placed on a small spot, 7 cm. from each edge, in the corner of a 46 x 57 cm. sheet. The compounds were then chromatographed in the long direction with phenol-water (100:39.5 by wt.). The papers were dried in a hood overnight to remove the phenol, and then chromatographed in the second direction with butanol-propionic acid-water (46.9:22:31.2). After they were dry, the chromatograms were sprayed with 0.1% ninhydrin in 95% ethanol and heated for 5 minutes at 95°C. to develop the characteristic, purple colors obtained with this reagent. Mixtures containing hexose phosphates and inorganic phosphate were also chromatographed and detected by the method of Hanes and Isherwood (1949) as modified by Bandurski and Axelrod (1952).

The locations of known amino acids following two dimensional chromatography were established with authentic samples. In all cases alanine was used as a reference standard (see Appendix II for the two-dimensional amino acid map prepared by this procedure).

One dimensional chromatography was done with both washed and unwashed Whatman # 1 filter paper, as well as with acid-washed Whatman 4lH paper. Propanol-ammonium hydroxide-water (6:3:1), butanol-propionic acid-water, or ethanol-ammonium acetate (7.5 volumes 95% ethanol:3 volumes 1 M ammonium acetate, pH 7.5), were used as solvents, the latter for the separation of phosphate esters in particular (Leloir and Pallidini, 1952). The one-dimensional Rf values for several compounds with different solvents and papers are tabulated in Appendix III.

Whatman # 1 paper was washed as follows: Large sheets, serrated at the lower edge, were placed in pairs in a large chromatography cabinet. One hundred and fifty ml. of 2 N acetic acid, containing 0.02% sodium EDTA, was added to each trough and allowed to migrate until all of it had run off the sheets. The paper was then washed twice with glass-distilled water in the same manner, and finally dried in the hood.

Propanol, butanol, and propionic acid were all distilled before use. The phenol used for quantitative chromatography was also freshly distilled, while that used for qualitative chromatography was a Merck reagent grade of crystals, stored in the cold until used.

When it was necessary to retain the proper color intensities of amino acid spots on chromatograms for later observation or photography,

they were sprayed with 0.5 N nickel sulfate, which converted the purple to a red color without loss of the correct, relative intensities of shading (Khaba and El'kin, 1955). The colors thus obtained were stable for several months, if protected from bright light.

Quantitative Determination of Amino Acids by Chromatography.--In order to obtain quantitative estimations of amino acids in plant extracts, aliquots were chromatographed in the two-dimensional system described. Following chromatography, the individual amino acid spots were cut out and determined spectrophotometrically by the ninhydrin method of Landua and Awapara (1949, Awapara, 1959; see Cantino, Lovett, and Horenstein, 1957). Curves prepared by treating known quantities of pure amino acids in the same manner were used as reference standards.

For quantitative determination of amino acids with one-dimensional paper chromatography of simple mixtures, the method of Kay, et al. (1956) was followed with slight modification. Propanol-ammonia was substituted for the solvent used by the authors, and sodium hydroxide was deleted from the 0.5% ninhydrin spray for color development. Propanol-ammonia yielded excellent separation of the amino acids in enzyme reaction mixtures with uniformly low blank paper values. The sodium hydroxide was recommended by Kay, et al. because it enhanced the color formation with taurine, but because it was found to depress the color intensity with glucosamine its use was undesirable in these experiments.

For chromatography of enzyme reaction mixtures, trichloroacetic acid (TCA) was used as the deproteinizing agent at a final concentration

of 5%. The TCA was removed before chromatography by extraction with ethyl ether in a small all-glass continuous extractor. Electrolytic desalting with a Reco electric desalter improved the quality of the chromatography but caused serious decomposition of glutamine.

Extraction of Soluble Amino Acids.--Wet mats of plant material were placed in a vacuum desiccator over calcium chloride immediately after harvesting, and stored at 0 to 2°C. until thoroughly dry. The dried material (100 mg.) was ground to a fine paste with a mixture of powdered-glass, carborundum, and 80% ethanol (250 mg. grinding mixture + 3 ml. ethanol per 100 mg. plants). The material was extracted three times (Tot. Vol. 14 ml.) and the pooled extracts taken to dryness, either by removing most of the solvent on a steam bath and then drying *in vacuo* over calcium chloride, or entirely *in vacuo* without heat. In both cases the final drying was carried out at 0 to 4°C. The extracted amino acids were finally re-dissolved in 2.0 ml. of either 20% ethanol or distilled water, the extracts centrifuged if necessary to remove insoluble residue, and aliquots chromatographed two-dimensionally.

Protein Determination.--Proteins were estimated by the turbidometric method of Stadtman, *et al.* (1951), with slight modification. The protein sample was diluted to 3 ml. with 0.5 M potassium chloride, 3.0 ml. of 5% TCA were added with mixing, and the turbidity measured after one minute in a Klett-Summerson colorimeter with a 580 mμ filter. A linear plot was obtained in the range 0.1 to 1.5 mg. protein. Crystalline serum bovine albumin (Sigma Chem. Co.) was used as a protein standard.

Determination of Ammonium Ion and Total Nitrogen.--Free ammonium ion was determined by direct Nesslerization with a commercial preparation of the Folin and Wu (1919) Nessler reagent (Harleco "Dry-Pack"). The samples were Nesslerized, allowed to stand for 20 minutes, and measured in the Klett-Summerson colorimeter with a 420 mμ filter. Total nitrogen was estimated by wet digestion of organic materials with sulfuric acid and hydrogen peroxide over a micro-burner, until a clear solution was obtained (Umbreit, Burris, and Stauffer, 1957). The digested samples were then diluted, the excess sulfuric acid neutralized with sodium hydroxide, and the samples Nesslerized and measured in the same manner as for free ammonium ion.

Inorganic and Total Phosphorus.--Inorganic phosphorus was determined by the method of Fiske and Subbarow (1925). Total phosphorus was estimated by a modified method using perchloric acid (and nitric acid or hydrogen peroxide if necessary) to digest samples of organic material (Allen, 1940). Before color development with the Fiske and Subbarow reagents, the excess perchloric acid was neutralized with sodium hydroxide. The completeness of the digestion procedure was routinely checked by the use of 3-phosphoglyceric acid as a standard.

Estimation of Hexose-phosphates.--Fructose-6-phosphate or mixtures of F-6-P and G-6-P were estimated by the anthrone method of Mokrasch (1954).

The commercial preparation of glucose-6-phosphate dehydrogenase (Sigma Chem. Co.) used to determine G-6-P in the presence of F-6-P

(Horecker and Wood, 1957), contained phosphohexoisomerase activity. However, the difference in the initial rates was used to establish the presence of the isomerase in homogenates containing F-6-P or G-6-P.

Glucosamine Determination.--Glucosamine and GA-6-P were originally determined by the Immers and Vasseur (1952) modification of the Elson-Morgan reaction. However, this method did not produce equivalent color values for GA-6-P as compared to GA and was, in addition, susceptible to interference by mixtures of amino acids and sugars (Horowitz, Ikawa, and Fling, 1950). The Dische and Borenfreund (1950) method for GA, involving deamination with nitrous acid and color formation with an indole-hydrochloric acid mixture, yielded equivalent color formation with both hexosamines. The sugars and amino acids in the reaction mixtures analyzed, produced only a negligible amount of color even when present in large excess, and this error was eliminated by running non-deaminated controls (hexosamines do not produce color without prior deamination), and by determining the optical density at two different wave lengths. For this reason it was possible to perform the Dische reaction directly on reaction mixtures, and it was used routinely for all the later determinations.

In order to determine the amounts of GA-6-P and GA in mixtures containing both, a micro method was devised utilizing paper chromatography to separate the two compounds and a colorimetric determination with the Dische reaction. Mixtures were chromatographed one-dimensionally on washed Whatman # 1 paper with propanol-ammonium hydroxide-water. The location of the hexosamine spots was established by spraying parallel,

duplicate strips with ninhydrin. The same areas were removed from the experimental strips (including paper blanks), cut into small pieces, and then placed in 12 ml. centrifuge tubes. The Dische reaction (using one-half the normal quantity of reagents) was then carried out in these tubes, the paper packed in the bottom of each tube, and finally the solutions poured into 5 ml. centrifuge tubes (tightly capped with parafilm to prevent evaporation) and centrifuged for 3 minutes at 1600 x g. to remove the filter paper fibers. The colored solution obtained (ca. 2.5 ml.) was then read in the Beckman Model DU spectrophotometer with appropriate standards. Linear standard curves were obtained for both hexosamines in the range from 0.01 to 0.05 μM.

Chitin Analyses.--The chitin content of material dried to constant weight at 95° C. was analyzed by a modification of the methods of Hackman (1954) and Tracey (1955). The samples were powdered in a mortar and approximately 20 mg. transferred to a 10 x 120 mm. Pyrex tube. One ml. of 1 N sodium hydroxide was added to each tube, a small glass bulb-condenser placed in the mouth to prevent excessive evaporation, and the contents digested in a steam bath for 9 to 10 hours. After digestion, the tubes were centrifuged and the soluble material discarded. The alkali insoluble residue was washed twice with water, once with 95% ethanol, and once with ether, in that order. After the residual ether had been removed in vacuo, 2 ml. of 6 N hydrochloric acid were added to each residue and the necks of the tubes sealed off. The samples were then digested for another 30 hours in the steam bath to hydrolyze the chitin. The tubes were cooled, opened, and placed in

a vacuum desiccator over potassium hydroxide pellets and calcium chloride to remove as much of the hydrochloric acid as possible. The contents were finally transferred to a volumetric flask, and aliquots analyzed by the Dische method for their hexosamine content. The µg chitin were calculated by multiplying the µM GA by the molecular weight of N-acetylglucosamine.

Melanin Estimation.--The melanin content of plants at various ages was estimated by digesting 10 mg. samples of dried plant material with 1 ml. of 0.5 N sodium hydroxide. The optical density of the slightly turbid solutions obtained (diluted 1:6) was measured at 5 points between 400 and 600 mµ in the spectrophotometer, as recommended by Schaeffer (1953). These data, when plotted as the logarithm of the optical density against the wave length, yielded the straight line slopes characteristic of the melanoid pigments.

Total Lipids.--The total lipid content of fresh plant material was determined by the method of Folch, Lees, and Stanley (1957), for animal tissues. To check on the efficiency of the chloroform-methanol extraction with fungal material a quantity of fresh Blastocladiella plant material (somewhat larger than was used in the experiments to be described) was extracted by the method of Folch, et al. The extracted residue was then refluxed for 20 hours with a fresh portion of the chloroform-methanol solvent mixture, and the quantity of lipid in this second extract determined. When this was done it was found that only 3.2% of the total extractable lipid remained after the initial extraction,

and the method was therefore considered applicable to the analyses of Blastocladiella material. No attempt was made to correct for the small quantity of γ-carotene which was extracted along with the lipids.

Preparative Procedures

Dowex-50 Resin Columns.--Dowex-50 resin (x8, 100 - 200 mesh; Bio Rad Laboratories; Reagent grade) was regularly regenerated before use with two complete cycles of acid and alkali, with thorough distilled water washes between each treatment. To obtain the resin in the hydrogen form, the last treatment was with 2 N hydrochloric acid; when the resin was required in the potassium form, the last treatment was with 2 N potassium hydroxide. Following regeneration the resin was washed exhaustively with glass distilled water before use.

Fructose-6-phosphate.--A fairly crude sample from Schwarz laboratories was purified by two precipitations as the alcohol insoluble, barium salt at pH 8.0. The remaining yellow color was removed by treating with Norite at 80°C., and the colorless material converted to the sodium or potassium salt by precipitating the barium with sodium sulfate, or by running the solution through a Dowex-50 (K^+) column, respectively.

A barium salt from the Sigma Chemical Co. gave clear, colorless solutions, and was converted to the potassium salt by the column procedure. This material assayed as 80.6% F-6-P by the anthrone method.

Due to the fact that all samples of F-6-P on the market also contained G-6-P, a sample of pure F-6-P was prepared from commercially

available fructose-1,6-diphosphate (FDP). The FDP (Schwarz laboratories, Inc.) was recrystallized 4 times as the cyclohexylammonium salt by the method of McGilvery (1953). After recrystallization, the salt was converted to the free acid by passage through a Dowex-50 (H^+) column. A sample, chromatographed in propanol-ammonium hydroxide-water, was found to be free of hexose-monophosphate esters and inorganic phosphate. The purified FDP was then hydrolyzed with 1 N hydrobromic acid, and the barium F-6-P salt isolated by the method of Neuberg, Lustig, and Rothenberg (1943). It contained 90.1% F-6-P and 0.15% inorganic phosphate. The barium salt was converted to the potassium derivative by the column method described above.

N-acetylglucosamine.--This compound was prepared from d-glucosamine hydrochloride (California Foundation for Biochemical Research) by the method of Roseman and Ludowieg (1954).

D-glucosamine-6-phosphate.--A few milligrams of GA-6-P were synthesized by the chemical method of Anderson and Percival (1956). Insufficient material was isolated to allow chemical characterization, but the product did have the same chromatographic properties as an authentic sample (prepared by the polyphosphoric acid method) which was very kindly provided by Dr. Saul Roseman (Distler, Merrick, and Roseman, 1958). With both the Hanes and Isherwood spray, and with ninhydrin, the synthetic compound gave a single spot with the same R_f as Roseman's compound.

Miscellaneous Preparations.--Tricalcium-phosphate gel was prepared according to the procedure of Keilin and Hartree (1938). The product contained 17 mg. dry weight of gel per ml. of suspension.

Solutions of protamine sulfate (Eli Lilly) were prepared containing 20 mg. per ml., and the pH was adjusted to 5.8 with 1 N Acetic acid.

3-Phosphoglyceric acid (Schwarz Lab. Inc.) was recrystallized two times by the procedure of Neuberg and Lustig (1942). Theoretical recoveries for total organic phosphate were obtained with the product. 1,2,4-amino-naphthol-sulfonic acid (Eastman Kodak) for the Fiske and Subbarow reaction was recrystallized by the method of the authors (1925). The ammonium sulfamate (Eastman Kodak, practical) for the Dische reaction was recrystallized from ethanol-water mixtures. Paradimethylaminobenzaldehyde was recrystallized by the procedure given by Tracey (1955).

Sources of Chemicals and Biochemicals

All of the inorganic chemicals used in the work reported here were of reagent or comparable grade.

The indole, anthrone, and acetylacetone used were products of Eastman Kodak. The barium- and dipotassium-salts of G-6-P were obtained from the Sigma Chemical Co. Ninhydrin was procured from either the Sigma Chemical Company or the Nutritional Biochemical Corp. The l-glutamine was a product of the California Foundation for Biochemical Research, and the l-glutamic acid of the Pfanstiehl Chemical Corp. All other amino acids were obtained from Nutritional Biochemicals Corp.

The glass beads used for homogenization in the Omni-mixer were obtained from the Minnesota Mining and Manufacturing Co.

EXPERIMENTAL

The Free Amino Acid Pools in Blastocladiella

As a preliminary approach to the problem of nitrogen metabolism and its relationship to differentiation in Blastocladiella it appeared that a study of the soluble amino acid pools might well provide some indication of the most profitable areas to investigate. Any obvious differences in these compounds between the two morphological forms, either qualitative or quantitative, should result from alterations in certain biosynthetic systems. If this were so, changes observed in specific pools would help to pin point those metabolic pathways which differ fundamentally between the alternative plant types.

To check the above, mature R.S. and O.C. plants derived from cultures grown for a period of several generations, were harvested, dried, and their free amino acids extracted. When these extracts were chromatographed two-dimensionally, striking differences were observed, both quantitative and qualitative (Fig. 2). It was obvious even from visual inspection of the chromatograms that the quantities of almost all the free amino acid pools in the R.S. plants were greatly decreased in comparison with the O.C. plants. This was not true, however, for glutamic and aspartic acids, and to a lesser extent for two or three of the unknown compounds falling in the same general area of the chromatograms. These all appeared to remain at the same level in both plant extracts. In addition to the quantitative changes, a new compound

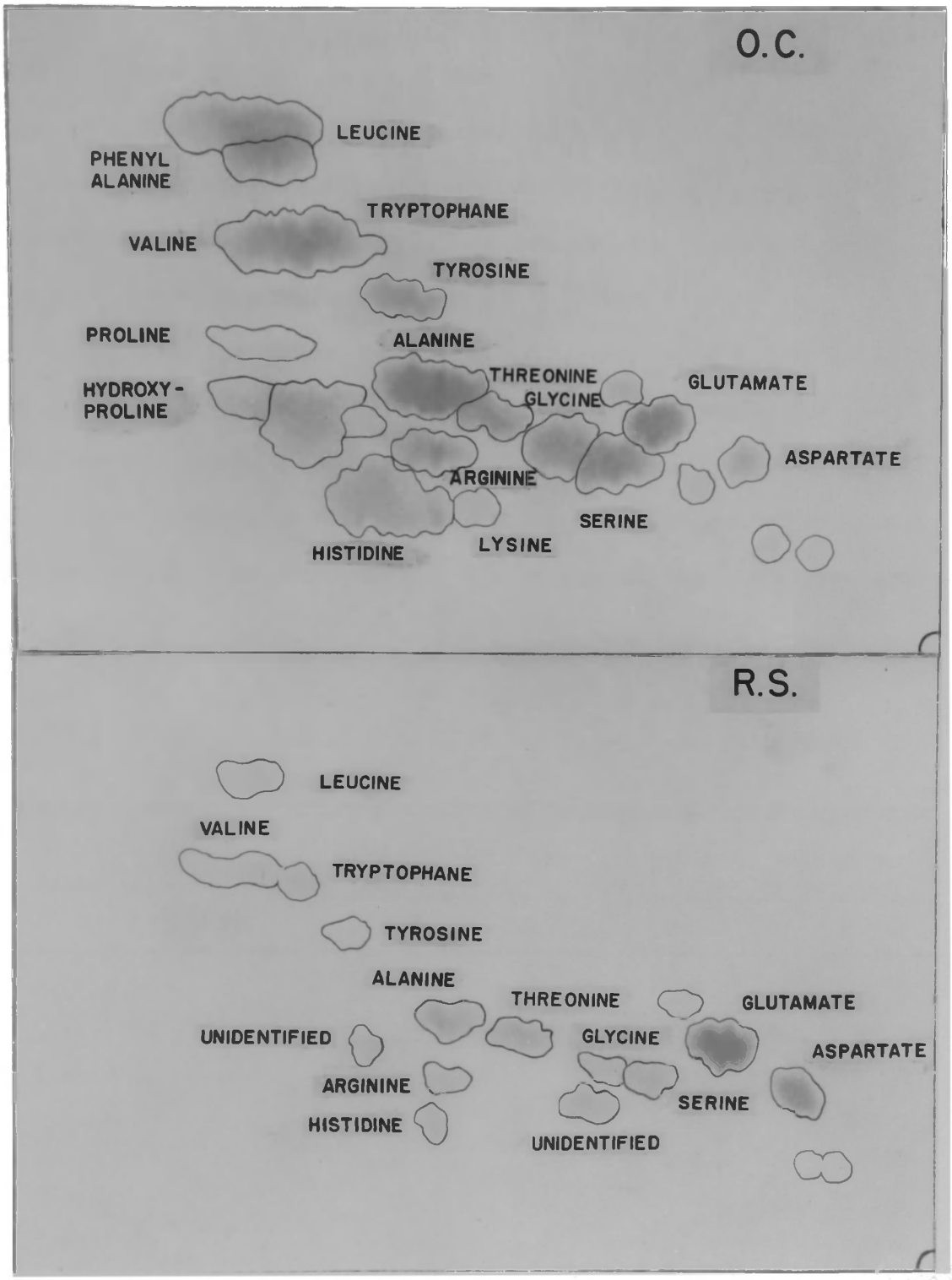


Figure 2

The Soluble Amino Acids of O.C. and R.S. Plants

An extract of 10 mg. dry weight of plant material chromatographed in phenol-water (horizontal) and butanol-propionic acid-water (vertical). The amino acids were detected with ninhydrin.

appeared in R.S. extracts below glycine (marked on the chromatogram as unidentified) which has been tentatively identified as asparagine. The unknown compound in the center of the triangle formed by serine, glutamate, and aspartate in the O.C. chromatogram, on the other hand, could not be detected in material from R.S. plants.

To serve as a check on the visual estimates, quantitative determinations were undertaken. Duplicate sets of chromatograms for each plant form were prepared and the glutamic acid, aspartic acid, and a few other well separated amino acids estimated by the method of Landua and Awapara (1949; Awapara, 1949). The results of these analyses are given in Table I.

TABLE I

ANALYSIS OF THE SOLUBLE AMINO ACID POOLS IN O.C. AND R.S. PLANTS OF BLASTOCLADIELLA

Amino Acid	μM Amino Acid per Gram Dry Weight		μg Amino Acid-N per Gram Dry Weight	
	O.C.	R.S.	O.C.	R.S.
Glutamate	29.2	30.2	408.8	422.8
Aspartate	11.8	13.2	165.2	184.8
Alanine	52.4	5.1	733.6	71.6
Lysine	16.4	8.8	459.2	246.4
Arginine	14.4	7.6	806.4	425.6
Threonine	15.6	9.6	218.4	134.4
Tyrosine	14.4	ca.0	201.6	ca.0
Total			2993.2	1485.6

These data corroborated the estimates for glutamic and aspartic acids. In fact, the concentrations of both compounds increased slightly in the R. S. material. At the same time the total amino acid nitrogen (as represented by the fraction analyzed) decreased by 50% in the R.S. When the change was calculated for all amino acids other than glutamic and aspartic, the drop amounted to 64%, a rather drastic change. Alanine and tyrosine showed the most severe decreases, but it was obvious from visual inspection that other amino acids (which could not be measured because of their overlapping positions) underwent changes of a similar magnitude.

The retention of the glutamic acid pool in the R.S. plants did suggest that it might indeed be worth investigating further. This seemed particularly pertinent since glutamate might have served as the link (e.g., by transamination, or the glutamic dehydrogenase system) between the putative locus of the bicarbonate effect, α-ketoglutarate, and the synthetic pathway for chitin synthesis. The position of glutamate in the latter will be made evident in the following sections.

Glucosamine Synthetase in Plant Extracts

The step in the biosynthesis of chitin in closest proximity to glutamic acid is that catalyzed by the enzyme glucosamine synthetase: Hexose-6-phosphate + l-glutamine ⟶ Glucosamine-6-phosphate + l-Glutamate. Therefore, this enzyme was assayed in R.S. and O.C. plants to see if there was any correlation between its activity and the level of the free glutamic acid pool. It was first necessary to establish that the reaction occurred with glutamine as a substrate, rather than

F-6-P and ammonia. For this purpose, a multiple-generation culture of O.C. plants was used since such material was much simpler to grow and homogenize than R.S. plants. Two grams wet weight of a 7 day old culture were ground in a glass homogenizer with 10 ml. 6.7×10^{-2} M. phosphate buffer, pH 6.8. Five-tenths ml. of the whole homogenate, 30 µM glutamine, 30 µM G-6-P, and 34 µM phosphate buffer, pH 6.8, in a total volume of 3.0 ml., were incubated at 32°C. for zero, one-half, and 2 hours. The following controls were incubated for the same time periods: complete system (a) with boiled homogenate, (b) minus homogenate, (c) minus glutamine, and (d) minus G-6-P. The reaction mixtures were inactivated and deproteinized at the indicated times with 0.5 ml. of 35% TCA.

The complete reaction mixtures contained a compound with low mobility (R_f 0.1 in butanol-propionic acid-water) which reacted with both the ninhydrin and Hanes and Isherwood sprays. This spot, presumably GA-6-P, showed a proportional increase with the time of incubation and was absent from all the control mixtures. In addition, incubation of the whole homogenate plus substrates, in the absence of phosphate buffer led to the production of a considerable quantity of free GA. This appeared to be due to phosphatase action upon the GA-6-P.

These results indicated that the synthetase enzyme (a) was present in Blastocladiella and (b) utilized glutamine as the source of amino groups in the synthesis of GA-6-P. To establish the location of the enzyme in the extract, the activity in whole homogenates was compared with that retained in supernatants after centrifugation at 500- and

22,000 x g. at 0 to 4° C. All these fractions appeared to have fully comparable activity as estimated by the size of the low mobility (R_f 0.16, propanol-ammonia) ninhydrin positive compound. This seemed to establish the "solubility" of the enzyme, and all further experiments were undertaken with the 22,000 x g. supernatants.

In the same experiment it was found that F-6-P had an activity equal to that of G-6-P in the synthetase reaction. Fructose-6-phosphate was therefore used in subsequent experiments because it was readily available, less expensive, and according to Blumenthal, et al. (1955), the preferred substrate for the reaction.

The Product of Glucosamine Synthetase Activity.--To establish with reasonable certainty that the reaction product was in fact GA-6-P, a partial purification was undertaken (Fig. 3). The barium precipitations were those conventionally used (Umbreit, Burris, and Staufer, 1957) to separate mixtures of phosphate esters. Fructose-6-phosphate and GA-6-P were the only compounds expected in the barium soluble alcohol insoluble fraction (BSAI), but when this material was redissolved and chromatographed both glutamine and glutamic acid were still present as contaminants. The result was somewhat surprising since Leloir and Cardini (1953) had used the procedure to separate G-6-P and GA-6-P from identical mixtures and did not report the presence of amino acids. On recourse to the literature it was found (Foreman, 1914) that glutamic acid does precipitate as an alcohol-insoluble barium or calcium salt.

To obviate the precipitation problem with amino acids the material, already twice precipitated as the BSAI salts, was run into a Dowex-50

Figure 3

GLUCOSAMINE-6-PHOSPHATE PURIFICATION PROCEDURE

REACTION MIXTURE CONTAINING TCA (20.5 ml.)

↓ Centrifuged → Protein discarded

SUPERNATANT - I

Extracted with 4 volumes ethyl ether to remove TCA.
The pH adjusted to 8.2 with KOH and phenolphthalein.
1.4 ml. 25% barium acetate added with mixing.

↓ Centrifuged → Precipitate discarded

BARIUM SOLUBLE SUPERNATANT

4 volumes of 95% ethanol added, placed at -18°C. for 2.5 hr.

↓ Centrifuged → Supernatant discarded

BARIUM-SOLUBLE-ALCOHOL-INSOLUBLE PRECIPITATE (BSAI-1)

Washed 3 times with 3 ml. 95% ethanol.
Redissolved in 2.5 ml. 10^{-1}N HCl, 2 X 10^{-2}N H_2SO_4 added to precipitate $BaSO_4$.

↓ Centrifuged → $BaSO_4$ discarded

SUPERNATANT - II

Alcohol precipitation as the BSAI salt repeated.

BSAI - 2

Redissolved in a minimal volume of dilute HCl and the barium removed with Na_2SO_4, and centrifugation.

↓

Solution run into a Dowex-50 (H^+) column (0.8 X 25 cm.) and a series of 10 ml fractions eluted with water.

↓

Fraction	Content[1]
1	F-6-P, inorganic phosphate
2	Negative
3 - 8	GA-6-P
9 - 20	Negative

[1]These results were obtained by chromatography and detection with ninhydrin and Hanes and Isherwood sprays.

(H^+) column and a series of fractions eluted with water (Wolf, Morita, and Nakada, 1956). The results are listed at the bottom of Fig. 3, and show good separation of the GA-6-P from the other compounds present in the reaction mixtures.

It was subsequently found that reaction mixtures which had been deproteinized with TCA, could be directly separated by the same column procedure without ether extraction to remove the TCA, with equally satisfactory separations. The amino acids which remained on the column were recovered when desired, by elution with 2×10^{-1} N ammonium hydroxide.

The GA-6-P fractions from the column were pooled and concentrated to a small volume in a flash evaporator at 30 to 35°C. When this material was co-chromatographed with an authentic sample prepared by Dr. Saul Roseman, the two compounds had identical mobilities (as determined by both ninhydrin and Hanes and Isherwood sprays) in three different solvents: butanol-propionic acid-water, propanol-ammonium hydroxide-water, and ethanol-ammonium acetate (c.f. Appendix III for R_f values).

In an attempt to isolate and further purify the enzymatically-generated GA-6-P, the pooled sample was treated with barium, the BSAI salt isolated, dissolved in 10^{-1} N hydrochloric acid, and freed of barium by the addition of sodium sulfate. This material was analyzed for its organic nitrogen and phosphorus content, along with the sample provided by Dr. Roseman. It was found to contain a phosphorus/nitrogen ratio of 1/1.2, as compared to 1/0.9 for Roseman's material. When the same

GA-6-P (that had been re-precipitated at pH 8.2) was again chromatographed, a second spot with an R_f of 0.26 (propanol-ammonia) was found which had been absent from the original column eluates. This material reduced alkaline potassium permanganate and contained organic phosphorus, but did not react with ninhydrin. It was concluded that this was a decomposition product from GA-6-P produced during the alkaline precipitation since it could be removed with Dowex-50 (H^+). The impurity, which was not F-6-P, appeared in the first fraction from the column. Its spectrum had a peak at 273 mμ, which agreed with Brown's report (1951) of an increase in absorption at that wave length when GA-6-P solutions were stored at pH 8.0. The decomposition did not occur when GA-6-P was stored in the refrigerator at low pH. It is presumed that this decomposition was the cause of the slightly high nitrogen values obtained in the analyses, since the ratio should theoretically have been one.

The spectrum of the colored product obtained in the Dische reaction with Blastocladiella GA-6-P was identical to that of GA. For both compounds the absorption decreased precipitously on either side of a sharp maximum at 492 mμ.

In conclusion, it was decided that the product of our enzyme reaction was GA-6-P.

Purification of Glucosamine Synthetase

The experiments described in the preceding paragraphs established the presence of glucosamine synthetase in cell free preparations of Blastocladiella, and the substrate and products of the reaction.

However, before beginning a study of the role of the enzyme during development it was essential to establish the optimal conditions for its activity. To do this a partial purification of the enzyme was attempted.

Purification Procedures.--A variety of conventional methods for enzyme purification were tried. However, the great majority of these resulted not only in a lack of purification but actual inactivation of the apparently very labile enzyme. Some of the more pertinent treatments and their effect upon synthetase activity are listed in Appendix IV.

The enzyme lost 10 to 20% of its activity on standing at 0° C. for several hours, and from 56 to 80% on 12 hours dialysis; both effects were accelerated by the presence of 10^{-3} M Versene. The addition of concentrated dialysate, or magnesium plus pyridoxal phosphate failed to restore the activity lost upon dialysis. Both glutathione (10^{-4} M) and F-6-P (9×10^{-4} M) had some stabilizing effect, but not enough to prevent significant losses in activity on standing, or upon dialysis. The activity was heat sensitive and, to a lesser extent, pH sensitive. All attempts to isolate the enzyme by ammonium sulfate fractionation met with failure. Although the greatest activity was always found in the fraction collected between 30 and 45% saturation with ammonium sulfate, the specific activity and/or total yield were in every case reduced by the treatment. If, in addition, the redissolved ammonium sulfate fraction was dialized against phosphate buffer (10^{-1} M, pH 7.0), a further loss of 75% in activity occurred.

The most satisfactory and reproducible purification was obtained by treating high speed supernatants, containing no phosphate buffer, with a protamine sulfate solution adjusted to pH 5.8 (0.17 mg. protamine sulfate/mg. protein). Under these conditions the glucosamine synthetase activity remained in the supernatant, and purifications ranging from 3.2 to 6.4-fold with nearly 100% recovery were obtained (c.f. Appendix IV).

The enzyme was effectively adsorbed from the protamine supernatant on tricalcium phosphate gel at pH 5.6. Attempts to elute the enzyme with a series of phosphate buffers of increasing molarity, or increasing pH, or both, failed to give adequate recoveries in any one fraction. However, in such series 10^{-1} M phosphate buffer at pH 7.0 always gave the best results, and single extractions of this sort did result in some purification.

Heppel and Hilmoe (1951) reported that the enzyme, inorganic pyrophosphatase, could be efficiently eluted from gel preparations by its own substrate, inorganic pyrophosphate. Therefore, a similar approach was tried with the synthetase enzyme from Blastocladiella using 5×10^{-3} M F-6-P as the eluting agent. The results were encouraging, and led to the trial of higher concentrations of both the commercial and synthetic F-6-P, as well as G-6-P. The results are shown in Table II.

It was quite evident from these data that the commerical F-6-P was superior to either the synthetic F-6-P or the G-6-P as an eluting agent. The reduced efficiency of the synthetic F-6-P elution remains

TABLE II

GEL ELUTION WITH HEXOSE-PHOSPHATES

Eluting Ester	Specific Activity[a] With Protamine Supernatant	Specific Activity With Gel Eluate	Percentage Increase in Specific Activity
G-6-P	8.59	16.9	97
F-6-P (synth.)	9.41	12.6	34
F-6-P (Sigma)	9.66	20.5	112

Conditions: Equal quantities of the gel (prepared by adsorption from the protamine supernatant) were eluted with 3 ml. (10^{-2} M) of the indicated ester. The eluates were assayed with 10 µM of the same hexose-phosphate, 20 µM glutamine, and 40 µM phosphate buffer, pH 6.5, in a final volume of 2.1 ml., for 1 hour ; T = 30.6°C. The reaction was stopped with 7% Na_2WO_4 (0.01 ml.) and 1 N HCl (0.06 ml.), and the GA-6-P determined by the Dische method. Specific Activity: µM GA-6-P/mg. protein/hr.

[a]Refers to the specific activity with the same ester as used for elution.

a puzzle since it had been as effective as the commercial F-6-P (and superior to G-6-P) in the glucosamine synthetase reaction with the protamine supernatant.

The pH Optimum of Glucosamine Synthetase.--An early determination of the pH optimum with F-6-P as the substrate had indicated a broad range of almost equal activity from pH 5.5 to 7.0. However, in the light of the activity demonstrated by G-6-P, the pH response for both compounds was examined at closer intervals (from pH 5.0 to 8.5) using the high speed supernatants. The pH optimum (Fig. 5) occurred at pH 6.5 to 6.7. An interpretation of the differences in the shape of the

curves, and their inverse relationship at higher pH's is found in the discussion. Due to the presence of F-6-P in the enzyme preparation purified with this ester, the pH response could only be determined using it as substrate. The results were essentially the same as those shown in Figure 5.

Because of the extreme lability of the enzyme, further attempts at purification were abandoned. The procedure which yielded the maximal final purification is shown in Figure 4 and the data obtained in Table III.

Time Course of the Glucosamine Synthetase Reaction.--Utilizing the enzyme purified in the above manner and both F-6-P and G-6-P as substrates, the relationship between GA-6-P formation and incubation time was established (Fig. 6). The lag obtained with G-6-P and its absence with F-6-P, was of importance in establishing which of the two served as the primary substrate for the reaction (see later discussion). The linearity of the response curve with F-6-P up to 30 minutes indicated that any time during this interval would be satisfactory for assaying the enzyme in plant homogenates.

Glucosamine Production vs. Enzyme Concentration.--The quantity of GA-6-P produced from F-6-P by the purified enzyme was a linear function of the protein concentration from 0 up to approximately 0.8 mg. of protein per tube (Fig. 7).

Substrate Specificity.--A series of controls set up to establish (a) the specificity of the partially purified enzyme, and (b) the absence of any non-enzymatic activity, are tabulated in Table IV.

Figure 4. Glucoasamine Synthetase Purification Procedure.

The results obtained by this procedure are given in Table III. The temperature was maintained between 0 and 4° C. throughout the purification. The gel suspensions were stirred intermittently by hand because mechanical stirring consistently reduced the recoveries obtained. All centrifugations but the first were for 5 minutes at 14,500 x g.

Figure 5. The Relation Between Glucosamine Synthetase Activity and pH.

Two-tenths ml. of a 22,000 x g. supernatant (6.3 mg. protein per ml.) were incubated with 20 µM F-6-P (or G-6-P), 20 µM glutamine, and 100 µM phosphate buffer, in a final volume of 2.0 ml., for 20 min. at 30.6° C. The reaction was stopped by the addition of 0.01 ml. 7% Na_2WO_4 and 0.08 ml. 1 N HCl. The GA-6-P was determined by the Dische method, and all values corrected for their unincubated controls.

Figure 6. Time Course of the Glucosamine Synthetase Reaction.

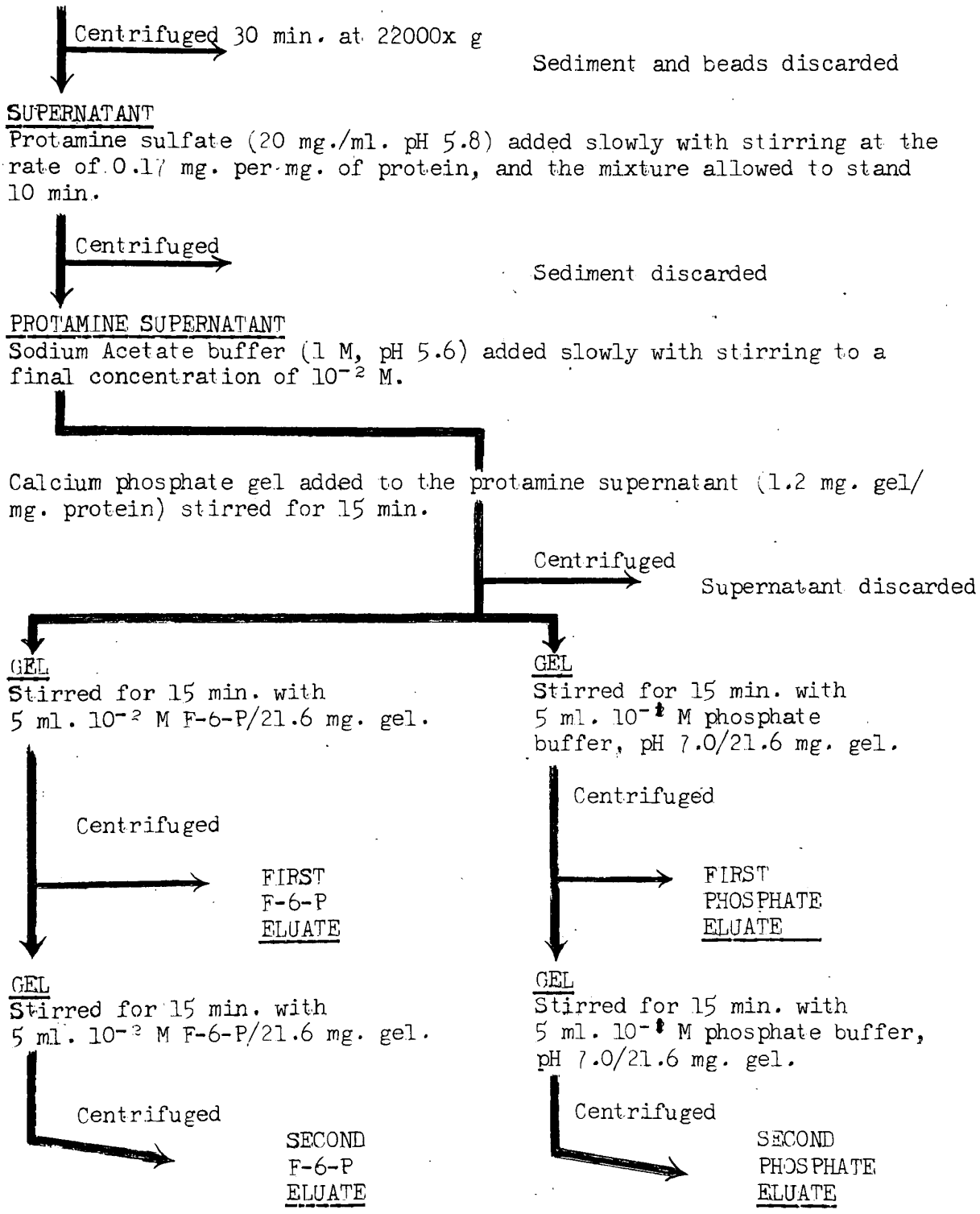
The enzyme (0.4 mg.) eluted from tricalcium phosphate gel with 10^{-1} M, pH 7.0, phosphate buffer was incubated and assayed under the conditions given in Fig. 5, except that the pH was 6.5 and the time of incubation varied.

Figure 7. The Effect of Glucosamine Synthetase Concentration on the Reaction Rate.

The enzyme eluted from tricalcium phosphate gel with 10^{-1} M, pH 7.0, phosphate buffer was incubated with 20 µM F-6-P, 20 µM glutamine, 100 µM phosphate buffer, pH 7.0, in a final vol. of 2.0 ml., for 20 min. at 30.6° C. See Fig. 5 for other details.

Figure 4

GLUCOSAMINE SYNTHETASE PURIFICATION PROCEDURE



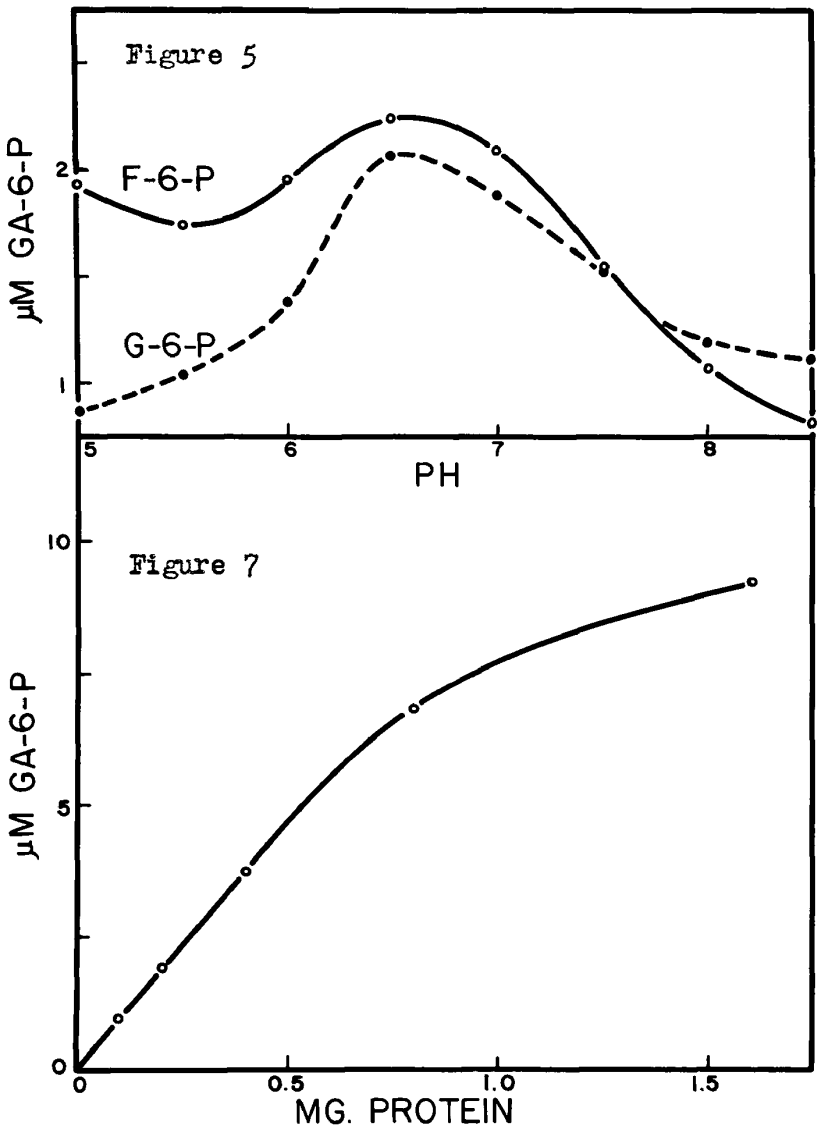


Figure 5
μM GA-6-P
F-6-P
G-6-P
2
1
5
6
7
8
PH

Figure 7
μM GA-6-P
10
5
0
0
0.5
1.0
1.5
MG. PROTEIN

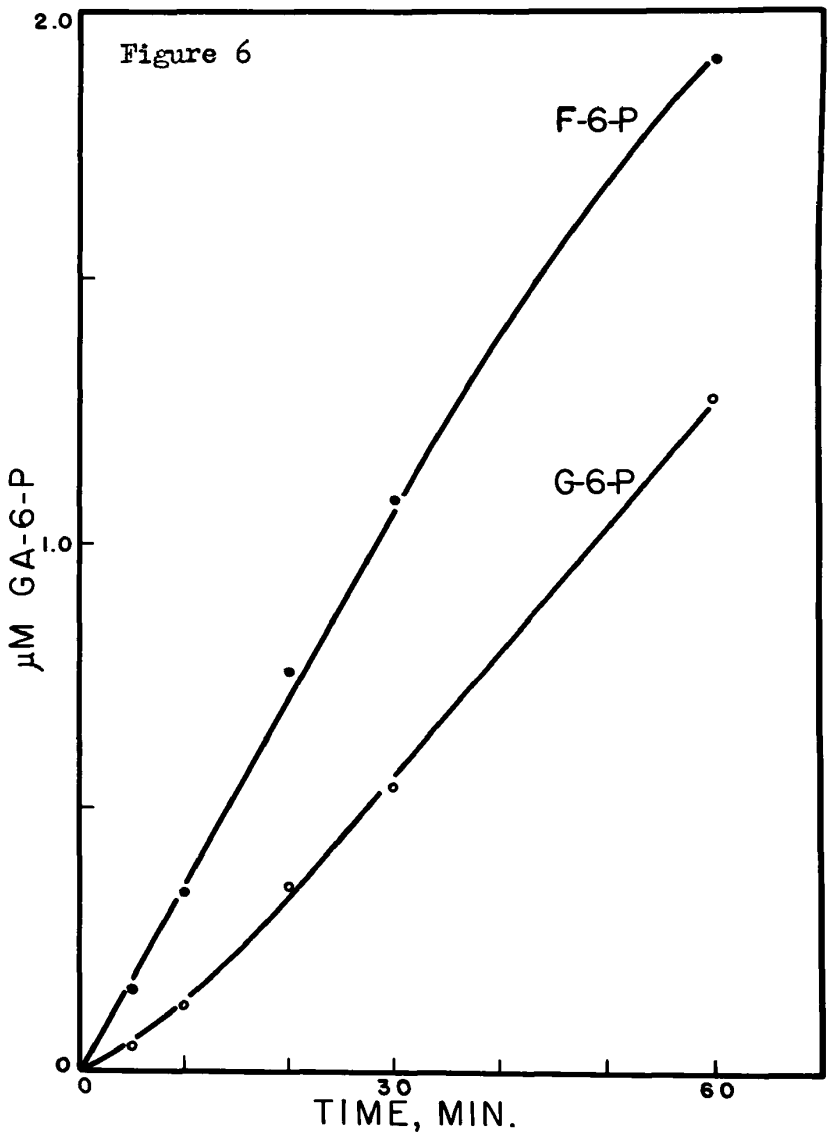


Figure 6
2.0
F-6-P
G-6-P
μM GA-6-P
1.0
0
0
30
60
TIME, MIN.

TABLE III

GLUCOSAMINE SYNTHETASE PURIFICATION

Fraction	Protein Conc., mg./ml.	Total Protein	Total Units of Activity	Specific Activity	Purification
Supernatant	5.15	56.8	215	3.79	
Protamine Supernatant	1.6	18.5	236	12.8	3.4 fold
Adjusted to pH 5.9	1.6	18.5	209	11.3	
First F-6-P eluate	0.25	1.24	67	53.6	14.1 fold
Second F-6-P eluate	0.07	0.35	25	72.5	19.1 fold
First Phosphate eluate	0.72	3.6	52	17.1	4.5 fold
Second Phosphate eluate	0.08	0.38	4	11.3	

Conditions: Each enzyme fraction was incubated for 1 hour with 30 μM glutamine, 30 μM F-6-P, 60 μM phosphate buffer, pH 7.0, and water to give a final volume of 3.0 ml; T = 30.3°C. The reaction was stopped with Tungstate-HCl, and the GA-6-P determined by the Dische method.

TABLE IV

CONTROL REACTION MIXTURES INACTIVE WITH THE PARTIALLY PURIFIED ENZYME

Enzyme	Substrates	Phosphate Buffer pH 6.5	Volume
0.25 ml.	20 μM glutamine	100 μM	2.0 ml.
0.25 ml.	20 μM F-6-P	100 μM	2.0 ml.
0.25 ml.	20 μM G-6-P	100 μM	2.0 ml.
0.25 ml.	20 μM glutamate, 20 μM F-6-P	100 μM	2.0 ml.
0.25 ml.	20 μM asparagine, 20 μM F-6-P	100 μM	2.0 ml.
0.25 ml.	10 μM NH_4Cl, 10 μM F-6-P, 10 μM ATP	70 μM	1.1 ml.
0.25 ml.(boiled)	20 μM F-6-P, 20 μM glutamine	100 μM	2.0 ml.

Conditions: The enzyme was a phosphate eluate (0.38 or 0.51 mg. protein/ml.). Each reaction mixture was incubated for 20 min. at 30.6°C., and included an unincubated control. The enzyme was inactivated and the proteins denatured with 10% Na_2WO_4 (0.01 ml.) and 1 N HCl (0.04 ml.), or by heating at 100°C. for 5 min. GA-6-P was determined by the Dische method.

From the negative results obtained with these mixtures it was concluded that the enzyme could only utilize glutamine as the amino-nitrogen donor, and F-6-P or G-6-P as the nitrogen acceptors in the reaction.

Stoichiometry of the Glucosamine Synthetase Reaction.--The recoveries obtained for both reactants and products, exclusive of the hexose-phosphates (see below), are shown in Table V. The results show a satisfactory, though not ideal, relationship between the disappearance of glutamine and the appearance of GA-6-P, GA, and glutamic acid. In the discussion of the early experiments (c.f. p. 38) it was noted that free (i.e., non-phosphorylated) GA appeared in reaction mixtures containing no inorganic phosphate. Upon incubating the ammonium sulfate fraction (Exp. 31, Table V) with sodium citrate buffer instead of phosphate buffer, 24.5% of the total product appeared as free GA. However, in no case did GA appear when inorganic phosphate was present in the reaction mixtures. It is obvious from the large increase in inorganic phosphate that a considerable quantity of the F-6-P was also lost by phosphatase action. Whether the dephosphorylation of both GA-6-P and F-6-P was the result of a single non-specific phosphatase or two specific enzymes is a moot point. Of interest in this respect is the fact that Blumenthal, Hemerline, and Roseman (1956) isolated a phosphatase from Neurospora crassa which was 25 times more active toward GA-6-P than it was toward other hexose-phosphates. The optimal pH range of 6.0 to 7.5 for the purified glucosamine phosphatase of Blumenthal, et al., overlaps that of the glucosamine synthetase and

TABLE V

STOICHIOMETRY OF THE GLUCOSAMINE SYNTHETASE REACTION

	Δ μM During Incubation				
	GA-6-P	GA	Glutamate	Glutamine	Inorganic Phosphate
Exp. 30	+ 4.6		+ 5.0	- 4.8	
Exp. 31	+ 6.8	+ 2.2	+ 9.9	- 7.1	+ 8.8
Exp. 65					
Crude supernatant	+ 1.8		+ 2.3	- 1.6	
Protamine step	+ 1.6		+ 2.2	- 2.2	
Gel eluate (0.1 M phosphate)	+ 5.0		+ 5.0	- 5.6	

Conditions: Exp. 30. A supernatant dialyzed 12 hr. at 0-2°C. vs. 10^{-2} M phosphate buffer, pH 6.9. 0.5 ml. enzyme, 30 μM F-6-P, 30 μM glutamine, 50 μM phosphate buffer, pH 6.9, incubated for 2 hr. in a final volume of 3.0 ml.; T = 31°C.

Exp. 31. The enzyme was a 30-50% ammonium sulfate fraction prepared from a supernatant dialyzed vs. 10^{-2}M phosphate buffer, pH 6.9, for 12 hr. at 0-2°C., and redissolved in 10^{-1}M sodium citrate buffer, pH 6.8. The incubation conditions were the same as those in Exp. 30, except that sodium citrate was substituted for the phosphate buffer.

Exp. 65. These were enzyme fractions obtained by the procedure given in Fig. 5. Each fraction was incubated with 20 μM glutamine, 20 μM F-6-P, and 100 μM phosphate buffer, pH 6.5, for 20 min. in a final volume of 2.0 ml.; T = 30.6°C.

Reactants and products were analyzed by the Dische method for glucosamines, and the method of Kay, et al. for the amino acids. All values were corrected for their unincubated controls.

such an enzyme could conceivably be present and active in Blastocladiella homogenates.

Attempts to establish a 1:1 ratio between the disappearance of F-6-P (or G-6-P) and the appearance of GA-6-P were only moderately successful, even with the partially purified enzyme. Table VI lists the results of analyses for changes in the sugar phosphates during a 30 minute incubation of the enzyme with F-6-P and G-6-P as the substrates. The data strongly suggested the presence of considerable phosphoglucose isomerase activity in the partially purified enzyme. To verify this, the control tubes containing only F-6-P were analyzed for the presence of G-6-P before and after incubation by measuring the rates of TPN reduction with glucose-6-phosphate dehydrogenase (Sigma). The results are given in Table VII.

TABLE VI

HEXOSE-PHOSPHATE CONVERSION DURING INCUBATION WITH THE PARTIALLY PURIFIED GLUCOSAMINE SYNTHETASE

Substrate	Δ μM During Incubation		
	GA-6-P	F-6-P	G-6-P
F-6-P	+ 1.1	- 4.0	+ 2.5
G-6-P	+ 0.5	+ 2.9	- 4.5

Conditions: The partially purified enzyme and reaction mixtures were as indicated for Fig. 6. The time of incubation was 30 min.

TABLE VII

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY FOR ISOMERASE ACTIVITY IN THE PARTIALLY PURIFIED ENZYME

Time in Minutes	Optical Density	
	Control	Experimental
2	0.015	0.042
4	0.024	0.063
6	0.032	0.077
8	0.037	0.087
10	0.045	0.094
15	0.061	0.112
25	0.095	0.146

Conditions: To detect phosphoglucose isomerase activity in the phosphate gel-eluate, 0.25 ml. of the enzyme (0.4 mg. protein) were incubated with 20 μM F-6-P and 100 μM phosphate buffer, pH 6.5, in a volume of 2.0 ml. for 20 min., and then heat inactivated. An unincubated control was prepared in the same fashion with enzyme boiled before addition. For the G-6-P assay, 1.2 ml. 10^{-1}M phosphate, pH 7.5, 0.3 ml. 10^{-1} M $MgCl_2$, 0.1 ml. 2.5 x 10^{-3}M TPN, 0.1 ml. of G-6-P-dehydrogenase (Sigma; 2 mg./ml.), and water to give a final volume of 2.97 ml. were added to each cuvette. The reaction was started by the addition of 0.03 ml. of the solution to be assayed and the reduction of TPN followed in the spectrophotometer at 340 mμ. The values in the table are corrected for the initial absorption of the complete system minus substrate.

Since this enzyme displays absolute specificity for G-6-P, the conversion of F-6-P to G-6-P by isomerase action was definitely established by the higher initial rate with the aliquot from the tube incubated for 20 minutes. Unfortunately, the presence of isomerase activity in the commercial preparation of glucose-6-phosphate dehydrogenase itself made an accurate estimate of the magnitude of the conversion impractical.

It should be noted, however, that the rates of reduction were equal after 10 minutes. Therefore, a rough estimate was obtained by assuming that the difference in optical density between the two after 10 minutes, when plotted as O.D. vs. time, reflected the TPN reduction due to the increased G-6-P in the tube incubated with glucosamine synthetase. This calculation was made in the following manner (Horecker and Wood, 1957):

$$\frac{0.051 \times 3 \text{ ml.} \times 66.7}{6.22 \text{ ml./}\mu\text{M}} = 1.64 \ \mu\text{M}$$

where 0.051 is the optical density difference between the curves, 6.22 ml./μM is the micromolar extinction coefficient of TPN at 340 mμ, 3 ml. is the volume in the cuvette, and 66.7 the dilution factor. The calculated value (1.64 μM) for the G-6-P which appeared was of the same order of magnitude as the decrease in F-6-P (2.15 μM) found by the anthrone method.

The presence of the isomerase in the purified glucosamine synthetase could therefore explain the capacity of G-6-P to serve as a substrate in the reaction. This interpretation was strengthened by the definite lag phase and reduced rate obtained with G-6-P as compared to F-6-P in the time course study (c.f. Fig. 6). Reference to Table VIII provides additional support for the function of F-6-P as the primary substrate for the Blastocladiella synthetase.

The 50% reduction in the G-6-P/F-6-P activity ratio during purification, therefore, established with reasonable certainty that F-6-P was the actual substrate for the Blastocladiella glucosamine synthetase, and that the reaction catalyzed by the enzyme was:

TABLE VIII

COMPARISON OF THE EFFICIENCY OF GLUCOSE-6-PHOSPHATE AND FRUCTOSE-6-PHOSPHATE AS SUBSTRATES FOR THE GLUCOSAMINE SYNTHETASE REACTION DURING PURIFICATION

Purification Stage	$\frac{\text{Specific Activity with G-6-P}}{\text{Specific Activity with F-6-P}} \times 100$
Supernatant	93%
Protamine supernatant	81%
Gel eluate (phosphate)	47%

Conditions: The enzyme fraction was incubated with 20 µM F-6-P, or G-6-P, 20 µM glutamine, 100 µM phosphate buffer, pH 6.5; T = 30.6° C. Final volume 2.0 ml. The reaction was stopped with 0.05 ml. 1 N HCl and 0.01 ml. 7% Na_2WO_4, and the GA-6-P analyzed by the method of Dische.

d-fructose-6-phosphate + l-glutamine ⟶ d-glucosamine-6-phosphate + l-glutamate

The pH optima and substrate requirements were the same as those reported for the enzyme from Neurospora (Leloir and Cardini, 1953; Blumenthal, et al., 1955). The data also established that the Blastocladiella enzyme was not the same as that reported for rat liver by Pogell and Gryder (1957), since the latter enzyme had a pH optimum between 7.4 and 8.0 and demonstrated an increasing specificity toward G-6-P on partial purification.

It should be noted in closing that the 19-fold purification attained resulted in an enzyme preparation with a specific activity (µM GA/mg. protein/20 min.) more than 242 times that reported by Leloir and Cardini (in µM GA/mg. protein/hr.), and ca. 40 times that reported

by Blumenthal, et al., the only other purifications published to date.

Studies of R.S. Ontogeny.

The purification of the enzyme outlined in the preceding section, was undertaken with the express purpose of determining optimal conditions for its assay in plant homogenates. With that information in hand, it became possible to proceed with a study of the enzyme at different stages in the life cycle of the fungus.

Glucosamine Synthetase Activity in Zoospores, O.C. Plants, and R.S. Plants.--As a first approach it was decided to compare the activity already determined for the mature thin-walled plants with that in the mature R.S. plants and in swarmers. To obtain a sufficient number of swarmers for an enzymatic assay, eighteen 15 cm. Petri plates of PYG agar were heavily inoculated with Blastocladiella swarmers. After 12 hours a dense suspension of spores was collected from each plate by a series of centrifugations, using the procedure described by McCurdy (1959). The swarmers were kept in an ice-bath at all times from this stage on. The cells were finally washed twice with 5 to 10 ml. of cold 5×10^{-2} M phosphate buffer, pH 6.5, by centrifugation at 1600 x g. for 15 seconds. The packed cells were resuspended in the same buffer (1 vol. cells: 9 vol. buffer), transferred to a small glass homogenizer, and ground until better than 95% of the cells were broken. The homogenate was centrifuged for 30 minutes at 22,000 x g. in a Servall Model SS-1 centrifuge at 2° C. The clear supernatant was then assayed for its glucosamine synthetase activity. The results of two such experiments are shown in Table IX.

TABLE IX

GLUCOSAMINE SYNTHETASE ACTIVITY IN ZOOSPORES

	Volume of Cells	Protein Concentration	Specific Activity With F-6-P	Specific Activity With G-6-P
Exp. 67	0.13 ml.	3.82 mg./ml.	2.2	1.6
Exp. 68	0.26 ml.	3.55 mg./ml.	4.3	3.2

Conditions: The supernatant (0.2 ml.) was incubated with 20 µM glutamine, 20 µM F-6-P, or G-6-P, and 100 µM phosphate buffer, pH 6.5, in a final volume of 2.0 ml., for 20 min.; T = 30° C. The reaction was stopped with 0.06 ml. 1 N HCl and 0.01 ml. 7% Na_2WO_4, and the GA-6-P determined by the Dische method. All values were corrected for unincubated controls.[1]
Specific Activity: uM GA-6-P/mg. protein/20 min.

To assay the synthetase activity in mature R.S. plants, two 1.5-liter cultures containing 2.38×10^{-2} M sodium bicarbonate were inoculated with swarmers and grown for 7 days at room temperature. The mats of mature resistant sporangia derived therefrom were homogenized in 5×10^{-2} M phosphate buffer, pH 6.5 in the Omni-mixer. Following centrifugation (22,000 x g., 30 min.), the supernatants were analyzed for their synthetase activity with the standard assay procedure (Table X). Dialysis of the supernatant decreased the activity considerably. In addition, the sediment consisting of the fragments of the resistant sporangia and other cellular debris was resuspended in buffer and mixed in equal proportions with the supernatant. This reconstituted mixture, containing the same quantity of supernatant as the unreconstituted mixture, was also assayed.

[1]With exception of the quantity of enzyme, this assay procedure was used for all subsequent determinations of glucosamine synthetase activity and will be referred to as the "standard assay."

TABLE X

GLUCOSAMINE SYNTHETASE ACTIVITY OF MATURE R.S.

	Protein Concentration	Specific Activity With F-6-P	Specific Activity With G-6-P
Exp. 69 Supernatant	2.0 mg./ml.	0.68	0.14
Exp. 70 Supernatant	2.3 mg./ml.	0.62	0.18
Exp. 70 Reconstituted		0.60	
Exp. 70 Dialyzed Supernatant[a]	2.0 mg./ml.	0.45	

[a] 2.0 ml. supernatant dialyzed against 200 ml. 5×10^{-2} M phosphate buffer, pH 6.5, for 7.5 hours at 2°C.

The failure of the reconstituted homogenate to demonstrate increased activity over the supernatant alone, established the exclusive presence of the enzyme in the soluble proteins of the supernatant. This agreed with the result obtained earlier for the O.C. plants.

The average specific activities for glucosamine synthetase in the three plant forms are listed in Table XI.

TABLE XI

A COMPARISON OF GLUCOSAMINE SYNTHETASE ACTIVITY IN ZOOSPORES, O.C. PLANTS, AND R.S. PLANTS

Plant Type	Specific Activity With F-6-P	Specific Activity With G-6-P
Zoospores	3.3	2.4
Thin-walled O.C. Plants	1.9	1.8
Thick-walled R.S. Plants	0.7	0.2

The differences in activity of the enzyme among the plant forms immediately raised the question of its relationship to the differences in the form and development of the organism. This, then, posed the problem: did the decrease in the enzyme activity in mature plants reflect its importance in the processes leading to the genesis of a particular form or, rather, just the residual activity after growth and synthesis had ceased? To decide which of these interpretations was correct, it was necessary to determine where the decrease occurred during development and, if possible, to correlate that knowledge with the changes in other processes associated with growth. Because of a basic interest in the genesis of the resistant sporangium, and the increased chitin synthesis involved in its development (Cantino, Lovett, and Horenstein, 1957), the studies which follow are concerned with this form only.

Cultural Conditions for Synchronized Growth.--In order to study the relative activity of the synthetase enzyme at different stages during the development of R.S. plants, two experimental conditions had to be satisfied: (1) The growth of the plants had to be well enough synchronized so that most, if not all, of the plants were at the same stage of growth at any given time; (2) Cultures had to be grown in a manner which permitted removal of a representative sample at desired intervals.

The use of swarmer suspensions partially satisfied the first requirement. It has recently been shown that such suspensions when placed in liquid media germinate in essentially synchronous fashion

(Turian and Cantino, 1960). In an attempt to satisfy the second condition a series of identical 3-liter flasks (containing 2.38×10^{-2} M $NaHCO_3$ before autoclaving) were inoculated with a uniform number of motile swarmers. Each flask was harvested at a different time. However, after several runs were made using the conditions described above, results indicated that neither of the two criteria was in fact being satisfactorily met. The first and more serious problem involved the condition of the plants grown in 2.38×10^{-2} M sodium bicarbonate. The concentration used was originally chosen because it ensured 100% R.S. formation on agar cultures. But in liquid cultures, although some plants developed in an apparently normal fashion, others appeared normal at first but showed increasing abnormality and degeneration after approximately 48 hours of growth. Morphologically, the most obvious changes were a pronounced abnormal thickening of the chitinous sporangial wall (Fig. 8), depressed pigmentation, and ultimately, a clumping of the sporangial contents. Therefore, a series of flasks were set up to establish the optimal conditions for a proper balance between R.S. formation and healthy growth in liquid media. As a consequence, it was found that 8.9×10^{-3} M sodium bicarbonate autoclaved in the PYG medium yielded the best results; cultures grown under these conditions consisted of better than 98% R.S. plants and these appeared entirely normal throughout their development.

The second problem encountered with the serial flask technique was unsatisfactory reproducibility in growth rates among replicate flasks, although all the conditions were made as nearly identical as possible,

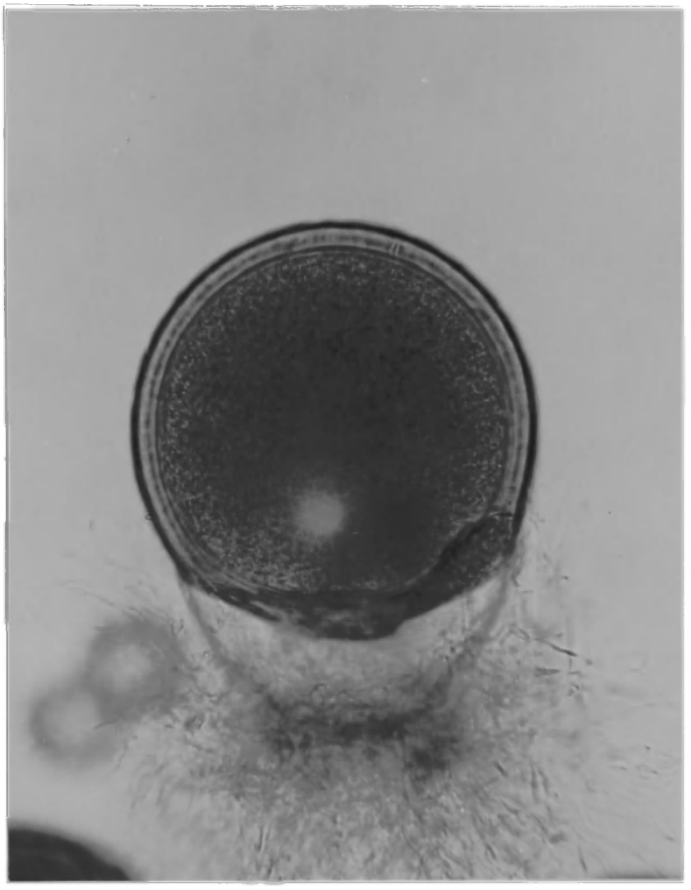


Figure 8

A Thick-Walled R.S. Plant Produced in Super-Optimal Bicarbonate Concentrations

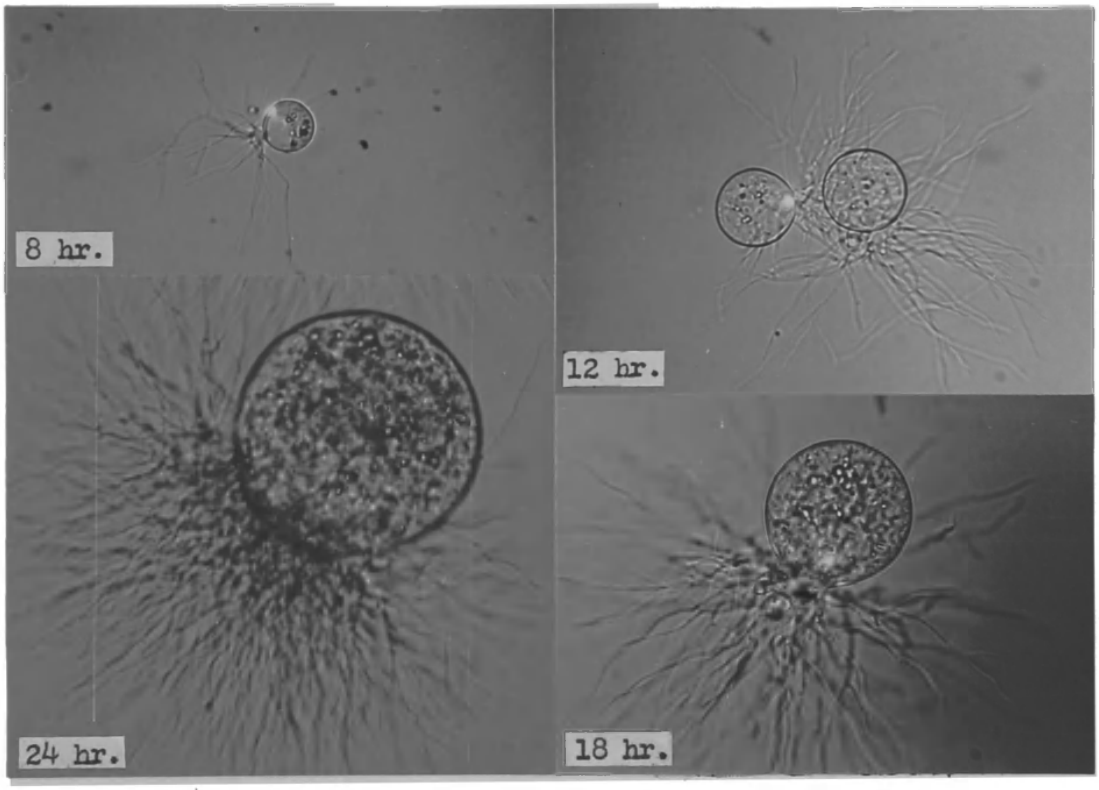
including the size of inoculum, temperature, and the rate of aeration. The degree of clumping of plants had a noticeable effect on growth. Several entangled plants always matured more rapidly than single ones, and this effect could be detected for even a single pair of plants. The variability in degree of clumping could not be eliminated without reducing the size of the inoculum to an impractical level. To obviate this difficulty flasks were used of sufficient size (12 liters) to accommodate enough medium (10 liters) for sampling throughout the entire growth period. The results obtained by this technique were excellent. In such cultures, the great majority of plants grew singly or at most in two's and three's throughout the generation time.

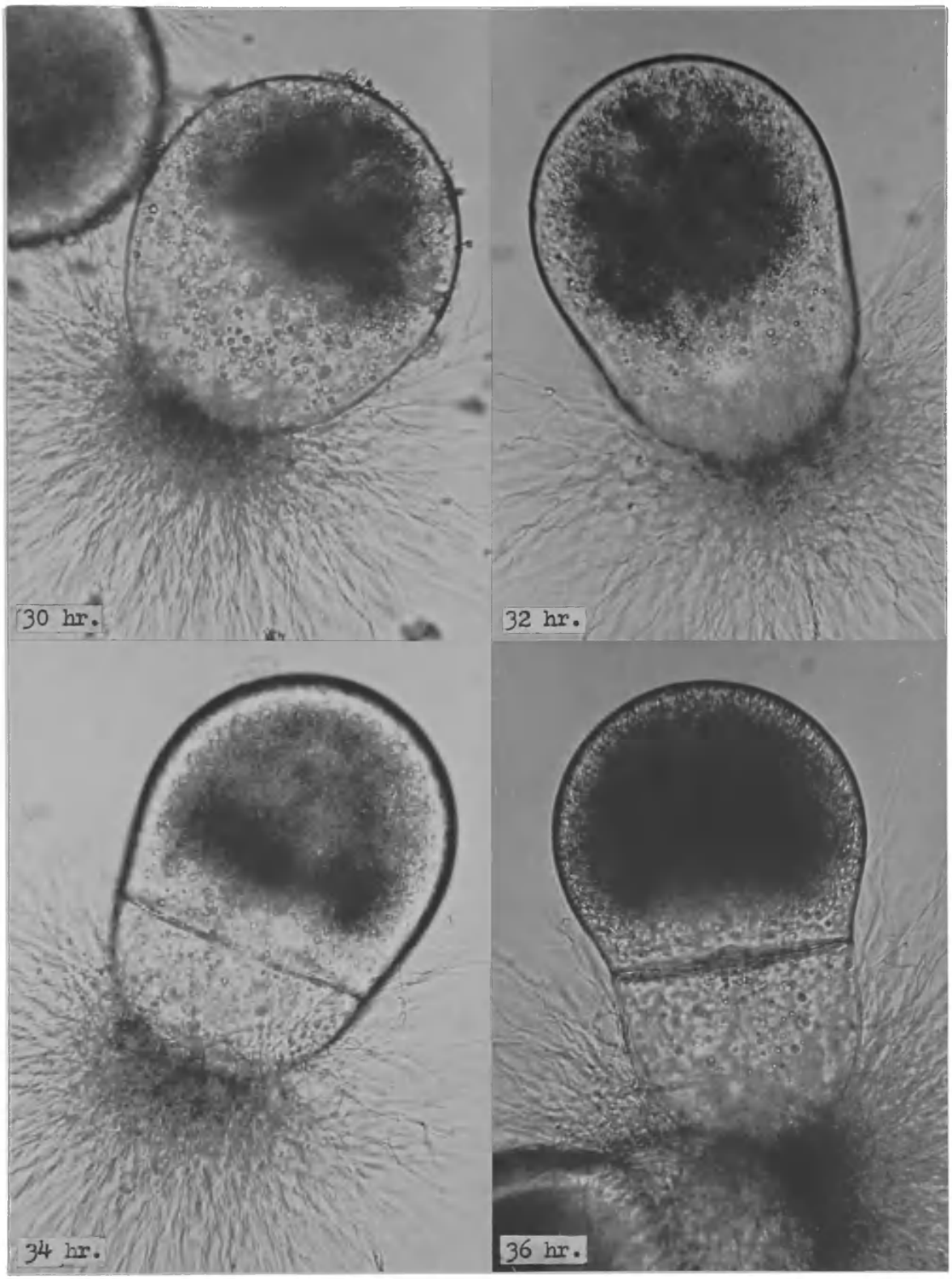
Growth Studies with Synchronized Cultures.--To establish that these cultures were as well synchronized as they appeared to be by visual examination, samples were removed for size measurement approximately every 6 hours up to 60 hours, and at less frequent intervals thereafter. The appearance of plants at these different stages of development is shown in Figure 9. Twenty plants from each sample were measured at random. At the earlier stages when they were spherical or ellipsoidal in shape, only the length and width was recorded. For the older developmental stages after the initiation of the sporangium, the overall length and the diameter of both sporangium and stalk were recorded (Table XII).

Calculation of the average volume per plant at each stage during growth was based upon the assumption that the shape of the plants was sufficiently near to that of a sphere so that a simplified calculation would not cause serious error. The volume of plants at different ages

Figure 9

Photomicrographs of R.S. Plants During Development in Synchronous Culture (X 350)

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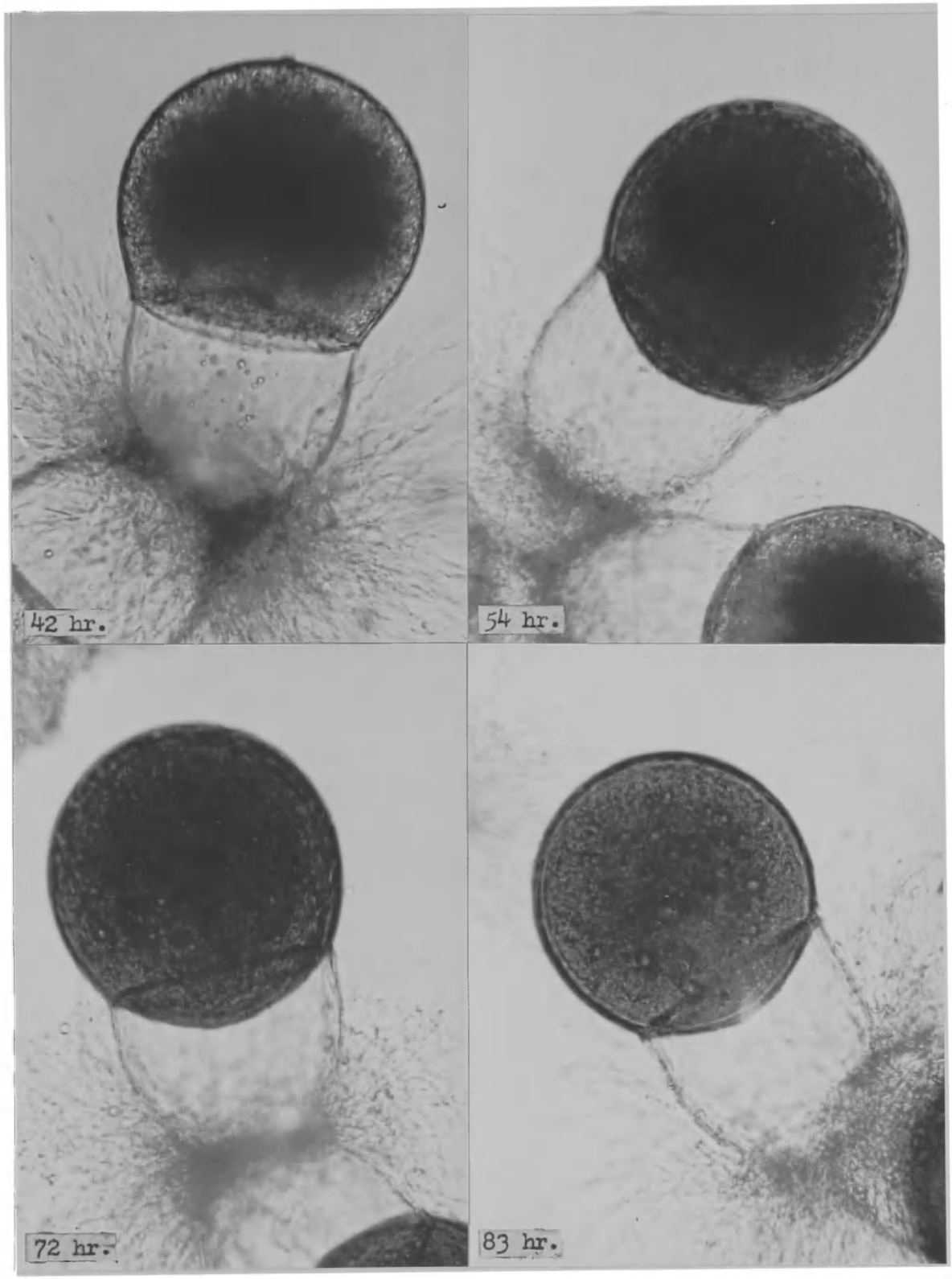
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72 hr.
83 hr.

TABLE XII

SIZE MEASUREMENTS OF DEVELOPING R.S.

Age in Hours	Length (μ)	Diameter of Sporangium (μ)	Diameter of Stalk (μ)
0 (Zoospores)	9	7	
8	16.5	16.4	
12.5	29.5	29.2	
18	51.4	51.1	
24	92.5	92.0	
30.5	193.7	142.6	
36	210.2	151.1	114.8
42	212.4	152.7	114.8
48	210.7	146.6	102.5
54	207.6	145.4	101.8
60	209.5	146.3	103.1
72	209.5	139.6	100.6
107	202.7	142.5	97.2

Each value represents the average for 20 plants measured at random. The values within any one sample were sufficiently consistent to make a statistical analysis unnecessary.

is plotted in Figure 10A-B. The observations that the plot of the relationship between volume and age was sigmoid, and that the logarithmic plot yielded a straight line were taken as additional confirmation of synchronized growth. It is important to note that the increase in size falls off rapidly after 30 hours and a decrease in size even seems to occur; the latter must be due to a certain amount of shrinkage during the maturation process, but its cause remains unknown.

To obtain one further parameter concerning synchronization of these cultures, the dry weight per thallus was determined as a function of

plant age. It was impossible to use 12-liter flasks for these determinations because a considerable quantity of plant material adhered to the upper portions of the flask and made quantitative recoveries by the siphon procedure unreliable. For this reason a series of 3-liter flasks were inoculated with a known quantity (i.e., few enough to prevent overcrowding) of swarmers and grown under identical conditions. The results are given in Table XIII.

The dry weights of plants at different ages were obtained from the data in Table XIII (Fig. 11A-B). The sigmoid and straight line relationships between dry weight/plant and time, and log dry weight/plant and time, respectively, were consistent with a satisfactory synchronization during the growth and development of the R.S. plants.

For purposes of comparison which will become evident in the discussion, an arrow has been placed on subsequent graphs indicating the age (36 hr.) where the increase in size of plants ceased.

Glucosamine Synthetase Activity During R.S. Development.--With conditions established for growing synchronized, reproducible, mass cultures of R.S. plants, the study of glucosamine synthetase activity during development was continued. The data from a typical experiment using such cultures are delineated in Figures 12, 13, and 14A-B. The culture flask, incubated at 24° C., was sampled by removing approximately one and one-half liters of thoroughly suspended plant material at five intervals between 21.5 and 83 hours. In order to obtain enough plant material for the 12 hour data, it was necessary to use a 4-liter flask

TABLE XIII

DRY WEIGHTS OF PLANTS AT DIFFERENT AGES DURING R.S. DEVELOPMENT

Age in Hours	Total Number of Plants	Total Dry Weight (grams)
0 (Zoospores)	532,972,510	0.0602
12	254,107,840	1.0297
24	3,493,983	0.3422
36	1,233,170	0.7730
48	1,233,170	1.0649
60	1,233,170	1.0314
84	1,438,699	1.1707

Conditions: The swarmers were harvested as previously described for enzyme preparations, washed with distilled water, and dried to constant weight.
The 12 hr. plants were grown in a 4-liter flask with PYG plus 8.9×10^{-3} M $NaHCO_3$, the remaining cultures were grown in 3-liter flasks of the same medium; all at 23 ± 1° C.

with a much heavier inoculum than could be used for the large flasks. From each aliquot of harvested plants a sample was taken for a dry weight determination. The remainder was homogenized in the Omni-mixer with 5×10^{-2} M phosphate buffer, pH 6.5, and the homogenate centrifuged for 30 minutes at 22,000 x g. The supernatant was separated from the sediment and an upper layer of lipidic material with a pipette. Each supernatant was then assayed for its glucosamine synthetase activity with the standard assay procedure, using approximately 0.6 mg. of supernatant protein per tube.

The averaged data from several series of cultures grown in single 3-liter flasks containing the higher concentrations of bicarbonate

Figure 10A-B. The Volume of an R.S. Plant During Development.

Plants were assumed to be spheres, and "average" diameters were derived from the data in Table XII, Fig. 10A; Volume per Plant. Fig. 10B; Log-Volume per Plant.

Figure 11A-B. The Dry Weight of an R.S. Plant During Development.

Data were calculated as the total dry weight per culture/ the number of plants per culture. Fig. 11A; Dry Weight per Plant. Fig. 11B; Log-Dry Weight per Plant.

Figure 12. The Specific Activity of Glucosamine Synthetase in R.S. Plants During Development.

Specific Activity: μM GA-6-P/mg. protein/20 min. (See context for details).

Figure 13. Total Glucosamine Synthetase per Unit Weight of Organism During R.S. Development.

One unit of synthetase: the quantity of enzyme mediating the production of 1 μM GA-6-P/20 minutes in the standard assay (see context). Total units synthetase per gm. dry weight: total gm. soluble protein x units per gm. protein/ gm. dry weight.

Figure 14A-B. Glucosamine Synthetase Activity per Plant During R.S. Development.

Fig. 14A; Synthetase Units per Plant (total units per gm. dry weight (Fig. 13) x gm. dry weight per plant (Fig. 11A). Fig. 14B; Log-Synthetase Units per Plant.

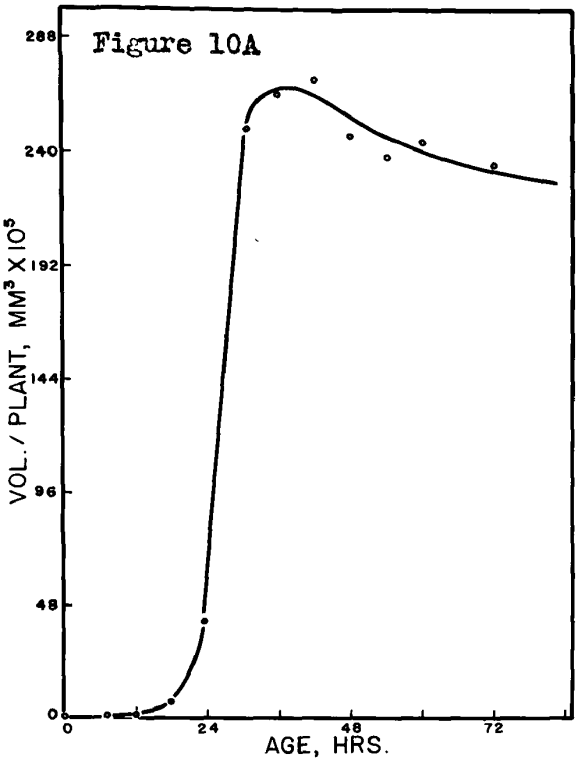


Figure 10A
VOL. / PLANT, MM³ X 10⁵
288
240
192
144
96
48
0
0
24
48
72
AGE, HRS.

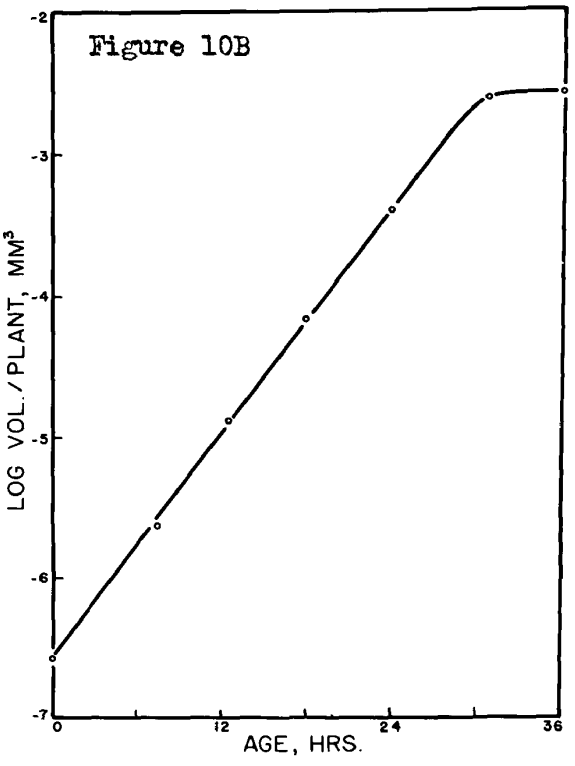


Figure 10B
LOG VOL. / PLANT, MM³
-2
-3
-4
-5
-6
-7
0
12
24
36
AGE, HRS.

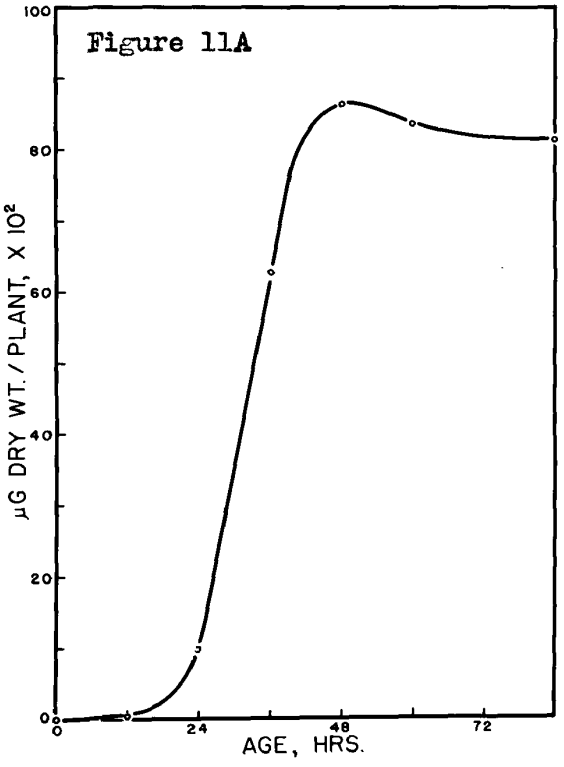


Figure 11A
µG DRY WT. / PLANT, X 10²
100
80
60
40
20
0
0
24
48
72
AGE, HRS.

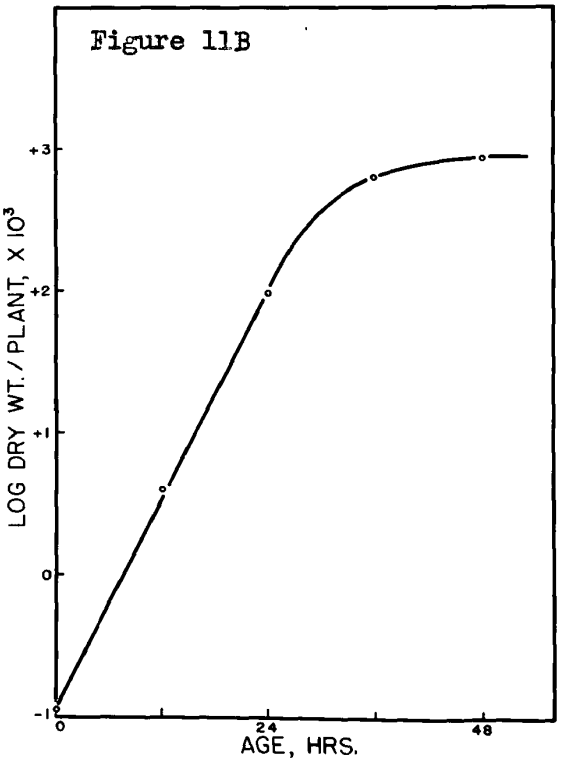


Figure 11B
LOG DRY WT. / PLANT, X 10³
+3
+2
+1
0
-1
0
24
48
AGE, HRS.

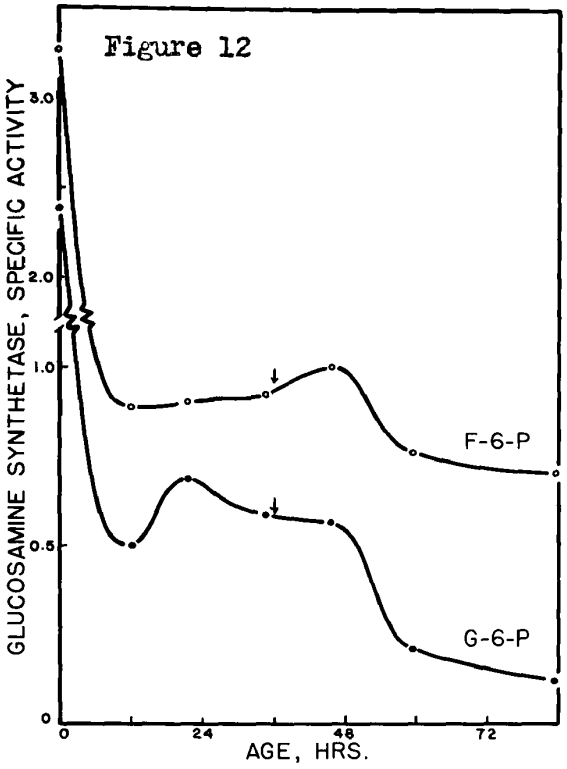


Figure 12
GLUCOSAMINE SYNTHETASE, SPECIFIC ACTIVITY
3.0
2.0
1.0
0.5
0
F-6-P
G-6-P
0
24
48
72
AGE, HRS.

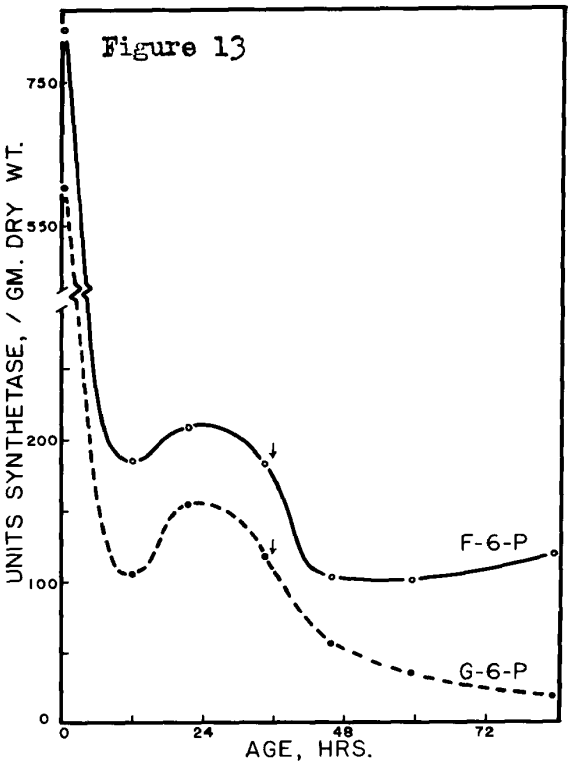


Figure 13
UNITS SYNTHETASE, / GM. DRY WT.
750
550
200
100
0
F-6-P
G-6-P
0
24
48
72
AGE, HRS.

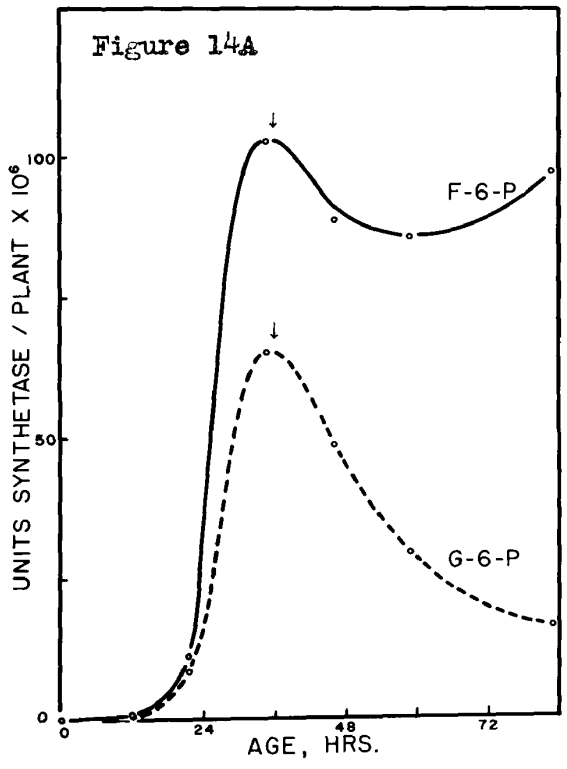


Figure 14A
UNITS SYNTHETASE / PLANT X 10⁶
100
50
0
F-6-P
G-6-P
0
24
48
72
AGE, HRS.

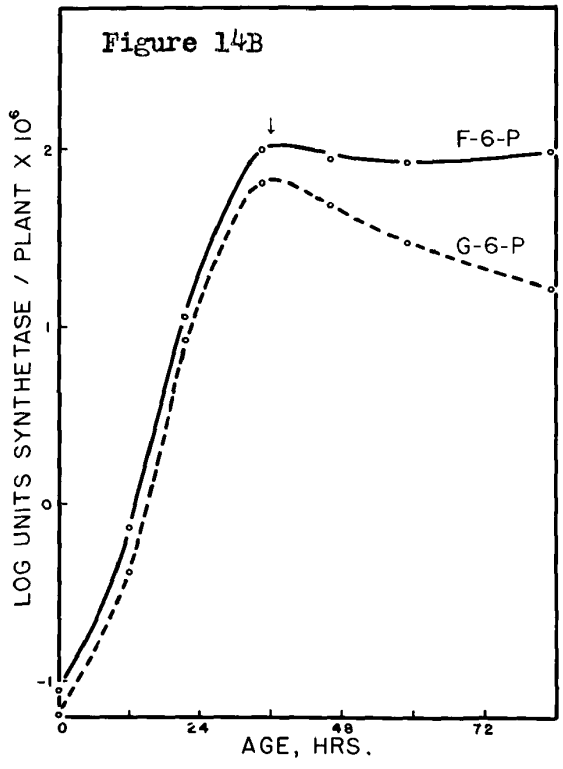


Figure 14B
LOG UNITS SYNTHETASE / PLANT X 10⁶
2
1
0
-1
F-6-P
G-6-P
0
24
48
72
AGE, HRS.

(2.38×10^{-2} M) in the medium, but harvested and analyzed in an identical manner to the large cultures, have been plotted in Figure 15 for comparison. The pattern of the curves is essentially the same in both cases; however, it is important to notice that the peaks in the curves are delayed almost exactly 24 hours by the 2.7-fold increase in the bicarbonate concentration. By microscopic observation it was established that the morphological changes associated with R.S. development were also shifted by the same amount.

The significance of the peaks in total and specific activity and their differential appearance with respect to the age of the plants will be discussed later. However, it is important to point out now that the steady decrease in activity found with G-6-P as the substrate for the synthetase enzyme, as compared to F-6-P, was quite striking. From the relative activities of the two substrates during the purification procedure, and from the rate studies, it had been concluded that the G-6-P was converted to F-6-P by phosphoglucose isomerase action, rather than itself directly involved in the reaction. If, indeed, this were the case the steady decrease in activity could be interpreted as reflecting a decrease in the activity of the isomerase during differentiation, thus gradually blocking the interconversion between the two hexose-phosphates. To test this hypothesis it was necessary to establish whether the phosphoglucose isomerase did decrease in activity during growth. The simplest method for doing this was to measure the reduction of TPN by glucose-6-phosphate dehydrogenase in Blastocladiella extracts, using each hexose-phosphate as substrate. The relative

activity of the two in such a system would indicate the activity of the isomerase since its presence would be required for the conversion of F-6-P to G-6-P. The glucose-6-phosphate dehydrogenase in turn had an absolute specificity for G-6-P and TPN. The method would only be valid if the dehydrogenase were present, and G-6-P thus served not only as a reference for the rates of reduction with F-6-P, but also as a control for the presence of the dehydrogenase itself.

Glucose-6-phosphate Dehydrogenase and Isomerase Activity During R.S. Development.--This experiment was done with swarmers, a 12 hour R.S. culture, and a large synchronized R.S. culture, all grown and harvested as previously described. With the resistant sporangial plants, a sample of the mat was used for a dry weight determination and the remainder homogenized in 5×10^{-2} M phosphate buffer, pH 7.0. The swarmers were harvested and homogenized as usual in the same buffer. One portion of the whole homogenate was centrifuged for 30 minutes at 22,000 x g. and used for protein and nitrogen determinations (see later). A second 3 ml. portion was dialyzed against 2 liters of the same buffer for 10 hours at 2° C. After dialysis the material was quantitatively recovered and diluted to a volume of 10 ml. with buffer. The diluted homogenate was centrifuged at 500 x g. for 10 minutes and the protein concentration of the supernatant determined. The rate of the glucose-6-phosphate dehydrogenase reaction was measured by following the reduction of TPN at 340 mμ (Table XIV). The quantity of protein added to each cuvette (ca. 0.04 to 0.1 mg.) was adjusted to yield rates of reduction that could be determined accurately, and which remained linear

for at least 10 to 15 minutes. The changes in the optical density (O.D.) per 10 minutes ranged from 0.24 to 0.84 in the assay system used. No TPN reduction was observed in controls without added substrate, indicating that the dialysis had removed endogenous substrates.

TABLE XIV

GLUCOSE -6-PHOSPHATE DEHYDROGENASE ACTIVITY DURING DEVELOPMENT OF R.S. PLANTS

Age in Hours	Mg. Protein Used in Assay	Rate of TPN Reduction as Δ O.D./min.[a]	
		With G-6-P	With F-6-P
0 (Zoospores)	0.045	0.054	0.028
12	0.102	0.020	0.013
24	0.055	0.029	0.024
36	0.056	0.045	0.037
48	0.048	0.044	0.034
60	0.051	0.049	0.036
83	0.037	0.070	0.046

Conditions: Each cuvette contained 0.6 ml. 10^{-1}M phosphate buffer, pH 7.5, 0.1 ml. 10^{-1}M $MgCl_2$, 0.2 ml. 2.5x 10^{-3}M TPN, 0.3 ml. 10^{-1}M F-6-P, or G-6-P, 0.05 to 0.08 ml. enzyme, and water to a final volume of 3.0 ml. The reaction was started by the addition of the enzyme, and the O.D. read each minute, beginning at 2 min., in a Beckman Model DU spectrophotometer at 340 mμ. The blank cuvette lacked substrate and TPN.

[a]The rate was calculated for the 3-6 min. interval (from plots of O.D. vs. time) because the rates of reduction with F-6-P, which displayed an initial lag, became linear and maximal after 2 min.

The specific activities obtained for the plants at each age are plotted in Figure 16, and an expression of the total units of dehydrogenase activity per plant as a function of age in Figure 17A-B.

From an examination of the curves it appeared that F-6-P did gradually lose some of its capacity to function in the coupled system. To this extent, it seemed to confirm the suggested loss in isomerase activity during development. However, it was evident from the comparative data in Table XIV that the decrease in isomerase activity could not adequately explain all the change in the capacity of G-6-P to serve as a substrate in the synthetase reaction.

TABLE XV

THE RELATIVE ACTIVITIES OF FRUCTOSE-6-PHOSPHATE AND GLUCOSE-6-PHOSPHATE IN THE GLUCOSAMINE SYNTHETASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ENZYME SYSTEMS DURING R.S. DEVELOPMENT

Age of Plants (hours)	(1) Ratio $\frac{\text{G-6-P}}{\text{F-6-P}}$ Activity in Synthetase Reaction	(2) Ratio $\frac{\text{F-6-P}}{\text{G-6-P}}$ Activity in Dehydrogenase Reaction	(2)-(1)
0 (Zoospores)	0.73	0.52	-0.21
12	0.57	0.66	0.09
24	0.74	0.84	0.10
36	0.63	0.82	0.19
48	0.53	0.77	0.24
60	0.33	0.74	0.41
83	0.17	0.67	0.50

The ratios were the same for both specific and total activity.

If the isomerase were the only limiting factor in the participation of G-6-P in the synthetase reaction the ratios in columns (1) and (2) should have been of the same order of magnitude. However, it was immediately obvious that this was not the case. A possible interpretation of the anomalous behavior of G-6-P will be discussed later.

Figure 15. Total Glucosamine Synthetase per Unit Weight of Organism During R. D. Development in Super-optimal Bicarbonate Concentrations.

Bicarbonate concentration: 2.38×10^{-2} M. See Figure 13 for calculations.

Figure 16. The Specific Activity of Glucose-6-phosphate Dehydrogenase in R.S. Plants During Development.

Specific Activity: rate of TPN reduction/mg. protein (see assay procedure Table XIV).

Figure 17A-B. Glucose-6-phosphate Dehydrogenase Activity per Plant During R.S. Development.

One unit of dehydrogenase activity: the quantity of enzyme mediating a Δ O.D. of 0.1/minute (see assay procedure Table XIV). The units per plant were calculated by the procedure described in Figures 13 and 14. Figure 17A; Dehydrogenase Units per Plant. Figure 17B; Log-Dehydrogenase Units per Plant.

Figure 18. The Chitin Content per Unit Weight of Organism During R.S. Development.

(See Materials and Methods for details)

Figure 19. The Chitin Content per Plant During R.S. Development.

µg chitin per mg. dry weight (Fig. 18) x mg. dry weight per plant (Fig. 11A).

Figure 20. The Melanin Content per Unit Weight of Organism During R.S. Development.

One unit of melanin: the quantity of melanin yielding an O.D. of 0.001 at 450 mµ (see context).

Figure 21. The Melanin Content per Plant During R.S. Development.

Units melanin per gm. dry weight (Fig. 20) x gm. dry weight per plant (Fig. 11A).

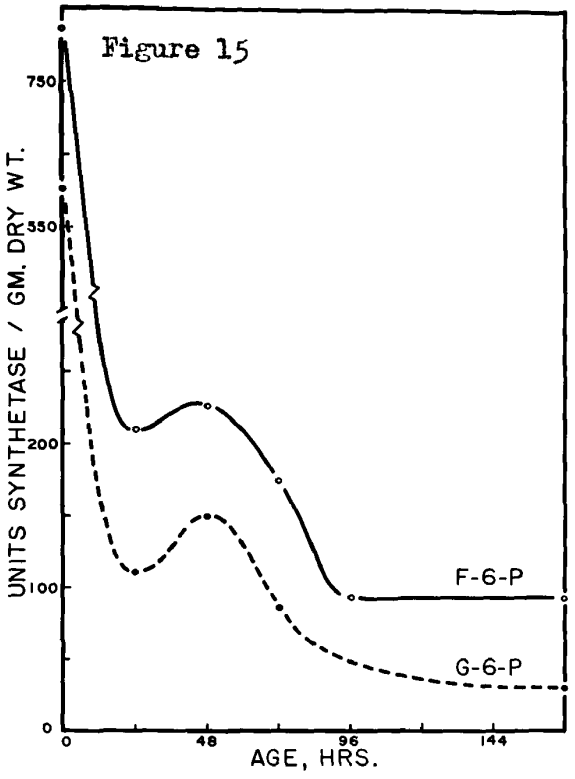


Figure 15
UNITS SYNTHETASE / GM. DRY WT.
750
350
200
100
0
F-6-P
G-6-P
0
48
96
144
AGE, HRS.

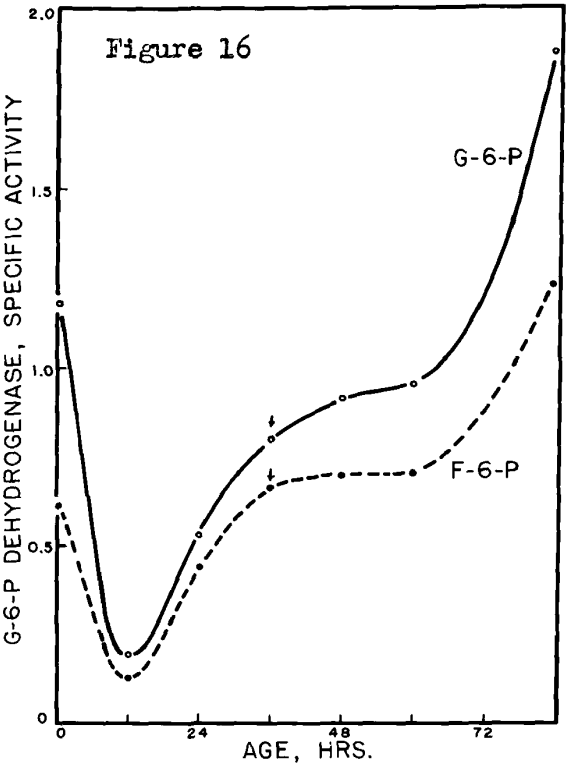


Figure 16
G-6-P DEHYDROGENASE, SPECIFIC ACTIVITY
2.0
1.5
1.0
0.5
0
G-6-P
F-6-P
0
24
48
72
AGE, HRS.

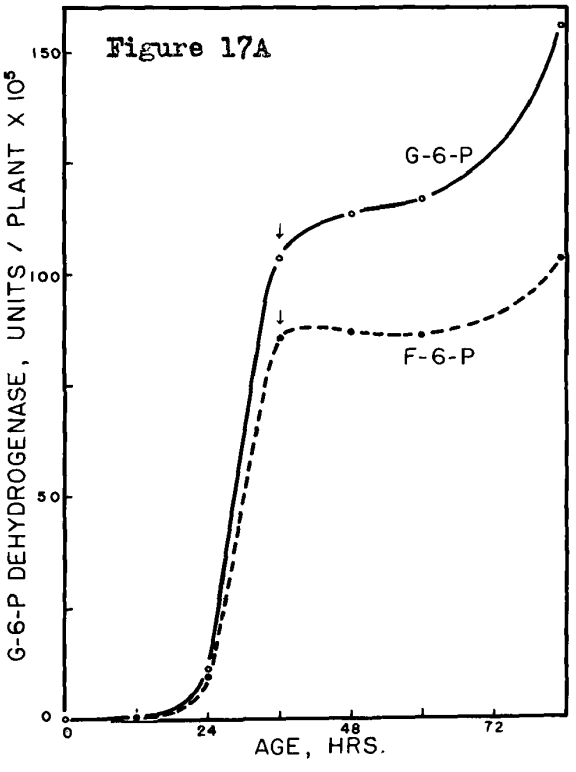


Figure 17A
G-6-P DEHYDROGENASE, UNITS / PLANT X 10⁵
150
100
50
0
G-6-P
F-6-P
0
24
48
72
AGE, HRS.

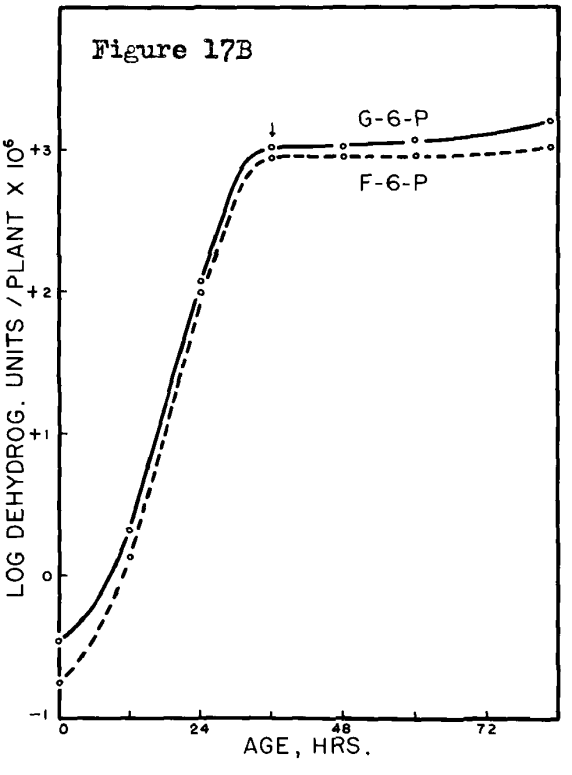


Figure 17B
LOG DEHYDROG. UNITS / PLANT X 10⁶
+3
+2
+1
0
-1
G-6-P
F-6-P
0
24
48
72
AGE, HRS.

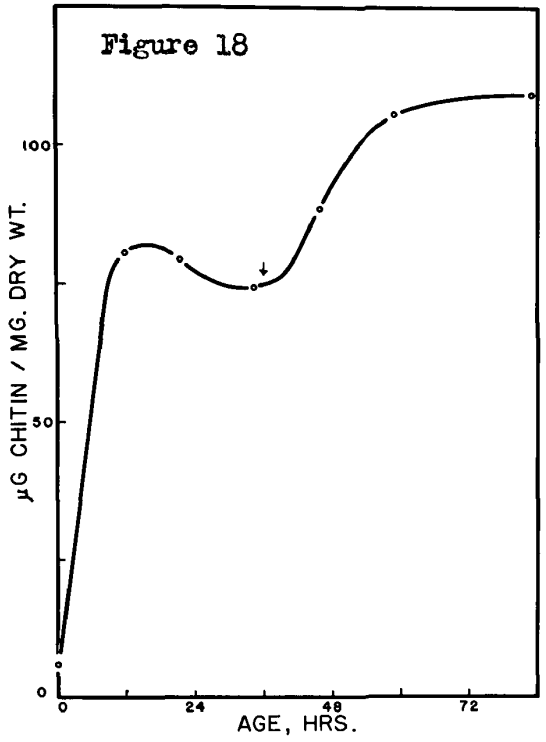


Figure 18
µG CHITIN / MG. DRY WT.
100
50
0
0
24
48
72
AGE, HRS.

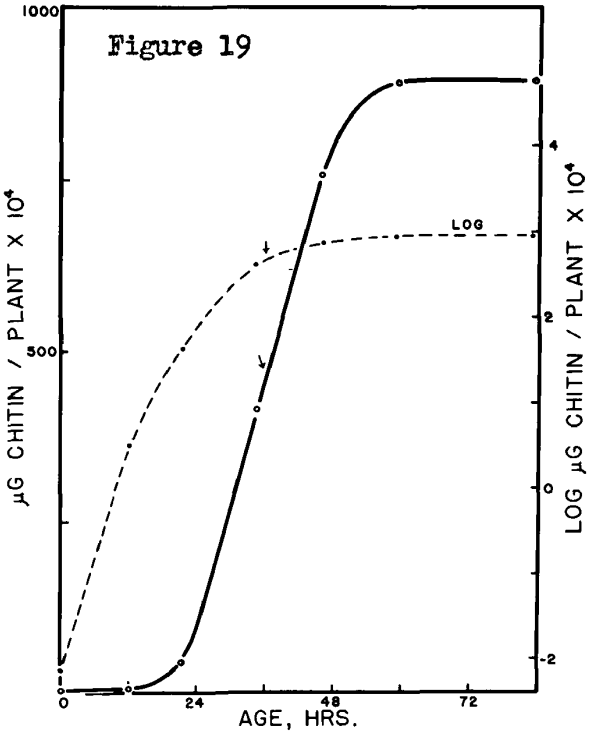


Figure 19
1000
500
0
µG CHITIN / PLANT X 10⁴
LOG
4
2
0
-2
LOG µG CHITIN / PLANT X 10⁴
0
24
48
72
AGE, HRS.

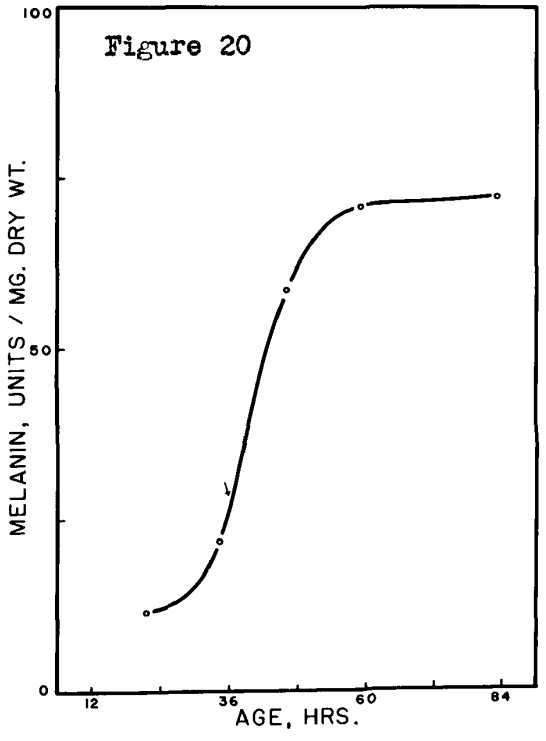


Figure 20
100
50
0
MELANIN, UNITS / MG. DRY WT.
12
36
60
84
AGE, HRS.

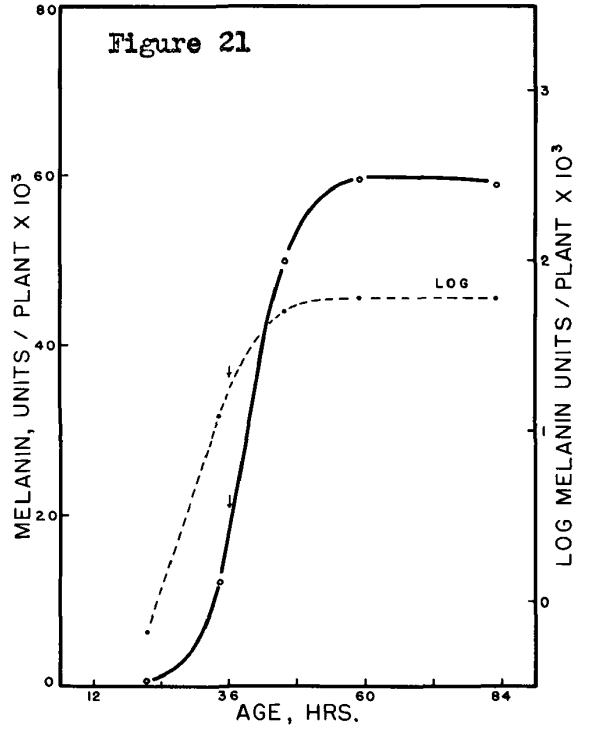


Figure 21
80
60
40
20
0
MELANIN, UNITS / PLANT X 10³
LOG
3
2
1
0
LOG MELANIN UNITS / PLANT X 10³
12
36
60
84
AGE, HRS.

Chitin Formation in Developing R.S.--Among other things, the study of glucosamine synthetase had been undertaken to detect any correlations between its activity and the free glutamic acid pools, and indirectly thereby, the site of bicarbonate fixation in the citric acid cycle. With the synthetase information available it became desirable to examine the other end of this biosynthetic pathway, namely the final product of the sequence, chitin. This could have accomplished two things: (1) it might have brought out any correlation between the rate of synthesis of chitin and the activity of the glucosamine synthetase, and (2) it might have established the pattern of chitin synthesis during differentiation.

The chitin analyses at several stages during the growth period are shown in Figure 18. It is worth emphasizing that the bimodality of the curve (Fig. 18) completely disappeared when the data were converted to a per-plant basis (Fig. 19)! Previous mention was made of the fact that plants grown in excessively high bicarbonate concentrations developed abnormally thick sporangial walls (c.f. p. 61). To verify these observations quantitatively, material from such cultures was analyzed for chitin (Table XVI). The results revealed that the higher bicarbonate concentration caused a 73 to 87% increase over the normal chitin content of the plants.

The characteristic production of the thick, chitinous wall in R.S. plants was always accompanied by the deposition of melanin in this structure, and by increased fat synthesis. Because of these relationships, a study of melanin and lipid production during R.S. formation was undertaken.

TABLE XVI

THE CHITIN CONTENT OF R.S. PLANTS GROWN IN MEDIA WITH DIFFERENT SODIUM BICARBONATE CONCENTRATIONS

Age of Plants	$NaHCO_3$ Concentration	µg Chitin per mg. Dry Weight	Appearance of Plants
5 days	8.93×10^{-3} M.	92.5	Normal
7 days	2.38×10^{-2} M.	160.3	Normal and thick-walled
5 days	2.38×10^{-2} M.	172.9	Abnormal and very thick-walled

Melanogenesis During R.S. Ontogeny.--It had already been established (Cantino and Horenstein, 1955c) that the colored material in the cell walls of Blastocladiella R.S. exhibited the properties characteristic of melanoid pigments. Therefore, the course of melanogenesis during the growth of the R.S. plants was established (Figs. 20 and 21). Eighty-six percent of the melanin synthesized was produced after growth in size ceased (i.e., after 36 hr.) but the final yield of melanin was reached at the same age (60 hr.) as was that for chitin.

Lipid Synthesis During R.S. Development.--The increase in the quantity of lipid during growth was very obvious in the living thalli microscopically, and in the homogenates of these plants as a surface layer after centrifugation. In mature R.S. the lipids appeared to coalesce into large globules (c.f. Fig. 9, 83 hr.). The analyses for total lipids (Figs. 22 and 23) do not include data for the spore stage

because it was impractical to collect enough material for an accurate determination. As with chitin and melanin, a considerable proportion of the total lipid was synthesized after the cessation of growth in size.

Nitrogen Transformation in Developing R.S. Plants.--Significant differences had been found between the soluble amino acid pools of O.C. and R.S. plants (c.f. p. 34). A previous report (Cantino, Lovett, and Horenstein, 1957) had also indicated a considerable difference in other nitrogen-containing fractions between the two types of plant. For this reason the distribution of nitrogen pools was re-evaluated at different stages during the development of R.S. plants (Figs. 25, 26, and 27).[1]

It was obvious that significant changes were occurring in the distribution of nitrogen during growth, and this was nowhere more apparent than in the soluble non-protein nitrogen. At 36 hours this fraction actually appeared to exceed the soluble protein nitrogen, although it rapidly decreased again. The fact that this non-protein fraction should have consisted primarily of amino acids made a chromatographic survey of the free pools of these compounds imperative. The results (Fig. 28) revealed a striking correlation between the levels of soluble, non-protein nitrogen and the quantities of extractable amino acids at different ages in ontogeny. It should be noted that in addition to the

---

[1]By analyzing supernatants before and after TCA precipitation it was also possible to check the accuracy of the TCA-turbidometric method used to estimate proteins. The analysis of dialyzed and undialyzed supernatants served to verify the data obtained by the TCA method and thus verify the soluble protein estimations. The correspondence of the curves for protein nitrogen by analysis and by calculation from the TCA protein determinations (Fig. 26), showed the latter to be a satisfactory method with Blastocladiella material.

Figure 22. The Lipid Content per Unit Weight of Organism During R.S. Development.

Figure 23. The Lipid Content per Plant During R.S. Development.

Micrograms lipid/mg. dry weight (Fig. 22) x mg. dry weight/plant (Fig. 11A).

Figure 24. Relative Enzymatic Activities per Unit Protein Nitrogen During R.S. Development.

Relative activity: total units of activity/plant ÷ total µg soluble protein nitrogen/plant.

Figure 25. Log Plots for Distribution of Nitrogen per Plant During R.S. Development.

See Fig. 27.

Figure 26. The Distribution of Nitrogen per Unit Weight of Organism During R.S. Development.

The total soluble nitrogen (TSN) was determined on supernatants (see context). Two methods were used to estimate the soluble protein nitrogen (SPN) and soluble non-protein nitrogen (SNPN): (1) the supernatants were treated with 2.5% TCA, the TCA-soluble nitrogen determined (SNPN), and the SPN obtained by difference (TSN-SNPN); (2) the supernatants were dialyzed exhaustively, and non-dialyzable protein nitrogen determined (SPN), and the SNPN obtained by difference (TSN-SPN). The µg nitrogen per mg. dry weight was obtained by dividing the total quantity in each fraction by the mg. dry weight used for homogenization. The data obtained by the two methods (two separate experiments) were averaged for the curves in the figure. The calculated protein nitrogen was obtained by assuming a nitrogen content of 16% for the proteins estimated by the TCA-turbidometric method.

Figure 27. The Distribution of Nitrogen per Plant During R. S. Development.

The µg TN, SPN, or SNPN per plant were obtained from: µg TN, SPN, or SNPN/mg. dry weight (Fig. 26) x mg. dry weight/plant (Fig. 11A).

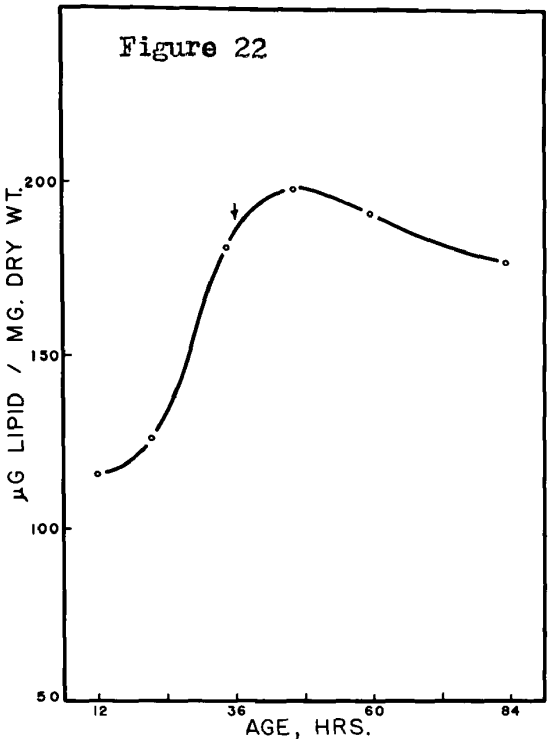


Figure 22
µG LIPID / MG. DRY WT.
200
150
100
50
12
36
60
84
AGE, HRS.

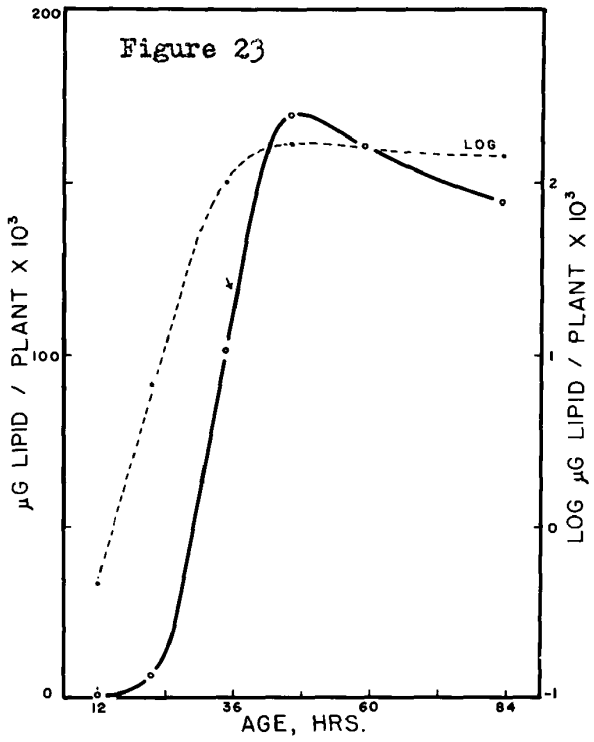


Figure 23
µG LIPID / PLANT X 10³
LOG µG LIPID / PLANT X 10³
LOG
200
100
0
2
1
0
-1
12
36
60
84
AGE, HRS.

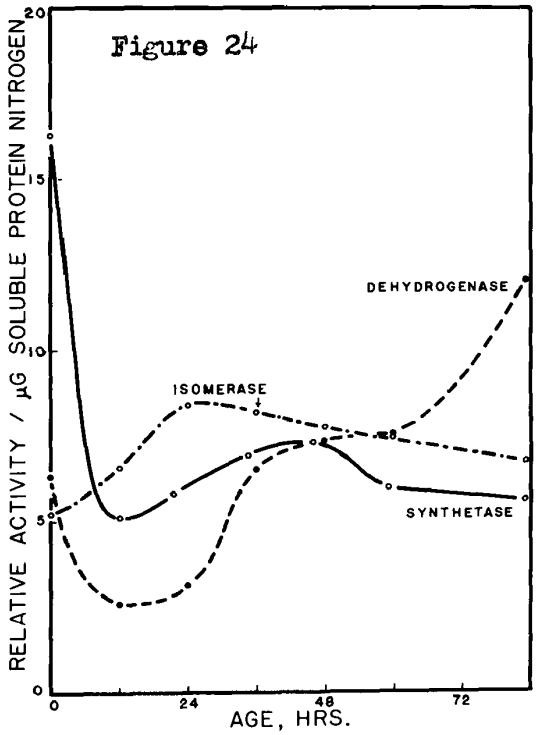


Figure 24
RELATIVE ACTIVITY / µG SOLUBLE PROTEIN NITROGEN
20
15
10
5
0
DEHYDROGENASE
ISOMERASE
SYNTHETASE
0
24
48
72
AGE, HRS.

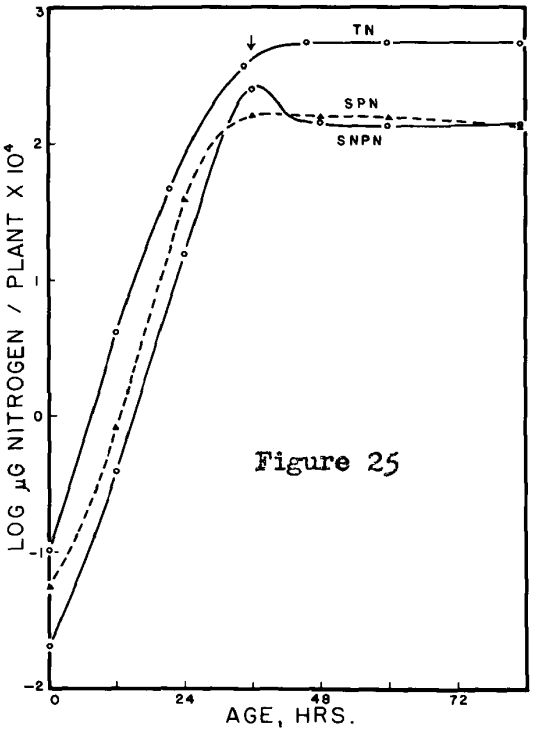

LOG µG NITROGEN / PLANT X 10⁴
3
2
1
0
-1
-2
TN
SPN
SNPN
0
24
48
72
AGE, HRS.

Figure 25

Figure 27

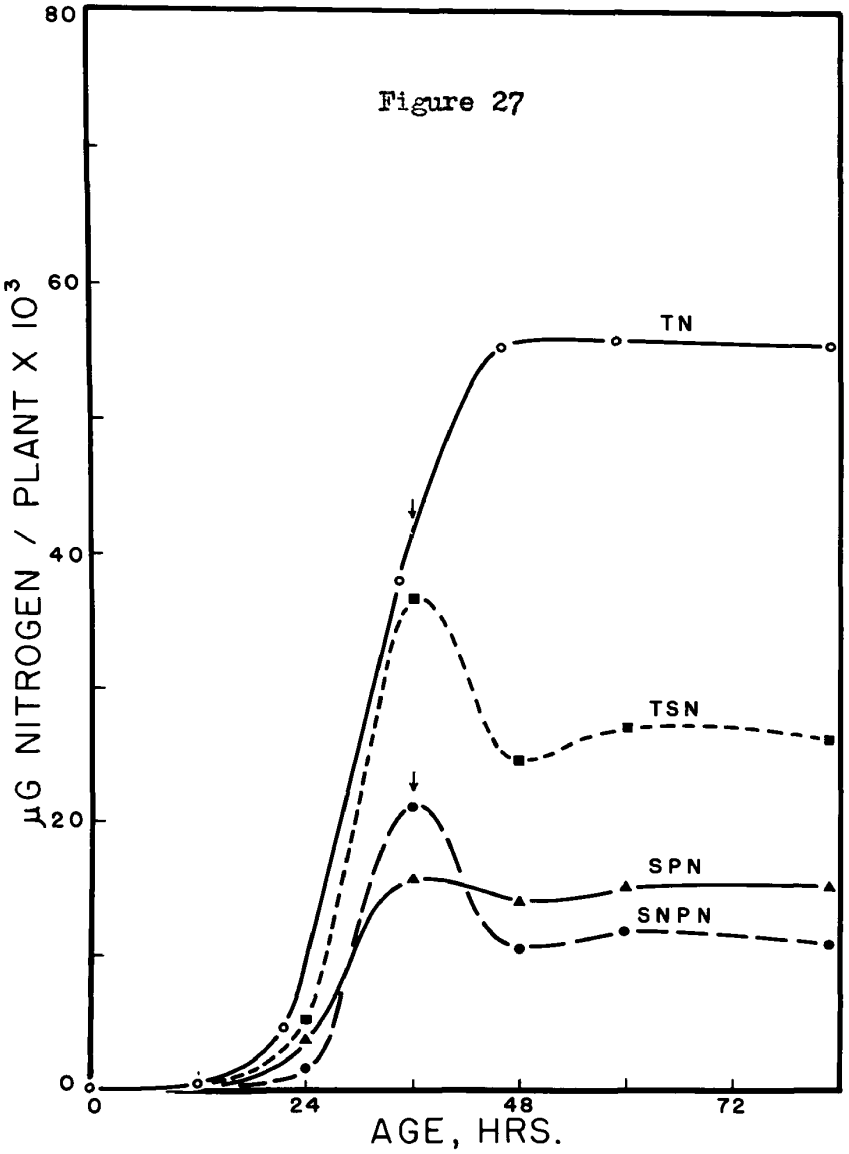


Figure 26

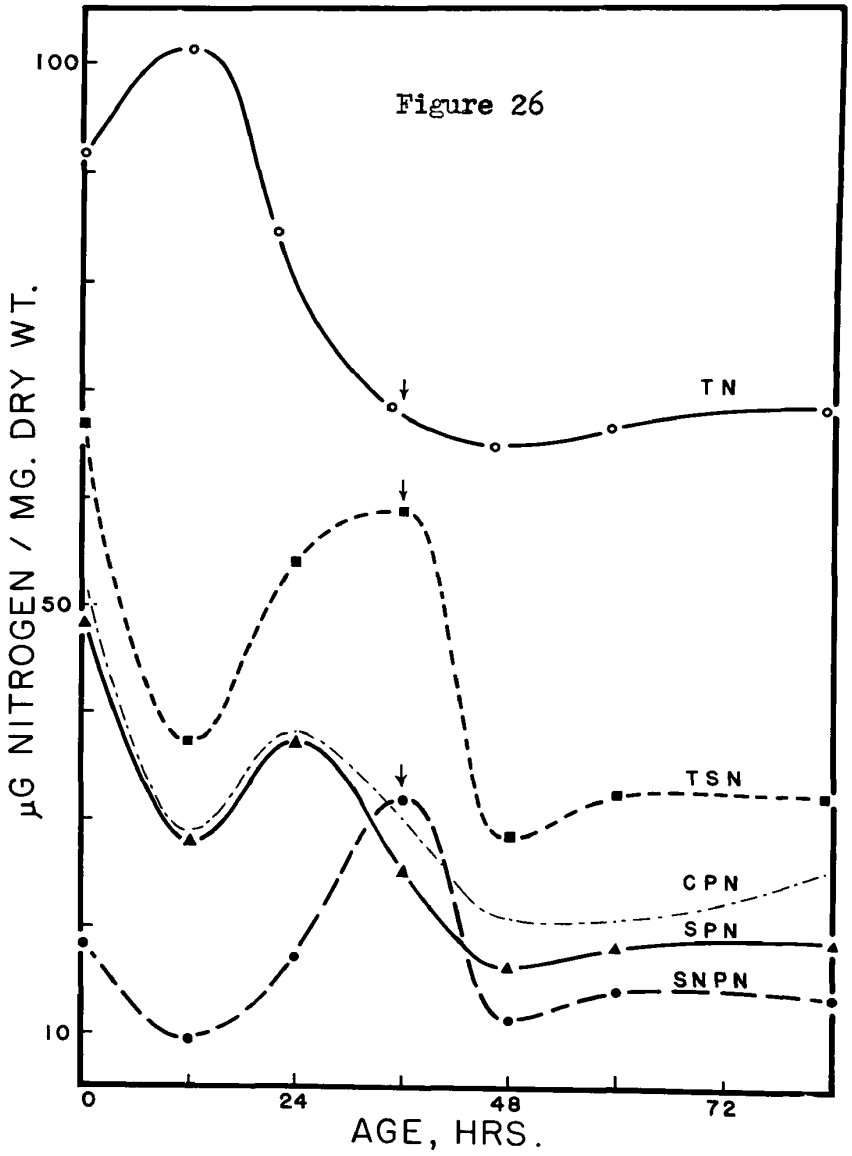
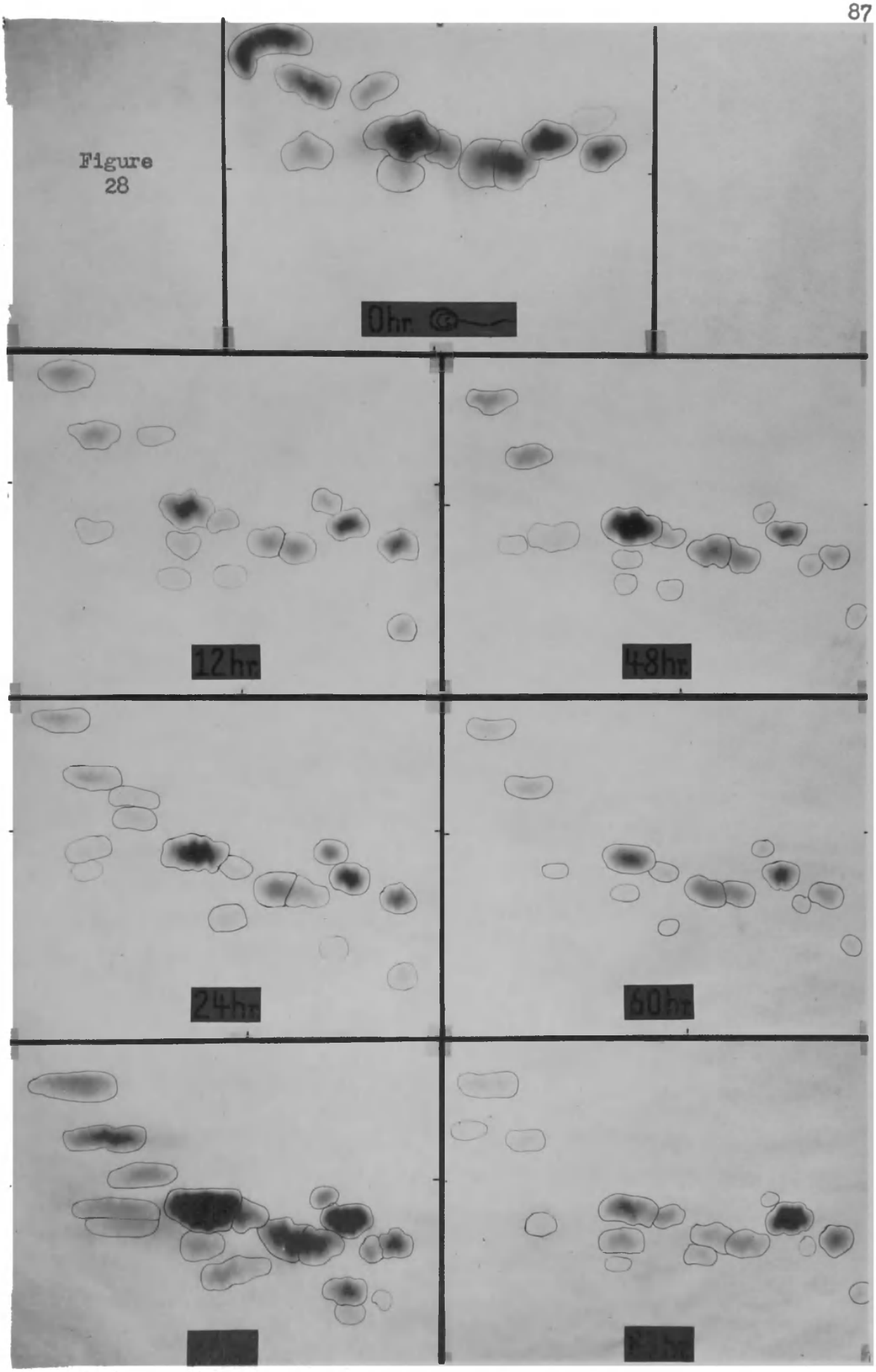


Figure 28. The Soluble Amino Acids of R.S. Plants During Development.

The zoospore chromatogram was prepared with an extract of ca. 3.42 mg. dry weight of spores; the 12 to 83 hour plant chromatograms, an extract of 5 mg. dry weight. Chromatography was carried out in the long direction with phenol-water, in the short direction with butanol-propionic acid-water, and the amino acids detected with ninhydrin.

Figure
28



general changes in the total amino acid levels, certain individual compounds increased or decreased differentially. In particular, the following should be mentioned: (1) the relatively high pools of both alanine and glutamate throughout; (2) the appearance of asparagine at 83 hours; and (3) the sharp rise in the glutamate pool at 83 hours. In short, a considerable reorganization of the pathways leading to and from the soluble nitrogen pools must have occurred during the differentiation process.

DISCUSSION

The Glucosamine-6-phosphate Synthesizing Enzyme in *Blastocladiella*

As a result of purification studies it was established that the GA-6-P synthesizing enzyme in *Blastocladiella* was very similar to the glutamine-F-6-P transamidase of *Neurospora*. However, the somewhat anomalous results obtained in its pH curve merit further comment (c.f. Fig. 5). At first glance it might be presumed that the relative increase in activity with G-6-P at high pH, and its decrease at low pH, was due to the presence of the phosphoglucose isomerase. The latter, whose pH optimum is around 9.0, loses better than 90% of its activity at pH 5.0 (Slein, 1955). The weak activity of the isomerase would indeed by expected to decrease the overall rate of low pH, and by the same token, it would explain the slight increase in activity with F-6-P at the same pH, i.e., little would be removed by isomerization to G-6-P. At pH 8.5, however, the G-6-P activity exceeds that with F-6-P as the substrate and this could hardly be due to isomerase alone. In reaction mixtures in which only F-6-P was present at the start, the concentration of the fructose isomer would always be higher than in those where it was being produced from G-6-P via the isomerase.

Since the unfractionated supernatant was used in these experiments, it appears possible that a second enzyme system in the crude homogenate utilized G-6-P in the same manner as the liver enzyme described by Pogell and Gryder (1957). The capacity of G-6-P to elute enzymatic activity from the gel preparations (in this respect even superior to

the synthetic F-6-P containing no G-6-P) provides some support for the idea of a second enzyme. The fact that the capacity of G-6-P to promote the reaction decreases during R.S. development, and that this cannot be explained by decreased isomerase action also suggests a second pathway to GA-6-P via this sugar. Only separation of the two (if there are two) types of activity from each other, and from the isomerase, will remove the problem from the area of speculation where it now rests securely.

Growth vs. Differentiation in Blastocladiella

The Point of No Return.--The photographic record and the linearity of the logarithmic growth curves during the first 30 hours suggest, first, that the plants are reasonably well synchronized and, second, that the rate of growth is constant during this period. Up to the age of 30 hours the plants appear identical with O.C. thalli at a comparable stage in their generation time. The first morphological indication of development toward R.S. plants can be seen (c.f. Fig. 9, 30 hr.) as an accumulation of cytoplasmic material in the terminal portion of the thallus, i.e., the region destined to become the resistant sporangium.

Although the logarithmic curves for the parameters investigated during this study are not all precisely linear for the period from 0 to 30 hours, the average rates (i.e., slopes) are actually quite similar to those for the dry weight and volume increases. Thus, in spite of some slight differences in the rates for individual parameters at different periods, this made it difficult to detect any effects associated

with differentiation rather than growth *per se*. For example, the maximal rate for chitin deposition *per plant* occurs from 0 to 12 hours, that for glucosamine synthetase and glucose-6-phosphate dehydrogenase activity as well as synthesis of non-protein soluble nitrogen (SNPN) and soluble protein nitrogen (SPN), between 12 and 24 hours, and that for melanin deposition from 24 to 36 hours. But, irrespective of these slight changes, the quantities and activities per plant of each of the above, as well as total nitrogen (TN), lipid, etc., increases steadily during the period of linear growth.

The capacity of such plants to revert to an O.C. type when they are removed from the bicarbonate, provided they are not more than *ca*. 36 hours old, proves that no irreversible changes essential to the structure and function of an R.S. plant have occurred up to that stage. By the same token, it can be assumed that any changes occurring thereafter are definitely associated with R.S. differentiation. Whatever the specific processes may be which cause formation of the septum and cessation of growth, they have a profound effect upon the whole cell which appears to reach its climax at 36 hours.

Ontogenetic Changes on a Per Plant Basis.--The value of expressing data in terms of the individual plant cannot be overemphasized when considering the problem of growth vs. differentiation. This approach has been found useful in studying the development of such diverse organisms as amoebae (Prescott, 1955), and higher plants (Brown and Robinson, 1955). In *Blastocladiella*, the activity of glucosamine

synthetase per gram dry weight reaches a maximum at approximately 24 hours, yet the activity per unit protein fails to reach its peak until about 48 hours. However, it can be seen that the total activity per plant attains its apex at 36 hours, then declines (c.f. Figs. 12-14). Two important conclusions can be drawn from these facts: 1) the 24 hour peak, **per unit dry weight**, results from a more rapid synthesis of the enzyme than of other protein constituents (c.f. log plot in Fig. 14B for increased slope); 2) the increased specific activity reflects a decrease in other soluble (enzymatic ?) proteins after 36 hours, rather than a net synthesis of glucosamine synthetase. This observation is important because it leads to the conclusion that the retention of this enzyme is required for the continued synthesis of chitin in the R.S. after 36 hours. The synthetase reaches its maximum quantity per cell at the stage when approximately 50% of the total chitin is still to be formed.

The behavior of the synthetase when G-6-P is used as substrate is more difficult to interpret. The peak in specific activity at 21 to 22 hours may mirror the highest level of isomerase activity reached at this age (c.f. Fig. 24). On both per weight and per plant bases, maximum synthesis of GA-6-P from G-6-P occurs at the same age as does that from F-6-P. The possibility that the decrease in G-6-P utilization relative to F-6-P after 36 hours was due to the declining activity of a second G-6-P specific enzyme has already been mentioned. There is, at present, insufficient information to warrant a definitive explanation of this phenomenon.

It is interesting to note that while both chitin content (c.f. Table XVI) and maximal specific activity (1.29 μM/mg. protein/20 min.) of the synthetase increases when plants are grown in the higher bicarbonate concentration, the amount of enzyme per gram dry weight does not. Although per-plant data were not obtained for this particular phase of the work, it can be inferred that the changes are the result of increased utilization by preferential diversion of substrates through that synthetic pathway.

The leveling off of the TPN-specific glucose-6-phosphate dehydrogenase activity at 36 hours, followed by a fairly large increase again after 60 hours, implies that during R.S. differentiation this system takes on added significance in cellular energy metabolism or the production of essential metabolites. Both TPN-specific glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activity are present in mature O.C. plants (Cantino and Horenstein, 1959) which have an endogenous Q_{O_2} of about 10 to 30 (McCurdy, 1959). On the other hand, the shift toward a typically oxidative pathway during differentiation of R.S. plants, suggested by the increase in glucose-6-phosphate dehydrogenase, is somewhat difficult to reconcile with the greatly reduced Q_{O_2} (0.1) of the mature R.S. plant (Cantino, Lovett, and Horenstein, 1957). The lack of cytochrome oxidase in mature R.S. plants (Cantino and Horenstein, 1955) precludes the use of reduced TPN via a terminal oxidase. Presumably it would also not be involved in the polyphenol oxidase system (which can be coupled to TPN specific reductions; Cantino and Horenstein, 1955) since no net synthesis of

melanin occurs after 60 hours. However, one obvious possibility would be its coupling with the reductive carboxylation of α-ketoglutarate. The actual amount of reduced TPN produced in the maturing R.S. plants and the extent of its utilization in synthetic reactions during ontogeny are at present unknown, but it would be an interesting area for future investigation.

The bimodal aspect of the relationship between age of thalli and their chitin content (c.f. Fig. 18) apparently results from a higher rate of chitin synthesis, as compared to other cell constituents, during the first 12 hours (c.f. rates in Fig. 19). Exactly the opposite relationship occurs during the period from 12 to 36 hours. Yet, it became a simple matter to interpret this apparently anomalous behavior when it was found that most of the other parameters of growth increase most strikingly (c.f. log plots) from 12 to 30 hours while the rate of chitin synthesis actually decreases.

Chitin deposition lies at the opposite end of the biosynthetic sequence which begins with the glucosamine synthetase reaction; at first glance, chitin synthesis might be expected to mimic the changes in activity of the synthetase. However, changes in any of the intermediate steps could, and probably do, alter this relationship. Furthermore, it is obvious that the rate of chitin synthesis would also be dependent upon the availability of hexose-phosphates and competition by other systems requiring the same substrates. Such an interpretation may be invoked to explain the discrepancy between the maximal rates for chitin accumulation from 0 to 12 hours, and for glucosamine synthetase

from 12 to 24 hours. These results make it manifestly clear that the specific activity of an enzyme *per se* only indicates its potential and cannot yield more than a partial understanding of the actual rate of the reaction *in vivo*. In fact the different methods of defining specific activity, e.g., on the bases of dry weight, nitrogen, protein, etc., often serve to confuse rather than clarify the situation.

But, notwithstanding the difficulties in interpreting its enzymological basis, it should be noted in conclusion that while chitin accumulation accounts for approximately 21% of the increase in dry weight after 36 hours, it contributes only about 11% to the final dry weight of the individual mature plant.

The synthesis of lipid exactly parallels the increase in dry weight (logarithmic plots) and reaches its maximum quantity at 48 hours (c.f. Fig. 23). It is again interesting to observe that lipid synthesis alone accounts for 24% of the increase in dry weight from 36 to 48 hours, and contributes almost 20% of the weight at this age. The subsequent decrease in fat from 48 to 83 hours accounts for 50% of the decrease in dry weight which occurs during this interval. The significance of the change is unknown, but it is possible that it is reutilized for synthetic processes, or perhaps converted to forms not readily extractable. The decrease is correlated with the appearance, by 83 hours, of the fairly large, distinct "lipidic globules" characteristic of the cytoplasm of mature R.S. (Fig. 9, 83 hr.). In any event, chitin and lipid together make up almost one-third of the total dry material in mature plants. The magnitude of the fat accumulation leads

to the supposition that this is for the "purpose" of efficient energy storage since R.S. can remain viable for several years (Cantino, unpublished).

The disruption of the citric acid cycle by bicarbonate (Cantino and Hyatt, 1953c) provides a possible explanation for the induction of increased fat synthesis in the developing R.S. plant. If acetyl-coenzyme-A was unable to enter the cycle by way of the condensing enzyme system, its accumulation could lead to the increased synthesis of fatty acids via the conventional pathway. The synthesis of over 90% of the fatty acid carbon from acetate has been shown by Ottke, et al. (1951), for Neurospora, and for yeast by White and Werkman (1947). Of even greater interest are the reports of bicarbonate- and reduced TPN-dependent systems for the synthesis of long chain fatty acids from acetyl-coenzyme-A in pigeon liver (Gibson, Titchener, and Wakil, 1958) and avocado (Squires, Stumpf, and Schmidt, 1958). The intriguing possibilities of such a synthesis in relation to the bicarbonate effect in Blastocladiella are obvious, particularly in view of the build-up of an active zwischenferment which could provide the necessary pool of reduced TPN in the developing R.S.

The synthesis of γ-carotene is characteristic of the R.S. since its presence cannot be detected in the normal mature O.C. plants (Cantino and Horenstein, 1956). The function of this pigment is unknown although it is produced by a very few (less than 2%) thin-walled plants of Blastocladiella, and occurs in the male gametes of the closely related genus Allomyces. Although the time course for γ-carotene

production was not established quantitatively, it appeared from visual inspection to roughly parallel the increase in lipid during growth of R.S. plants. The formation of β-carotene in Mucor heimalis involves the utilization of acetate carbon (Grob and Butler, 1956). This presumably occurs via the formation of mevalonic or dimethylacrylic acid from acetyl-coenzyme-A, followed by polymerization into larger isoprenoid units. Such a synthetic sequence in Blastocladiella could be favored by the backing up of acetate, or acetyl-coenzyme-A, in the same fashion as was suggested for induced fat production. Evidence that this may be so stems from the observations (Cantino and Hyatt, 1953b) that a mutant of Blastocladiella with a lesion in the Krebs cycle accumulates large quantities of γ-carotene.

A functional polyphenol oxidase system in mature R.S. plants and its coupling (established in vitro) with the reductive carboxylation of α-ketoglutarate has been described by Cantino and Horenstein (1955). In the present study it was determined that the in vivo deposition of melanin occurs most rapidly during the period from 24 to 36 hours, but the age at which the polyphenol oxidase first appears is still unknown.

Nitrogen Metabolism and Differentiation.--The increase in total nitrogen per plant (as a logarithmic function) closely parallels that for its dry weight (c.f. Figs. 25 and 11). On the other hand, a decrease in soluble nitrogen and an increase in total nitrogen per unit dry weight occurs during the first 12 hours of growth. The difference between the soluble and total quantities in swarmers (ca. 25 µg./mg. dry weight) is of the same order of magnitude as the nucleic acid

nitrogen at this stage (Turian and Cantino, 1960).[1] Before the swarmers germinate, these nucleic acids are associated almost entirely with two distinct structural units, the nucleus (DNA), and the nuclear cap (RNA) which would be expected to sediment with the cellular debris upon centrifugation.

During the first 12 hours of growth, the increase in total nitrogen is largely due to chitin synthesis. On the other hand, the apparent decrease in soluble protein per milligram dry weight during this period may be an artifact due to the incorporation of protein into the rapidly expanding wall and rhizoidal system of the young plants. Plants homogenized at 12 hours do not fragment but merely crack open to release their protoplasts. The shapes of the sporangia and rhizoids remain remarkably intact and it is possible that the proteins in the rhizoids and some of those in or on the sporangial wall were not recovered.

A decrease in the free amino acid pools also occurs in the first few hours and it is assumed that this results from both uitlization and dilution due to rapid growth. A most striking phenomenon is the rapid increase in the soluble non-protein nitrogen (SNPN) between 24 and 36 hours (c.f. Figs. 26 and 27). The corresponding increase in the free amino acids demonstrates that the rise is largely due to changes in these pools. On a dry weight basis, the SNPN doubles during this interval while the soluble protein nitrogen (SPN) decreases.

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[1]The swarmers contained ca. 203 to 287 µg. total nucleic acid/mg. dry weight. If a nitrogen content of 16% is assumed for Blastocladiella nucleic acid (based on yeast data), the above quantity of nucleic acid would contribute ca. 32 to 46 µg nitrogen/mg. dry weight.

Once again, however, the changes are more easily understood when considered on a per plant basis. This approach reveals that a differential increase in amino acids vs. protein occurs, the process reaching its climax at 36 hours. At this stage the SNPN exceeds the SPN by about 34 to 35%, and the two together comprise all but a small fraction (6%) of the total non-chitinous nitrogen of the plant.

The large change in the free amino acid pools could, of course, result from uptake of nitrogen from the medium, the breakdown and reorganization of proteins, or both. That the two processes may be involved is strongly suggested by the results of electrophoretic studies (Cantino, Lovett, and Horenstein, 1957); three out of four soluble protein fractions in O.C. plants were absent in R.S. plants, while the latter contained a soluble protein not found in O.C. plants. The reversibility of the system up to approximately 36 hours would suggest that these changes must occur either during (or after) this time, or they must be easily and rapidly reversible.

Failure to detect an increase in the soluble proteins from 36 to 48 hours, concomitant with the 50% decrease in SNPN, clearly implies conversion of the latter to chitin, insoluble protein, and/or purines and pyrimidines and nucleic acids. Although insufficient data are available, certain general conclusions can be drawn concerning the fate of this fraction. At the very most, the increase in chitin during this period could only account for 26% of the decrease in SNPN. Also during the same time interval, the increase in total nitrogen is 1.4 times the decrease in SNPN, indicating that nitrogen uptake is still occurring.

This obviates any necessary correlation between the increase in chitin and the decrease in the SNPN pools. It does, however, mean that these soluble pools are being utilized more rapidly than they are being replenished.

Finally, consideration of the ultimate function of a mature R.S. permits an educated guess concerning the form of the insoluble, non-chitinous nitrogen compounds produced during the 36 to 48 hour period. The R.S. plant eventually uses essentially all of its protoplasmic contents to produce motile swarmers. If the insoluble, non-chitinous nitrogen is calculated as the equivalent amount of nucleic acid per milligram dry weight of protoplasm (see footnote on p. 98), a value of about 200 μg is obtained. This is exactly the order of magnitude actually obtained by analysis of swarmers, a remarkable correlation indeed! Thus, the data suggest that a considerable proportion of the SNPN is used for the synthesis of nucleic acids.

The subject matter just discussed provides some explanation of the changes in the overall level of the free amino acid pool during the growth and differentiation of an R.S. plant. It is more difficult to ascribe a role to the changes which particular amino acids undergo. The almost complete disappearance of tyrosine (an established substrate for the polyphenol oxidase in Blastocladiella) after 36 hours, may well be due to its rapid oxidation for melanin synthesis. The relatively large alanine, and small glutamate, pools at 48 hours correspond to the peaks in both isocitritase and glyoxalate-alanine transaminase activities which occur at the same time (McCurdy, 1959), and which are known

to provide a potential pool of glycine for biosynthesis in Blastocladiella. This is consistent with the notion proposed above that nucleic acid synthesis, and therefore purine and pyrimidine synthesis, occur after 36 hours. Glutamate could play a two-fold role in the system: first, as the source of the alanine amino group by transamination with pyruvate (McCurdy, 1959); and second, by amidation, as the source of the glutamine required for purine synthesis. Aspartic acid, which also decreases sharply after 36 hours, is involved in the synthesis of both purines and pyrimidines in other organisms and might play a similar role in Blastocladiella.

The disproportionate increase in the glutamate pool at 83 hours is interesting but, for the time being, inexplicable. It is of course possible that the increase is an indication of its activity in various transaminations, i.e., that it serves as a nitrogen bottle neck or funnel for nitrogen transformations, and that it increases in concentration when no longer actively utilized. A rather unique role for glutamate in Blastocladiella was earlier suggested by the fact that it was the only amino acid able to serve as the primary source of nitrogen in a synthetic medium (Barner and Cantino, 1952). The in vivo synthesis of glutamate from glucose-U-C^{14} without apparent mediation of the citric acid cycle, and its slow equilibration with α-ketoglutarate (Cantino and Horenstein, 1956) also suggest that this amino acid displays unusual behavior in Blastocladiella.

General Conclusions.--The process of R. S. development can be summarized as follows: The first observable effect of the bicarbonate

inducing agent is a reduced growth rate, presumably by a disruption of the oxidative metabolism of the citric acid cycle and its associated cytochrome system. With this exception no other changes are detected until 24 to 36 hours after spore germination. At about 30 hours the morphologically distinguishable migration of cytoplasm toward the site of the future sporangium occurs. This is followed by changes in enzyme synthesis and other cellular processes indicating a fundamental reorganization within the cell. The culmination of these processes by ca. 36 hours is accompanied by the irreversible commission of the plant toward R.S. formation. Following this stage, a period best described as the maturation phase ensues, during which further changes in enzymes and storage products occur. The result is the round, melanin-pigmented, thick-walled, lipid- and γ-carotine-containing R.S., lacking cytochrome oxidase as well as most of the citric acid cycle enzymes, and displaying a very low respiratory activity. This structure surmounts an essentially empty and lifeless lower stalk.

As might perhaps be expected, each new bit of information gained in a study of this kind raises more new questions than it answers old. However, at each stage of our accumulating knowledge we are in a position to frame these questions with increasing sophistication and precision.

Probably the most fundamental question posed by our present knowledge is the following: since it is known that the process leading to R.S. formation becomes increasingly irreversible from about 24 to 36 hours, what system or systems are responsible, how are they formed, and at what stage do they begin to evolve toward this condition? It will

obviously be impossible to completely answer this question for some time, but it is instructive to consider the possible mechanisms by which such a situation may come to exist.

Weiss (1949) has suggested that a cell could be considered statistically in terms of the "ecology of an organized molecular population," with all the interactions and interdependencies which the term ecology implies. If such a concept is accepted, growth could be defined in Weiss's terms "as the increase in the size of a given population without essential change in character"; and, "differentiation, as a progressive change in the composition of the molecular population including the appearance of new, and the disappearance of old, species." Such a definition necessarily implies that any morphological changes must be preceded by differentiation at the molecular level.

In the instance of Blastocladiella the first morphologically detectable differentiation is the cytoplasmic migration at 30 hours. It must be assumed that some molecular changes had already occurred previous to that time. The only obviously new parameter so far demonstrable before 30 hours is a slight amount of melanin synthesis beginning at about 24 hours. However, it is known that this process can be uncoupled from R.S. differentiation without otherwise essentially altering its progress (Cantino, 1953). Tentatively, therefore, it can be presumed that it is not, of itself, important in causing the plant to differentiate. The fact that melanogenesis is, nevertheless, a characteristic of the R.S. developmental sequence, leads to the conclusion that its initiation must result from even earlier submicroscopic

events. Thus it can be stated with reasonable certainty that some change, or changes, involved in R.S. differentiation must occur before 24 hours of age, although no time limit can be placed as to the earliest alteration.

Having at least narrowed down the limits, our attention can be addressed to the first part of the question concerning the systems involved and how they came to exist. With the limited knowledge at our disposal this can, at best, only be done in very general terms.

At least one primary effect of bicarbonate has already been described, i.e., the operation of the SKI cycle and disruption of the normal citric acid cycle. How, then, can this lead to the differentiation of an R.S. plant? Essentially two possibilities exist, both based on the assumption that the bicarbonate mechanism results in the piling up of intermediates; it has already been demonstrated for α-ketoglutarate that such piling up does in fact occur. The first is that there is an increased utilization of specific pre-existing, or constitutive, enzymatic pathways leading to an overall quantitative shift in the intracellular balance. The second is that in addition to the constitutive systems, new and unique enzymatic sequences may be induced, and certain old ones eliminated, by the presence of increased levels of endogenously produced intermediates.

The evidence to date strongly suggests that the second alternative more adequately describes the situation in Blastocladiella. At least one enzyme system, the polyphenol oxidase, is formed de novo during R.S. development, and the formation of a second, for γ-carotene

synthesis, is probable. Other pathways which are known to be altered, or could possibly be altered, by intracellular intermediates have already been mentioned. The reversibility of the Blastocladiella system up to a critical point appears to distinguish it from that in bacteria where an enzyme once induced is not broken down again but merely diluted out of existence by subsequent growth and reproduction (Hogness, Cohn, and Monod, 1955). The intracellular changes in this organism appear to be much more akin to the state of "dynamic equalibrium" found for mammalian cells (Heimberg and Velick, 1954; Velick 1956). This supposition is supported by the complete loss of several constitutive enzymes, and the changes in the glucosamine synthetase, glucose-6-phosphate dehydrogenase and isomerase enzymes (c.f. Fig. 24). It should be noted with respect to Figure 24 that after 36 hours there is no increase in the soluble protein, nor does growth occur after 48 hours. Despite this, the levels of the few examples studied do change quantitatively; both increases and decreases occur, i.e., the proteins are presumably being synthesized at certain periods and degraded at others.[1]

A portion of the original question posed has been answered; part remains unanswered. However, though it is evident that there are large gaps in our knowledge of the cellular processes in Blastocladiella, the concept of a dynamic equilibrium, influenced and rechanneled by the effects of bicarbonate, has proved useful as a working hypothesis.

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[1]The argument that these changes could equally well occur as the result of the inhibition, or vice versa, of existing and potentially catalytic proteins cannot be ignored. This possibility could only be eliminated by labeling experiments with isotopic tracers during different stages of development.

It is only a logical extension of this idea to suggest that these effects lead to an increasingly altered metabolism, the process eventually becoming autocatalytic and irreversible. The latter presumably occurs when a key intermediate, or synthetic pathway, reaches a critical threshold and, by so doing, shifts the whole equilibrium in a new direction. Until such a threshold is reached, the system could remain reversible by removal of the stimulus, i.e., bicarbonate.

The technique described herein for growing reasonably synchronized large scale cultures of Blastocladiella emersonii has made feasible a direct approach to the problem during the early critical periods of growth. The results obtained by this technique have provided a frame of reference to serve as a guide in developing the experimental approaches most likely to yield answers to some of the manifold questions associated with morphogenesis in Blastocladiella.

SUMMARY

1. The enzyme glucosamine synthetase (glutamine-F-6-P transamidase) was purified ca 19-fold from extracts of Blastocladiella emersonii and some of its properties studied.

2. A method was developed which made possible, for the first time among the Phycomycetes, the study of physiological and morphological processes during the well synchronized growth of Blastocladiella from zoospores to mature resistant sporangial plants.

3. The enzymes glucosamine synthetase, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase, as well as several other nitrogenous and non-nitrogenous cellular components were studied during the course of resistant sporangial development.

4. The significance of the changes in the cellular components as they related to the structure and function of the developing organism was discussed. An attempt was made to integrate the physiological and morphological processes involved in the differentiation of the resistant sporangium.

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APPENDICES

APPENDIX I

List of Abbreviations

AG-1-P N-acetylglucosamine-1-phosphate
AG-6-P N-acetylglucosamine-6-phosphate
AG-1,6-DP N-acetylglucosamine-1,6-diphosphate
ATP ..... Adenosinetriphosphate
BCP ..... Bromcresol purple
DNA ..... Desoxyribose nucleic acid
DPN ..... Diphosphopyridine nucleotide
EDTA ..... Ethylenediaminetetraäcetic acid
F-6-P Fructose-6-phosphate
FDP Fructose-1,6-diphosphate
G-1,6-DP Glucose-1,6-diphosphate
G-6-P Glucose-6-phosphate
GA ..... Glucosamine
GA-6-P Glucosamine-6-phosphate
GA-1-P Glucosamine-1-phosphate
Pi ..... Inorganic phosphate
RNA ..... Ribose nucleic acid
SPN ..... Soluble protein nitrogen
SNPN ..... Soluble non-protein nitrogen
TCA ..... Trichloroacetic acid
TN ..... Total nitrogen
TSN ..... Total soluble nitrogen
TPN ..... Triphosphopyridine nucleotide
UDPAG ..... Uridinediphosphate N-acetylglucosamine
UDPGA ..... Uridinediphosphate glucosamine

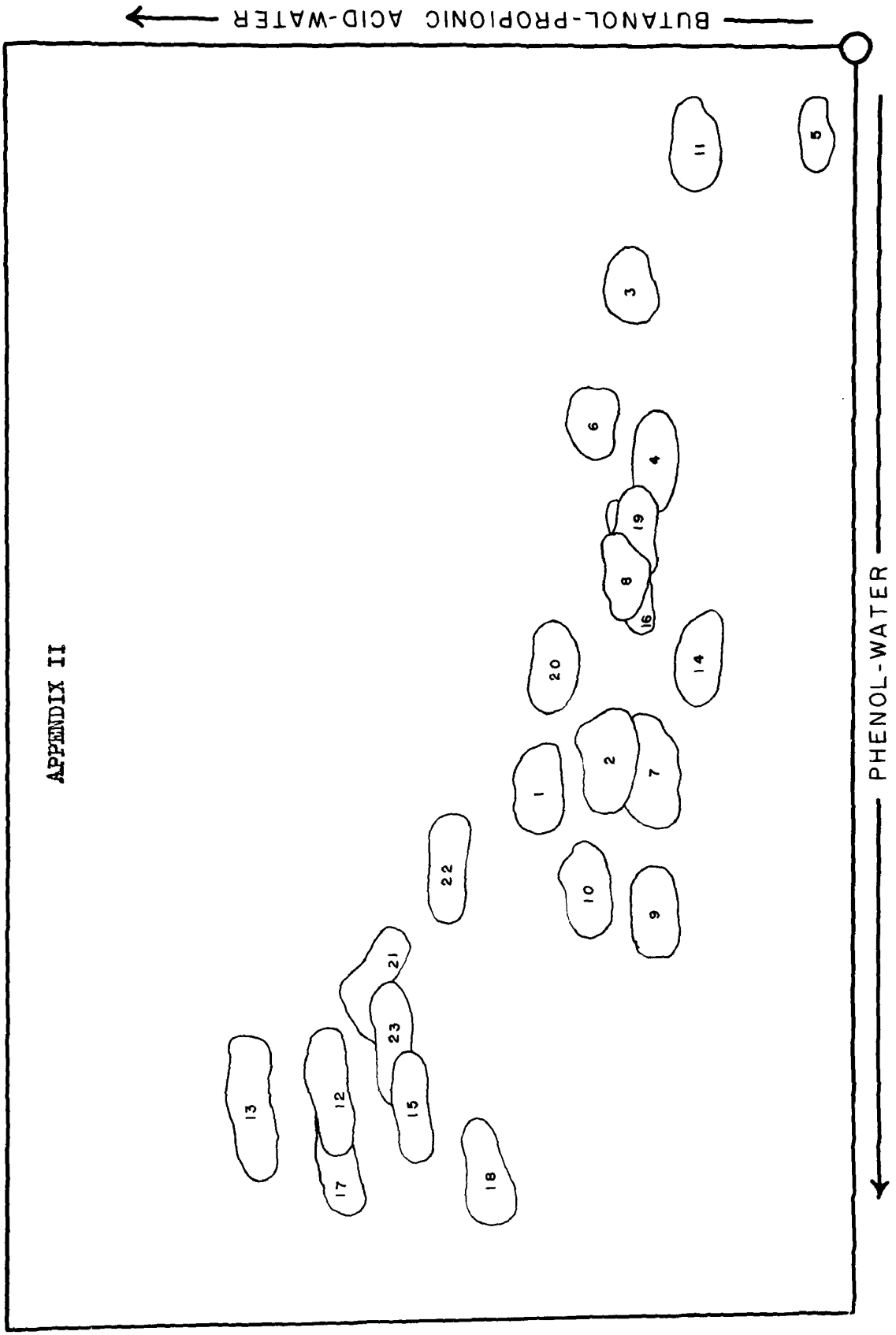
APPENDIX II

Two-dimensional Chromatographic Map of Amino Acids

Key

1. L-alanine
2. L-arginine
3. L-aspartic acid
4. D-glucosamine
5. D-glucose-6-phosphate
6. L-glutamic acid
7. L-glutamine
8. L-glycine
9. L-histidine
10. L-hydroxyproline
11. Inorganic phosphate
12. L-isoleucine
13. L-leucine
14. L-lysine
15. DL-methionine
16. N-acetylglucosamine
17. L-phenylalanine
18. L-proline
19. DL-serine
20. DL-threonine
21. L-tryptophan
22. L-tyrosine
23. DL-valine

APPENDIX II


BUTANOL-PROPIONIC ACID-WATER
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APPENDIX III

CHROMATOGRAPHIC MOBILITIES OF SELECTED COMPOUNDS

Solvent System	Propanol-ammonia-water			Butanol-propionic acid-water	Phenol-Water	Ethanol-ammonium Acetate	
Paper[1] / Compound	W1	WW1	W41H	W1	W1	W1	W41H
F-6-P (Schwarz)	.16, .22	-	-	.04, .10	-	-	.45
F-6-P (Synthetic)	-	.46	.19	-	-	-	-
FDP	-	.20	-	-	-	-	-
G-6-P	.18	.43	-	.05	.07	-	-
GA-6-P (Roseman)	.16	.30	.17	.03	-	.18	.38
GA-6-P (Blastocladiella)	.16	-	.18	.03	-	.18	-
Roseman & Blastocladiella GA-6-P co-chromatographed	.16	-	-	.03	-	-	-
GA-6-P (Synthetic)	-	.32	-	-	-	-	-
Inorganic phosphate	-	.42	-	-	-	-	.40
1,3,4-triacetyl-N-acetyl-GA-6-P	-	.39	-	-	-	-	-
Glucosamine	.56	-	.55	-	.32	-	.58
N-acetylglucosamine	.56	-	-	-	.37	-	-
Glutamic acid	.31	-	.29	-	.27	-	.50
Glutamine	.42	-	.41	-	.57	-	.58

[1]Key: W1 = Whatman # 1 filter paper; WW1 = washed Whatman # 1 filter paper; W41H = Whatman # 41H filter paper.

APPENDIX IV

Summary of Attempts to Purify Glucosamine Synthetase

Treatment	Result as Percentage of Previous Specific Activity*
Fresh plant mat frozen at -18°C. overnight	100
Fresh plant mat frozen at -18°C. 9 days	23
Supernatant stored 8 hr. at 0°C., 2 x 10^{-2}M phosphate buffer, pH 6.8	90
Supernatant stored 13 hr., 0°C., 2 x 10^{-2}M phosphate buffer, pH 6.8	83
Supernatant stored 13 hr., 0°C., 2 x 10^{-2}M phosphate buffer and 10^{-3}M Versene	52
Supernatant (2 x 10^{-2}M phosphate, pH 6.8, 10^{-3}M Versene) heated at 50°C. for 5 min	16
As above, but pH adjusted to 5.9 with 1 N acetic acid, then heated at 50°C. for 5 min	63
As above, but pH adjusted to 6.0 with 1 N acetic acid, then heated at 50°C. for 5 min	68
As above, but pH adjusted to 5.0 with 1 N acetic acid, then heated at 50°C. for 5 min	0
Supernatant dialyzed vs. 2 x 10^{-2}M phosphate buffer, pH 6.8, overnight at 2°C	19-44
Supernatant dialyzed vs. 2 x 10^{-2}M phosphate buffer, pH 6.8, 10^{-3}M Versene, overnight at 2°C	0-52
Treated as above, then added back concentrated dialysate	39
Treated as above, then added back magnesium and pyridoxal phosphate	56
Supernatant (2 x 10^{-2}M phosphate, pH 6.8) incubated with 9 x 10^{-4}M glutathione for 8 hr. at 0°	75
Supernatant (2 x 10^{-2}M phosphate, pH 6.8) incubated with 9 x 10^{-4}M F-6-P for 8 hr. at 0°	96
Supernatant dialyzed vs. 5 x 10^{-3}M F-6-P and 2 x 10^{-2}M phosphate, pH 6.8, for 12 hr. at 0°C	63
Supernatant dialyzed vs. 10^{-4}M glutathione, 10^{-3}M Versene, 2 x 10^{-2}M phosphate, pH 6.8, for 12 hr. at 0°C	84
Supernatant (2 x 10^{-2}M phosphate, pH 6.8) treated with protamine sulfate, centrifuged and supernatant assayed.	190-299
As above, but with a mature first generation culture	404
Supernatant (1st. generation plants, homogenized in dist. water) treated with protamine sulfate (pH 5.8) 0.17 mg. /mg. protein, centrifuged and the supernatant assayed	318-643
A protamine supernatant cut at 80% $(NH_4)_2SO_4$, the precipitate redissolved in 10^{-1}M phosphate, pH 6.8, assayed	60

*Refers to the specific activity of the immediately preceding step.

Appendix IV - continued

Treatment	Percentage
As above, but treated with 100% saturation of $(NH_4)_2SO_4$.....	54
A protamine supernatant fractionated with 80% saturation of ammonium sulfate, the precipitate redissolved in 10^{-1}M phosphate, pH 6.8, and dialyzed against the same buffer for 2 hr. at 0°C.....	25
Protamine supernatant cut with 80% saturation ammonium sulfate, the precipitate redissolved in 10^{-1}M phosphate, pH 6.8, and recut with 35% saturation ammonium sulfate, the precipitate dissolved in the same buffer, then assayed.....	135
Protamine supernatant dialyzed vs. 90% saturated ammonium sulfate(adjusted to pH 6.8), precipitated proteins redissolved in 10^{-1}M phosphate, pH 6.8, and assayed.....	59
Protamine supernatant treated with tricalcium phosphate gel (3.7 mg./mg. protein), eluted with 5 x 10^{-1}M phosphate buffer, pH 7.5.....	74
Protamine supernatant adjusted to 5.6 with M sodium acetate, treated with tricalcium phosphate gel (0.85 mg./mg. protein), gel eluted with 10^{-1}M phosphate, pH 7.0.....	128
Protamine supernatant, as above, treated with tricalcium phosphate gel (1.2 mg./mg. protein), gel eluted with 5 x 10^{-3} F-6-P.....	149
Supernatant (distilled water), stored overnight at 0°C.....	24
Protamine supernatant, stored overnight at 0°C.....	9
F-6-P gel eluate, stored overnight at 0°C.....	48