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pH INDUCED AGGREGATION IN CHLORELLA

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

One of the major technical difficulties associated with the use of unicellular alga for biomass production is harvesting. When grown on a large scale, photoautotrophic unicellular algae such as Chlorella cannot be economically separated from the medium. However, if aggregated or clumped, these cells can be readily separated from their growth medium.

The phenomenon of aggregation in Chlorella vulgaris Beijerinck has been observed to be inducible by alkaline pH (9.5) and has been investigated structurally, morphologically and biochemically.

The pattern of the pH-induced aggregate formation exhibits two stages. The first phase (0-24 hours) is characterized by a 7-fold increase in cell volume. Cellular chlorophyll, protein and DNA content also increase significantly, probably representing the prevention of autospore release. Also characteristic of this first phase are changes in cell wall metabolism as evidenced by an increase in cellulase specific activity and polysaccharide content. Changes in cell wall morphology cannot be detected.

The second stage (24-96 hours) is characterized by clustering of the enlarged cells. Electron micrographs taken during that stage reveal the existence of a number of autospores within a modified, multilayered mother cell wall. The pectin content of cells at that stage is twice that of cells grown at pH 6.3. In addition, there are changes in distribution among the different pectin fractions as a result of the

alkaline treatment.

The results are interpreted as the repeated failure of the autospores to completely detach from their original mother cell walls, thus forming clusters which represent several generations of cells. In the final stage, modification of the polysaccharides in the cell wall results in the adsorption of the clusters to each other.

Under alkaline conditions, an alteration of certain events of the cell cycle seems to occur, resulting in the prevention of autospore release. The alkaline-pH sensitive events occur during the first 8-12 hours of the cell cycle. Transfer of the culture to an alkaline pH after that time, does not prevent autospore release. These results suggest the existence of a "point of no return" in the life cycle of Chlorella. In addition, the ability to synthesize protein and DNA increases gradually when a culture is transferred from alkaline pH back to the low pH.

Under alkaline conditions the normal diurnal fluctuation in cellulase specific activity and polysaccharide content does not exist. Thus, their decrease in the second part of the cycle is prevented. It is suggested, that the alkaline pH treatment amplifies and prolongs the diurnal increase in cellulase activity resulting in an increase in the flexibility of the mother cell wall sufficient to prevent autospore release by mechanical rupturing.

להורי

רבקה ואליהו מליס

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INTRODUCTION

I. The cell cycle of Chlorella

Chlorella is a green unicellular alga, belonging to the phylum Chlorophyta, class: Chlorophyceae, order: Chlorococcales, family: Chlorococcaceae.

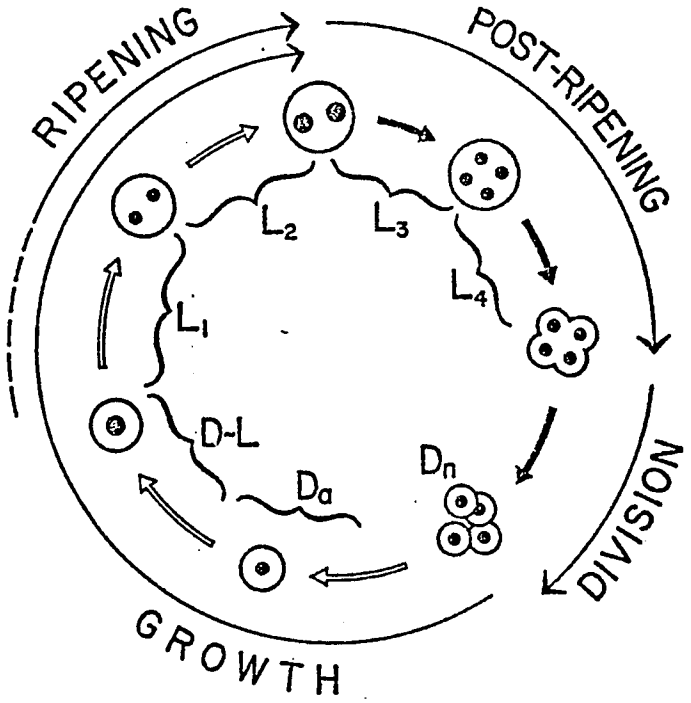
Figure 1 is a schematic representation of the cell cycle of this alga as described by Tamiya (1963b). Daughter cells (Dn) are produced in the dark from a mother cell. Upon illumination these cells continue to develop into L cells using the energy derived from photosynthesis. Thus, an L_1 cell is an unripe L cell which is enlarged but incapable of undergoing cell division when incubated in the dark. An L_3 cell is an L cell which can divide completely when incubated in the dark, and the L_4-stage is that which immediately precedes the liberation of daughter cells. The "growth" phase starts from the Dn stage, which extends to the L_3 stage and requires light. It appears to begin somewhere around the D-L stage and terminates at the L_3 stage. The "post-ripening" phase, which immediately precedes division and does not require light, begins at the L_3 stage and lasts until the L_4 stage. For a more complete description of the cell cycle see Appendix AI.

It was suggested on the basis of experiments with different cell types, that preparation for division involves a series of processes which must be completed before division can occur (Mazia, 1961). These processes are assumed to

Figure 1

THE CELL CYCLE OF CHLORELLA

Schematic representation of the change of cell status (cell size and nuclear pattern) at different stages during the life cycle of Chlorella ellipsoidea. The white arrows indicate light-dependent processes, and the black arrows show the transformation which occurs independently of light (in darkness). From Tamiya (1963b). For further explanation see text.


RIPENING
POST-RIPENING
DIVISION
GROWTH
L1
L2
L3
L4
D-L
Da
Dn

occur either in parallel (Mazia, 1961) or as alternative pathways with some steps in parallel and others in series (Campbell, 1964). The time when the cell has finished its preparation for division is called by Mazia, the "point of no return." At that time a special protein necessary for cell division is formed, and from then on, cell division is not influenced by protein synthesis inhibitors. However, in Chlorella, Moberg et al. (1968) using puromycin, p-FPhe, or DNP, could not show a transition effect, but demonstrated the necessity of protein synthesis at least at the later stages of the cell division process. With chloramphenicol, a transitory effect on cell division was shown. However, the mode of action of chloramphenicol in Chlorella is still not satisfactorily understood (Morris, 1966), which makes it difficult to interpret the results.

Synchrony

Wanka (1965) showed that light is necessary up to a certain stage (about 8 hours), if the cells are to divide. After this stage, light is no longer necessary, indicating that some photochemical process is linked to the preparation for cell division and demonstrating a "point of no return."

One of the earliest and best known synchronous systems is that of Tamiya et al. (1953) who found that alternating cycles of light and darkness would synchronize Chlorella. Tamiya's group also carried out biochemical studies on synchronously grown Chlorella (Tamiya et al., 1953; Tamiya,

1963a; Tamiya, 1963b; Tamiya, 1966). There are a number of ways to produce synchronous cultures. One is to start with Ds cells (the small daughter cells, Fig. 1) which are collected by differential centrifugation of a 7-9 day culture (Tamiya and Morimura, 1964). The Ds cells are then illuminated for 31 hours at 21°C until they have reached L_3 stage. After 16 additional hours in the dark, cell division and separation is completed and all the cells are in the Dn stage. Dn cells are almost identical with the Ds cells except that they are a little larger and can grow faster in the light. This sequence is called the DsLD cycle. It is then followed by a DLD cycle of 15 hours in light and 7 hours in the dark. There is, therefore, a degree of selection and of growth inhibition of these Chlorella cultures after starvation in the dark.

Another synchronization system was developed by Schmidt (Schmidt, 1966; Hase and Schmidt, 1968; Schmidt, 1969). In his procedure, a random culture of a higher temperature strain of Chlorella pyrenoidosa is brought into synchrony by four alternating cycles of light and dark (11 hours each) at 38.5°C (Baker and Schmidt, 1963). The culture is then left in continuous light where it performs a series of synchronous cell releases. After each release, the culture is diluted to its original cell concentration. Recently, however, a method of continuous dilution has been developed which gives a higher yield and eliminates any shock which

might occur as a result of the periodic dilution (Senger and Bishop, 1969). From the point of view of cell cycle this system has two great advantages over Tamiya's system: 1. The culture is synchronous for at least three cycles without any changes in the environment; and 2. Nuclear division occurs later in the cell cycle and is more synchronous.

Other ways of synchronizing Chlorella were also described in reviews by Pirson and Lorenzen (1966), Senger and Bishop (1969), Sitz et al. (1970), and Sorokin (1964). Most of these methods also involve alternating cycles of light and dark with cell release taking place in the dark.

Changes in content of various cell constituents during the life cycle of Chlorella

Using synchronized cultures of Chlorella ellipsoidea the content of the bulk protein, lipid, and carbohydrate was investigated (Hase et al., 1957). In terms of the percentage of dry weight, the protein content, like that of total nitrogen content, remained relatively constant (47-54%) showing only a slight decrease at earlier stages of the growth phase. The percentage of carbohydrates (2-23%) and lipid (19-29%), fluctuated, decreasing temporarily at stages L_1 or L_2 and again decreasing markedly at L_4 and Dn. The decrease of these substances (especially carbohydrates) at the L_4 stage (2%) seems to be due partly to their conversion to other cell constituents and partly to their utilization as substrates of respiration in the dark (Lorenzen and Ruppel, 1960). Among the carbohydrates, starch forms the

major component throughout the life cycle of Chlorella ellipsoidea. Sucrose and other polysaccharides, which are present in considerably smaller amounts, show a temporary decrease in quantity during the earlier stages of the L cells. Using paper chromatography, the existence of various sugars such as glucose, fructose, xylose, galactose, ribose and trioses and their phosphate esters were demonstrated (Hase et al., 1957).

Throughout the algal life cycle, the synthesis of RNA paralleled the synthesis of protein, except during the transformation of L_3 cells into L_4 cells, where RNA decreased, while protein synthesis increased slightly (Lorenzen and Ruppel, 1960). On the other hand, only slight DNA synthesis took place during the growing phase and occurred rapidly during the phases of ripening and post-ripening. Essentially the same results were obtained for another strain of Chlorella pyrenoidosa (Pirson et al., 1959).

II. The effect of environmental conditions on the life cycle of Chlorella

Effect of pH

The acid tolerance of numerous Chlorella strains was studied by Kessler (1967), who demonstrated species-specific differences which appear to be genetically determined. Chlorella saccharophila, with a lower pH limit of 2, shows the greatest acid tolerance and C. minutissima, with a lower pH limit of 5.5 displays the greatest acid sensitivity. The role of pH as a factor determining the

composition of fresh-water phytoplankton communities was emphasized by Merilaenian (1967) and Patrick (1968).

The extent to which the internal pH of a living cell can be influenced by that of the external medium is still a matter of uncertainty. Since the enzymes involved in the various metabolic processes have different pH optima, it would be expected that any shift in internal pH would exert a selective influence upon the rates of those processes. Effects of two kinds, coupled with changes in oxidation reduction potentials, may well be related to differences between the paths of carbon in photosynthesis, dark assimilation and respiration. Pigment changes may occur as a result of variation in pH. Starosta (1970) studied Ankistrodesmus under carefully controlled pH conditions on a glucose medium in the dark and found that exposure to a low external pH caused a substantial transformation of violoxanthin to zeaxanthin. An increased synthesis of secondary carotenoids in Chlorella zofingiensis exposed to low pH has been shown to be due to an acid inhibition of nitrate reductase and nitrite reductase so that nitrogen-deficiency resulted (Kessler and Czygan, 1965). Carotenogenesis in streptomycin-bleached Euglena is favoured by an acid pH with an optimum at pH 6 for dark-grown cells and pH 4 for light-grown cells (Dolphin, 1970). The rates at which polyphosphates and several organic phosphates are synthesized are also influenced markedly by pH. Nitrogen

fixation by blue-green algae is also pH dependent (Fogg et al., 1973). pH may also affect the state and mobility of heavy metals, as illustrated by an increase of Cu^{++} toxicity in Chlorella when pH decreases (Kanazawa and Kanazawa, 1969). Similarly, the action of organic inhibitors depends in many cases on pH. In Anacystis, for example, maleic hydrazide is mutagenic at pH 5 but not at pH 8 (Gupta and Kumar, 1970). Depending upon the pH of the medium, either high or low affinity uptake of hexoses was reported for Chlorella (Komor and Tanner, 1975). The effect of pH on respiration was described by Nielsen (1955), and Ried et al. (1962). Frank (1940) postulated an influence of pH upon the permeability of the chloroplast membrane. The pH dependence of dissociation rates and the ionic state of polar inorganic and organic compounds affects the availability of many algal nutrients such as CO_2, iron (Stengel, 1970) and organic acids (Cook, 1965). pH also exerts an effect upon the electrical charge of the cell wall surface (Hegewald, 1972), on ion transport systems at the plasmalemma, and on associated membrane potentials. Although changes in these properties alone may cause conspicuous changes in metabolic rates, some observations also point to a direct influence of pH upon metabolic activities such as mentioned above. It should also be noted that most experiments are not carried out under static pH conditions and that the hydrogen ion concentration of a synthetic nutrient solution is not necessarily identical

with the pH of the same medium gassed with a CO_2 in air mixture (Soeder et al., 1964; 1966).

Two factors can alter the pH of an algal culture: the unequal cellular absorption of cations and anions, and the bubbling of the culture with CO_2-enriched air (Galloway and Krauss, 1961). Shifts of pH in response to ion removal are known. The degree of ionization of many constituents of the medium, including dissolved O_2, is determined by pH. Thus, factors such as membrane permeability, concentration of carbon dioxide and its distribution among the species, H_2CO_3, HCO_3^- and CO_3^{--}, and the value of the photosynthetic quotient, are difficult to control and distinguish from direct influence of pH changes upon the metabolic steps (Oullet and Benson, 1952).

Most algae are exposed to H_2CO_3 and its ions HCO_3^- and CO_3^{--}. Water in equilibrium with air at 15°C contains about 10 μM of dissolved CO_2. The amount of HCO_3^- and CO_3^{--} increases with pH. All algae seem to be able to take up free CO_2, which readily diffuses across cell membranes. Not all algae can take up HCO_3^-. However, uptake of HCO_3^- at rates sufficient to support net photosynthesis requires active transport (Raven, 1970). Thus, when HCO_3^- is the form of inorganic carbon which enters an algal cell, it must be converted to CO_2 before it can be used in photosynthesis. This reaction is catalyzed by carbonic anhydrase (Loeblich, 1970). Work with Elodea indicated that light-induced pH changes may be attributed to uptake of CO_2 or HCO_3^- from

the surrounding medium (Ruttner, 1960). Light-induced increase in the pH of a suspension of whole cells of Chlamydomonas reinhardi required net photosynthesis (Atkins and Graham, 1971).

The role of CO_2 in the regulation of photosynthetic and respiratory metabolism in plants is known (Hogetsu and Miyachi, 1977). In Chlorella, for example, after autotrophic growth in high CO_2 (5.5%) transfer to a CO_2 concentration about ten times lower than the concentration in air results initially in low rates of photosynthesis characterized by the absence of the Calvin cycle of CO_2 fixation (Graham and Whittingham, 1968). An induction period is necessary before normal photosynthesis rates are established and CO_2 fixation occurs predominantly via a β-carboxylation mechanism. Cells grown in air (0.03% CO_2) do not show this effect. Graham interpreted these observations as showing the requirement for the enzyme carbonic anhydrase which has substantial activity in air grown cells, but could not be detected in cells grown in 5% CO_2 (Graham and Reed, 1971).

It was also reported (Hauschild et al., 1964; Ogasawara and Miyachi, 1970) that the quality of light affects the distribution of absorbed carbon in various products of photosynthesis. Blue light-induced incorporation of CO_2 into amino acids and organic acids, and the incorporation of CO_2 into citrulline brought about by low intensity light are operated by a mechanism independent of ordinary photo-

synthetic CO_2 fixation. Carbon dioxide also has other effects. Both the induction of the delay of cellular development in Chlorella fusca and Chlorella vannielii (Soeder et al., 1966; Sorokin, 1962), and the excretion of the bulk of organic substances are also associated with exogenous carbon dioxide (Maksimova, 1974). For more detailed information on the effect of environmental conditions see Appendix AII.

III. Cell wall of Chlorella

Structure and composition

The cell wall of Chlorella is similar to the cell wall of higher plants (Northcote, 1972) and contains, in many strains, an outer layer of sporopollenin (Atkinson et al., 1972). This substance is known for its occurrence in pollen grains and other spores, and is defined as one of the most resistant and stable of all known organic materials (Faegri and Inverson, 1964). Sporopollenin is presumably of some survival value to the algae that possess it, and in this regard it was shown (Atkinson et al., 1972) for Chlorella that it can pass unharmed through the digestive system of a snail. By contrast, the presence of a trilaminar wall component does not protect Scenedesmus from digestion by parasites (Schnepf et al., 1971a, b). Efforts to produce naked protoplasts by enzymatic degradation of the cell wall have only recently met with success (Braun and Aach, 1975).

The wall is composed of two distinct parts : an organized microfibrillar structure embedded in a continuous matrix which could be separated intact from one another. The general analysis of the cell wall (Northcote, 1958) reveals a composition of 27% protein, 18.1% lipid, 15.4% alpha-cellulose, 31% hemicellulose, 5.2% ash.

The protein content of the wall is high compared with analyses of other plant cell walls from soft tissues. This protein may be structural in function, specifically active as part of the synthetic system of the cell wall constituents, or both.

The presence of amino sugar is of interest in relation to the protein content of the cell wall and might explain the presence of glycoprotein. Northcote and Goulding (1958) demonstrated the presence of glucosamine in a hydrolyzate of cell walls of Chlorella, its content changing during the course of the life cycle (Mihara, 1961). According to Mihara (1961) the substance is derived from chitosan which forms one of the constituents of the wall of algal cells. Using synchronized cultures of Chlorella ellipsoidea he showed that an increase in the content of glucosamine occurred in three steps: in proportion to the growth of D cells into larger cells; second, corresponding to the formation of autospores within ripened cells; and third, parallel to the growth of newly-born daughter cells. Around the L_2 stage an abrupt and temporary stop in the increase of

glucosamine occurred indicating the existence of some change in the property, probably in the permeability of the cell wall at that stage. Indeed, a most active uptake of water as well as some nutrient ions occurs around the same stage.

Uronic acid constituents of the plant cell wall are usually associated with middle lamella or intercellular material which is absent in this unicellular algae, and thus were found in very low concentrations.

The microfibrils have a diameter of 30-50A° and are irregularly interwoven in a continuous network throughout the wall. Two main directions of microfibrils are apparent under the electron microscope, lying at right angles to one another. There are no differences in the general arrangement and orientation of the microfibrils on the inner and outer surfaces of the wall, and there appear to be no local concentrations of microfibrils. In these respects, the wall resembles the primary cell wall of higher plants rather than the secondary wall. Chemically, the microfibrils correspond to the alpha-cellulose fraction and hence, contain polysaccharide that is composed of monosaccharides other than glucose (Northcote, 1958).

The matrix has a granular appearance and is continuous over the cell surface. Chemically it is related to substances soluble in dilute sodium hydroxides. These are hemicellulose, proteins, amino sugars and possibly lipids. Impure hemicellulose contains a high proportion of the

nitrogen present in the original cell wall. The walls digested with the snail enzyme lose 70% of their alpha-cellulose, 43% of their lipid, and only 13% of their hemicellulose, so that very little of the protein of the wall is removed. The material left shows no microfibrillar structure but resembles the granular matrix, and when sectioned, these digested walls have a laminated appearance. Two distinct layers can be seen, one near the outer edge and one near the inner edge, separated by a space of 100A°, which indicates a local concentration of some of the materials of the matrix in those outer and inner lamellae. The cellulose phase of the wall as isolated by the analytical procedure is composed of polysaccharides made up of galactose, arabinose, mannose, xylose and rhamnose in addition to glucose. Hydrolysis of the hemicellulose fraction of the cell wall gives rise to galactose in relatively large amounts and also mannose, arabinose, xylose and rhamnose.

Cell wall enzymes

Many enzymes have been reported to be associated with the cell wall. Keegstra and Albersheim (1970) have shown that β-glucosidase and β-galactosidase are associated with cell wall suspension of cultured sycamore cells. Nevins (1970) has suggested that a number of glycosidases are present in the walls of bean hypocotyl, indicating that some polysaccharide-degrading enzymes are associated with the wall of intact tissues.

Cellulose, like starch, is a compound of high molecular weight which, upon complete hydrolysis, yields β-D-glucose molecules. According to available evidence, the enzymatic hydrolysis of cellulose can be considered a random attack on the β(1→4) linkage. The cellulose molecule is reduced to cellodextrins and eventually to cellobiose, a disaccharide composed of 2 glucose units. The enzymes involved in the random hydrolysis of cellulose to cellobiose have not as yet been characterized, but have been grouped under the general term cellulase. The β(1→4) link of cellobiose can be hydrolyzed by the enzyme cellobiase.

$$\text{cellulose} \xrightarrow{\text{cellulases}} \text{cellodextrins} \text{-----} \text{cellobiose} \xrightarrow{\text{cellobiase}} \text{glucose}$$

It was shown in a system for Trichoderma viride (Li et al., 1965) that the cellulase system is able to convert a crystalline amorphous and chemically derived cellulose to glucose. This is a multienzymatic system, composed of 3 enzyme components and all three play essential roles in the overall process of converting cellulose to glucose. Enzymologically they differ only slightly in activation energies for hydrolysis of both cellulodextrins and carboxymethylcellulose, but are conspicuously different in mode of action, Km values for the cellodextrin series, optimum substrate chain length, the capacity to attack aryl-β-glucosides and pH optima.

For a more complete description of cell wall biosynthetic cellulases and pectin composition see Appendix AIII.

IV. Aggregation in algae

Several forms of aggregation of algal cells have been described. One is the palmelloid state that occurs in the life history of many flagellates and is named after the normal structure of the tetrasporine green alga Palmella (Fritsch, 1965) and is found in Chlorophyceae, Xanthophyceae, Chrysophyceae and in Dinophyceae. The palmelloid state refers to a change in the normal aggregation of cells in a multicellular complex, or to a massing of cells through cell divisions of normally unicellular species into aggregates of shorter or longer duration. One of the basic characteristics of the palmelloid state is the production of mucilaginous material that causes the cells in the aggregate to become embedded in this matrix and is also characterized by loss of motility. The palmelloid state, when a temporary phase of development, is usually considered an environmental response, but conditions contributing to its onset or reversion to normal are poorly understood.

Another form of aggregation reported for Chlorella is that found in induced cells grown heterotrophically on glucose in the dark. Under these conditions the cells grow in volume beyond their usual maximal size. These "giant cells" result from a delay in the completion of the cellular division process (Higashiyama, 1967). For a more complete description of "gigantism" in Chlorella see Appendix AIV.

For Anabaena, a changed environment that could involve

autointoxication, depletion of carbon dioxide, and change in pH of the medium are thought to be the factors involved in the transition (Bausor and Agona, 1973). In the blue-green algae Chlorogloea fritchii, irregular sorts of clumps of cells result from a decrease in carbon and nitrogen in the medium, as well as in response to reduced light and increased temperature (Hilary et al., 1976).

Another phenomenon of clumping of cells is their adhesion to artificial surfaces, a topic that has received considerable attention (Golueke and Oswald, 1965; Curtis, 1962; Houghton, 1970; Jones and Morrison, 1969). Two mechanisms were suggested to explain this phenomenon: the contact relationships of a cell might depend essentially on the surface charge of the cell and the charge on the surface to which the cell adheres. On the other hand, it has been suggested that cells produce materials to "coat" surfaces, which are responsible for their adhesive behavior (Nordin and Tsuchiya, 1967; Bausor and Agona, 1973). It seems that both processes (those entailing surface charge, and those entailing the production of cellular "cement" substances) are involved in the process of cellular adhesion.

The biochemical events that characterize the different intermitotic phases in Chlorella vulgaris might be expected to alter the physical characteristics of the cell surface. It has been suggested that such changes take place in the Chlorella cell as it passes through its life cycle (Sitz,

et al., 1970). The adhesion of Chlorella vulgaris to glass surfaces increased in the presence of thymidine and colcemid (Zaidi and Tosteson, 1971), which appear to arrest the cells in the G2 phase of the cell cycle, in which they are more adhesive.

PURPOSE

Many characteristics of Chlorella described previously, its very rapid growth rate, the existence of a wide spectrum of strains, and its ability to grow autotrophically, make it an important candidate for algal biomass production (bioconversion of solar energy) (McGary, 1971; Oswald, 1976; Arad et al., 1976). However, the major unsolved problem in unicellular algal farms is the harvesting of the algae in a way that does not consume more energy that it produces. Centrifugation requires much energy; filtering is a more likely possibility (Doellinger, 1978). When aggregated or clumped, single algal cells could be readily removed from their growth medium.

With this in mind, it is not surprising that aggregates of Chlorella cells that were observed in an alkaline medium attracted our attention, and led to our desire to describe this phenomenon from morphological and physiological standpoints and to understand the mechanism involved.

MATERIALS AND METHODS

Culture Technique

Organism and media

The *Chlorella* strain used was isolated in 1976 at Beer-Sheva, Israel, and identified by Dr. I. Friedman (Florida State University) according to the monograph of B. Fott and M. Novakova (1969) as *Chlorella vulgaris* Beijerinck *var vulgaris* fa. *vulgaris*.

For comparison in one experiment *Chlorella sorokiniana* 7 11 05 (Cambridge) was used and cultured under its optimal temperature 39°C. *Chlorella* cells were cultured in a N-8 basal medium as described by Soeder (1964) (see Appendix G). In order to increase the pH of a culture, sodium bicarbonate ($NaHCO_3$) was added to a final concentration of 4 gm/l.

All algal stocks were monitored for contamination by plating 0.1 ml aliquots onto nutrient agar plates. The stocks were maintained on slants that were composed of N-8 medium which was 2% in bacto-agar.

Batch culture technique

Cultures were grown in conical tubes (200 ml) at 26-30°C. Filter sterilized air was pumped into the conical tubes via a network of glass tubing to agitate the culture and to prevent the sedimentation of the cells. Continuous illumination was provided by a series of fluorescent tubes at an intensity of approximately 10,000 lux at the surface

of the culture tube. The pH of the culture was maintained at 6.3 (low) by the addition of CO_2 to the air stream to give a final concentration of 1.5%. Other regulation of the pH was effected by altering the percent of CO_2 in the air stream.

Continuous culture technique

Experimentally a continuous culture can be controlled in two ways:

1. Chemostat - in which the growth rate depends upon the concentration of a substrate that is limiting and in which the substrate concentration is determined by the control of the dilution rate. Thus, cell concentration in the steady-state depends upon the limiting factor.
2. Turbidostat - in which cell concentration is maintained at a certain level and in which each addition of biomass that contributes to the optical density is diluted out of the culture by the introduction of fresh medium. Thus, the dilution rate is determined by the growth rate. When there are no limiting factors in the system, the growth rate and cell division in the steady state are only a function of the genetic characteristics of the organism. Thus, the maximal growth rate (μ max) in a steady state can be achieved only in the turbidostat, since in a chemostat μ max cannot be maintained. For this reason, continuous culture in the turbidostat was used.

Continuous cultures were grown in cylindrical tubes of 500 ml under identical conditions to those described for

batch cultures (N-8 4 gm/liter $NaHCO_3$ and continuous illumination of 10,000 lux, 28°C). The different pH's were achieved by varying the concentration of CO_2 in the air stream. A constant optical density was maintained at 90-100 klett units (k.u.) by automatic bleeding and the addition of sterile medium supplied from 2.5 l reservoirs. Excess algal solution above a certain volume was removed from the tube and collected in reservoir flasks.

When the algal culture reached the higher limit of optical density (100 k.u.) an electromagnetic valve was opened enabling the introduction of fresh media from the reservoirs into the tube. This valve closed when the optical density of the culture reached a lower limit. A constant volume was maintained in the culture by the fact that the positive hydrostatic pressure of the aeration system could only be vented by a U-shaped exit tube set at a desired level. A steady state was established when a culture specific growth rate continued within a ±2.5% value for 3-4 days.

The specific growth rate (μ) was calculated from the volume of the effluent collected as a result of dilution. As a measurement of growth, the doubling time (Td) of the culture was c

$$\mu(h^{-1}) = \frac{\text{Volume of Bleeding (ml)}}{\text{Time (h) x Volume of Tube (500 ml)}}$$

$$\mu(h^{-1}) = \frac{\text{Volume of Bleeding (ml)}}{\text{Time (h) x Volume of Tube (500 ml)}}$$

$$Td(h) = \frac{\ln 2}{\mu}$$

Synchronized culture technique

In order to achieve synchronization of the cultures, a method similar to that described by Lorenzen and Hesse (1974) was utilized. A regime was used of alternating light and dark periods in which the culture was diluted to 7.5×10^6 cells/ml in dark immediately prior to the onset of the light period. Following repetition of this procedure for 3 times, the culture was considered synchronized. Synchronization was verified by monitoring cell size which is known to be the smallest at the beginning of the cycle.

Any method which imposes artificial synchrony on a culture might change some physiological events in the cell cycle. Selection of synchrony is based upon changes in cell density in the different stages of the cycle. The cell cycles observed following selection synchrony appear to be normal provided that separation procedure does not affect cell metabolism. However, the yield (percent of synchrony) is lower than is produced using induction synchrony (Mitchison, 1971). The dark phase in the light-dark regime acts as an inducer of the rhythm (Zeitgeber). If the dark phase is reset by a small quantity of light the rhythm fades out (Lorenzen and Hase, 1974) and lowers the productivity of succeeding cycles to a minimum value (Soeder, 1966).

Culture growth

Culture growth was monitored in three different ways:

1) Direct determination of cell number, achieved micro-

scopically using a Petroff-Houser counting chamber and a microscope equipped with phase optics.

2) Indirect determination by a turbidometric method, using a Gilford Spectrophotometer at 560 nm or a Klett Somerson colorimeter equipped with filter No. 54.

3) Dry weight determination, was accomplished by filtering 20 ml algal samples through preweighted filters (47 mm diameter and 1.2 μ pore size, Millipore Corporation). The filters plus the filtrate were dried in an oven at 80°C for 1 hour and reweighed after cooling in a dessicator for 20 minutes.

The limitation of all these methods is that they do not measure the viability of the cells. Thus, cell counting, O.D. and dry-weight determination would also include dead cells. However, from the shape of the growth curve one can assume that the cells were alive.

Inhibitors

Cycloheximide

The inhibitory effect of cycloheximide on protein synthesis in eucaryotic cells is well documented (Ennis and Lubin, 1964). In preliminary experiments, it was found that the protein content of cells grown in a medium containing cycloheximide at pH 9.5 was not affected by this treatment. However, the cells grown at pH 6.3 did have their protein content diminished. Therefore, cells were routinely treated with cycloheximide (Sigma) at a final concentration of 35 μM

at pH 6.3 for 2 hours, centrifuged at 5,000 rpm for 10 minutes, the precipitate washed with N-8 medium and resuspended in N-8 medium to give the original volume, and the pH of the culture adjusted to 9.5.

Puromycin

To confirm the observed inhibition of protein synthesis and to avoid possible side effects associated with cycloheximide application, an alternate protein inhibitor, Puromycin, (Sigma) was used (Nathans, 1964). Following a similar protocol to that described for cycloheximide treatment, the cells were subjected to a medium containing a final puromycin concentration of 0.5 mM for 2 hours at low pH before transferring to a higher pH.

Based upon subsequent results, the duration of exposure to the inhibitors used (2 hours) proved sufficient to effect a decrease in net protein accumulation.

It is important to note that the inhibitor effect is on protein content and is not proved to be on protein synthesis. Side effects of these inhibitors, as on membrane permeability, might cause leakiness of cells, thus result in a decrease in total protein content.

DCMU 3-(3,4-dichloro)-1-1-dimethyl urea

The cultures were exposed to 20 μM DCMU for 2 hours in low pH (as previously described) and then centrifuged, washed and resuspended in N-8. The DCMU (Sigma) stock solution (2mM) was prepared by dissolving the inhibitor in

ethanol. Control cultures were treated with an equal amount of ethanol.

Darkness - was achieved by covering the conical tube with aluminum foil so that no light could penetrate through the protective surface enclosure.

Sedimentation Test

The cultures were diluted to an optical density of 80 klett units (k.u.), and the tubes left in a vertical position for 60 minutes at room temperature. The optical density of the medium at the top of the test tubes, representing the amount of light absorption by the algal cells which did not settle out, was then determined. The limitation of this method as a measure of aggregation is discussed in the Results.

Measurements of Cell Diameter

Cell diameter was determined by using a calibrated eye piece. For each determination, the diameter of at least 200 cells was measured. Cell diameter was confirmed by measuring cells from photographs taken with a light microscope equipped with Nomarsky optics.

Electron Microscopy

Using a modified method of Gergis (1971), Chlorella cells were harvested and washed twice with phosphate buffer

(pH 7.4, 0.2 M). To achieve fixation, the cells were then suspended for 3 hours in 3% gluteraldehyde. The fixed cells were washed 3 times with the buffer for a period of 30 minutes and then suspended in 1% OsO_4 for 50 minutes, and rewashed as described above. The cells were then added to melted 2% Agar and after solidification, dehydrated in a graded water - ethanol series (20-100% ethanol) and embedded in Epon. Sections were cut on an ultramicrotome equipped with a diamond knife, double stained for 10 minutes each with 5% uranyl acetate and lead citrate and subsequently coated with carbon. The prepared specimens were examined with an Elmiscope (type Jeol 100B).

In all the sections observed the plane of the section was assumed to be totally random, and therefore representative of all possible orientations. This ensured that there would be no bias in the determination of the number of autospores per mother cell. Pictures were taken from three different experiments all giving the same results.

Determination of Enzyme Activities

Cellulase

The level of cellulase activity was monitored according to a modification of the method of Mandels and Reese (1964).

Enzyme preparation

A sample of Chlorella cells (80 ml) was centrifuged at 7000 x g for 10 minutes. The resulting supernatant solution

was discarded, the pelleted cells resuspended in 10 ml of phosphate buffer (0.1 M pH 6.3) and broken by vigorous agitation for 60 seconds in a Braun Homogenizer, with glass beads of 0.5 mm diameter. The supernate was collected and the beads washed with 5 ml of buffer. The wash solution was combined with the original supernatant solution and utilized as the crude enzyme preparation.

Assay procedure

To 100 mg of the substrate alpha-cellulose (Sigma) were added 3 ml of enzyme preparation and the resulting mixture was incubated for 2.5 hours at 37°C with constant agitation. The reaction was terminated by removal of the residual substrate through centrifugation. The amount of reducing sugars released (product) was assayed by the procedure of Park and Johnson (1949) (see Appendix B). Endogenous activity was found to be less than 2% of the total activity.

Limitation of the assay procedure would be that considerable digestion is required before detectible product is formed. By the general method used, the very end products - cellobiose or glucose - are measured. Thus, the assay actually measures the end products of many enzymatic activities. Therefore, all results reported later should be considered the lowest detected cellulase activity and the actual amount might be greater.

Glucostat

A quantitative colorimetric determination of glucose

was performed using the glucostat semi-micro method of Meyers et al. (1960) as follows: A 2 ml sample of Glucostat reagent was mixed with a sample from the cellulase assay and allowed to stand for 10 minutes at room temperature prior to the addition of one drop of 4 N HCl to stabilize the resulting yellowish color which was quantitated as the absorbance at 425 nm. The concentration of an unknown was determined by comparison with a standard curve (0-400 mg % glucose).

Viscometric method for the determination of cellulase activity

The viscometric method for the determination of cellulase activity was a modification of the method described by Almin and Eriksson (1967) and Lewis and Varner (1970). The enzyme preparation was created as described previously. Carboxymethyl cellulose (C.M. cellulose type 7HF kindly supplied by Hercules Powder Co., Wilmington, Del.) was prepared by stirring a set quantity of the C.M. cellulose in a known volume of 0.1 M phosphate buffer, pH 6.3 until a final concentration of 1.33% (w/v) was obtained.

Enzyme activity was measured in the following manner: 1 ml of the C.M. cellulose solution was incubated with 2 ml of the enzyme solution at 37°C. The viscosity of the assay solution was measured at 0, 2.5 hr and 5 hr after the initiation of the reaction. Draining time through a calibrated 0.1 ml or 0.2 ml pipette was used as a measure of viscosity at room temperature of 22.5°C. The pipette was calibrated using different viscosities (concentrations) of C.M. cellu-

lose created by the dilution of a stock solution 4, 5, 8 and 10 fold (Bull, 1964) (see Appendix C, Fig. 21).

The contribution of the enzyme preparation itself to the viscosity was tested and found to be negligible. However, the contribution by other macromolecules other than the added cellulose (like DNA) to the viscosity was not tested.

Ions

The separate ions treatments were achieved by the addition of: $MnCl_2$, $MgCl_2$, $CaCl_2$, $Co(No_3)_2$ H_2O, $CaCl_2$, $FeCl_3$. $6H_2O$ to the reaction mixture in final concentrations of 2mM from a concentrated stock solution of 20mM in 0.1 M phosphate buffer pH 6.3.

Lectins

The different lectins treatments were achieved by the addition of each lectin to a reaction mixture in a final concentration of 10 or 50 µg/ml, from a concentrated stock solution of 250 µg/ml dissolved in 0.1 M phosphate buffer pH 6.3 (Lis and Sharon, 1973). The following lectins (all from Sigma Company) were used: from Castor Bean type II (galactose affinity), from Castor Bean type IIA (galactose affinity), from Garden Pea (affinity for glucose and mannose) and concanavalin A (primarily glucose affinity).

Malate dehydrogenase (MDH)

Enzyme preparation:

The cells were prepared in a manner identical to the protocol described for cellulase with the following alter-

ations: The crude extract was centrifuged in the Fisher International Clinical Centrifuge at its highest velocity for 5 minutes, and the supernatant used as the source of Malate Dehydrogenase.

Assay procedure

The assay used to determine MDH activity was that developed by Ferguson (1967). In general, the reaction mixture contained 1.25 ml of 0.2 M tris-HCl buffer, pH 7.6, 0.5 ml of NADH (0.6 mM NADH in 0.2 M tris-HCl buffer, pH 7.6, freshly prepared), 1.0 ml of 9 mM oxaloacetate (freshly prepared), and 0.25 ml of enzyme preparation which was added last. The contents of the cuvette were then mixed by inversion and the recorded rate of decrease in optical density at 340 nm monitored over a period of at least 2 minutes. MDH activity is measured as the rate of oxidation of NADH in the presence of an excess of oxaloacetate.

$$OAA + NADH \xrightarrow{MDH} Malate + NAD$$

The activity was expressed as umoles NADH consumed/min/mg protein. A decrease of 1 optical density unit at 340 nm in a 1 cm light path represents the loss of 160 μmoles of NADH.

All results should be taken as a measure of a net effect of several isozymes of malate-dehydrogenase. The intracellular localization of this enzyme was not tested, however, its activity in the particulate fraction (cell wall enriched) was found to be low (Table 20).

Preparation of a Particulate Fraction Enriched with Cell Walls

A fraction enriched with cell walls was prepared according to the description given by Northcote and Goulding (1958). A sample of cells was centrifuged for 10 minutes at 1500 x g. The supernate was discarded, the cells diluted in 10 ml of phosphate buffer (0.1 M pH 6.3), and broken using a Braun Homogenizer as previously described. The supernatant solution was collected, the beads washed twice with 5 ml of phosphate buffer, and the supernatant solution and washes combined ("whole homogenate"). A sample of 7 ml was removed from the "whole homogenate" and the remainder centrifuged at 200 x g for 5 minutes. The supernatant solution from this centrifugation was collected ("supernatant 1"), the sediment washed twice with the same phosphate buffer (8 ml each time), centrifuged as before, and the supernatant solutions and washes combined ("supernatant 2"). The remaining sediment was diluted with 20 ml of N-8 medium. This was the "particulate fraction enriched with cell walls."

The degree of breakage was not determined but from light microscopic examinations it was assumed to be greater than 90%.

To increase the pH of the "particulate fraction enriched with cell walls", 4 gm/l sodium bicarbonate were added and the tube agitated for 2 hours under the same light and temperature conditions used for algal growth experiments.

Chlorophyll determination

A 10 ml sample of a culture was centrifuged in Fisher International Clinical Centrifuge at its maximal velocity for 5 minutes. The supernatant solution was decanted and 5 ml of 90% methanol and a few grains of $MgCO_3$ were added to the sediment. The tubes were then incubated at 75°C for 10 minutes, after which they were cooled to room temperature, recentrifuged as before, and the optical density of the supernatant recorded at 663 and 645 in a Gilford Spectrophotometer. The quantity of chlorophyll was calculated according to the formula of Arnon (1949):

$$\text{Quantity of Chlorophyll } (\mu g/ml) = (A_{645} \times 26.2 \times .791) + (A_{663} \times 8.02 \times 1.086)$$

DNA Determination

At specific points during the cell cycle, 10 ml of algal cells were transferred by pipette to 13 x 100 mm test tubes and centrifuged in a Fisher International Clinical Centrifuge at its highest speed for 5 minutes. The resulting supernatant solution was immediately decanted, and 5 ml of methanol was added to the algal pellet to facilitate chlorophyll and lipid extraction, heated at 75°C for 10 minutes, cooled to room temperature, and the tubes then recentrifuged, as previously described, for 5 minutes. The supernates containing the chlorophyll extract were decanted and the pellet air dried. Determination of DNA was according to the method of

Tustandoff and Bartley (1964). The dried pellet was subsequently treated with 3 ml of 0.5 M Perchloric acid ($HClO_3$) and incubated at 70°C for 1 hour. After cooling the acidified reaction mixture to room temperature, the hydrolyzed DNA was mixed with 4 ml of freshly-made diphenylamine reagent (DPA). In preparing the DPA reagent, 1.5 g of DPA was dissolved in a solution containing 100 ml of glacial acetic acid and 1.5 ml of concentrated sulfuric acid (H_2SO_4) and stored in total darkness at 2°C. On the day of use, 0.1 ml of a 16 mg/ml solution of aqueous acetaldehyde was added to each 20 ml portion of DPA reagent utilized. Following vigorous agitation, the reaction mixture was incubated for 24-30 hours at 30°C. Any particulate matter was then separated from the supernatant by centrifugation of the solution in a Fisher International Clinical Centrifuge at highest speed for 5 minutes. The absorbance of the supernatant solution was then measured at 600 nm in a Gilford Spectrophotometer using a 1 ml cuvette. Blanks were prepared using acid-extracted DNA from algal cells that had been subjected to identical experimental protocols, with the exception that in lieu of DPA treatment, the samples were supplemented with 4 ml of distilled water. Calf thymus DNA (Worthington Biochemical Corporation) was utilized as the standard for the colorimetric determination of DNA. A stock solution, prepared by dissolving 0.2 mg of DNA in 5 mM NaOH was stored at 0°C, and for this stock, working standards were prepared by mixing a

measured volume of the stock standard with 5mM NaOH to effect a concentration range of 0-120 ug DNA/final volume. Each standard (0.6 ml) was mixed with 2.4 ml of 0.5 M HCl and incubated at 70°C for 1 hr. Upon cooling to room temperature, 4 ml of DPA reagent was added to each tube. After agitating the reaction mixture, the tubes were incubated for 24-30 hr at 30°C. One ml aliquots of the solution were removed and transferred to cuvette as described previously for measurement of absorbance at 600 nm.

In light of the fact that some changes in cell wall were observed in cells grown at alkaline conditions, it is possible that DNA extraction from aggregated cells might be different than from control cells. Efforts were made to standardize the procedure in a way which would insure a high degree of reproducibility.

The results of a duplicate sample of the same experiment fell between 4%, and of similar samples of different experiments within 10%. Values of μg DNA/cell that were determined are similar to those determined by Tamiya. In addition, the color reaction might also measure deoxyriboses other than those present in the DNA.

Protein Determination

Protein determination was performed according to the method of Lowry (1951) with the following modifications: Either a 0.5 ml sample of broken cells or 5 ml of intact cells were centrifuged in the Fisher International Clinical Centrifuge at its highest velocity and 4.5 ml or 5 ml of 0.1

N NaOH was added. The mixture was then boiled for 20 minutes, cooled to room temperature, and recentrifuged as before. One ml aliquots of the supernatant solution were then used for protein determination as follows.

To a 1 ml sample, 3 ml of C reagent were added (C reagent was prepared from 0.5 ml of $CuSO_4$-1% + 0.5 ml of 2% Sodium Potassium Tartarate dissolved in 49 ml of Na_2CO_3-2%), mixed, and left at room temperature for 10 minutes after which 0.3 ml of Folin reagent was added (Folin reagent was prepared from Folin and Ciocalteu's phenol reagent 2N from Sigma diluted 1:1 with distilled water), mixed and left for 30 minutes at room temperature after which the absorbance at 650 nm was measured. Bovine Serum Albumin (BSA)(Sigma) was utilized as a standard. All results were compared to values obtained using BSA as a standard and corrected accordingly.

Staining

Ruthenium Red

A quantity of Ruthenium Red to achieve a final concentration of 30 mg/l was dissolved in a mixture of ammonium acetate (0.01 M pH 8.6) and added to a sediment of *Chlorella* cells previously pelleted by centrifugation. The cells were mixed with the Ruthenium Red solution and the adsorption of the dye observed after 3-4 hours under the light microscope.

Ruthenium Red staining, although not specific to pectic

substances only is the standard procedure for pectic substances in the cell wall (Jensen, 1962) and in fruits (Ben Arie, 1969). Ruthenium Red is specific for carboxyl groups and thus will stain other "plant slimes" with such a group, and also mildly oxidized cellulose, which has such groups. Certain phospholipids and fatty acids might also be stained by it.

Ruthenium Red precipitation reaction is primarily related to the number of ionizable COOH groups available if the molecular weight is high enough (Luft, 1971).

Histochemical method for pectin determination

The histochemical method for pectin determination was performed according to the method of Gee et al. (1959). Chlorella cells were centrifuged as described above for Ruthenium Red, resuspended in acetone, washed 3 times with methanol, centrifuged and resuspended in the esterification reagent (0.5 N HCl in dry absolute methanol), and incubated for 12 hours. Following centrifugation and resuspension in methanol, the washed cells were recentrifuged as previously described and the pellet immediately resuspended for 5 min in a solution containing 5 ml of NaOH and 5 ml of hydroxylamine reagent. Ethanol (96%) and 12 N HCl (5 ml of each) were then added for an interval of 5 minutes, followed by centrifugation and the resuspension in 10 ml $FeCl_3$, 2.5% in HCl 0.1 N. The development of the dye was observed after 10 minutes under the light microscope.

Stain for polysaccharide (PAS)

Samples of algal cells in different stages of their cell cycle were put on glass slides and left to dry overnight. After fixation the following staining procedure was employed:

1) The slides were left in carnoy fixative for 5 minutes.
2) Extraction of chlorophyll was achieved by exposure to a 95% ethanol solution for 5 minutes.
3) The slides were left for 5 minutes in the Periodic acid solution and rinsed in water.
4) The slides were left for 5 minutes in Fuchsin-Schiff

reagent and rinsed in water.

5) Bleaching was for 2 minutes (X2).

6) Finally the slides were rinsed in water and air dried.

Samples were read in a microspectrophotometer at 550 nm. Sugar calculation was made using the following formula:

$$\text{Sugar amount} = E\, r^2$$

$$\text{Where } E = \log \frac{I_o}{I_1}$$

I_o = background
I = amount of sugars
r = cell radius in μ

For a detailed description of the solution formulas see Appendix D.

Extracellular enzyme treatments

Cells that were fixed on the slides were treated with the enzymes: Cellulase -β-1, 4 glucan glycanohydrolase-type 1 (Sigma), Pectinase - Polygalacturonase, Poly-α-, 1,4 galacturonide glycanohydrolase - (Sigma), Protease type V (Sigma), D-Amylase (Sigma). All enzyme solutions were prepared in 0.1 M phosphate buffer pH 6.3 in final concentration of 1 mg/ml, and the slides left in enzyme solution for 16 hr, or as mentioned in each experiment) after which the slides were rinsed with water and stained with PAS as described previously.

Pectin Extraction and Determination

Pectin was extracted according to the method described

by Ben Arie (1969) and delineated below.

Preparation of insoluble materials in acetone and ethanol (AIS)

A culture sample of 500 ml was centrifuged at 5000 x g for 10 minutes, the supernate decanted prior to resuspension of the sediment in 50 ml of 70% ethanol boiled for 20 minutes (to remove all traces of water), and cooled to room temperature. The sample was then centrifuged as before and resuspended in 70% ethanol for washing purposes. The sediment was resuspended twice, in 50 ml of acetone, centrifuged and rewashed in acetone. The resulting white powder was dried in the oven at 50°C overnight and the weight determined the following morning. The sample was then maintained dessicated at 0°C for further analysis.

Water-Soluble Fraction (WSP) (containing primarily pectic acid)

To 300 mg of powder, 10 ml of distilled water was added and the mixture agitated for 1 hour at room temperature. After centrifugation, another 50 ml of water were added to the sediment, and the mixture was reshaken, recentrifuged and both supernates were combined in a common vessel.

EDTA-Soluble Fraction (VSP) (containing primarily calcium pectate)

The sediment was resuspended and extracted on a shaker with EDTA (0.5%) (pH 6.0). After twice centrifuging and resuspending, the supernatant solutions were combined.

EDTA-Insoluble Fraction (ISP) (containing primarily protopectin)

A portion of the sediment was deesterified with 0.5% of

EDTA adjusted to pH 11.5 with NaOH. After 30 minutes incubation at 30°C the pH was adjusted to 5.5 with glacial acetic acid, 100 mg of Pectinase (Sigma) added and the solution stirred for 60 minutes at room temperature.

Pectin determination

The determination of pectin concent was effected by using the Carbazole reagent (McComb and McCready, 1952).

The absorption spectra for color produced from galacturonic acid, pectin acid, polygalacturonide methyl ester and methyl galacturonide methyl ester were found to be identical. This indicates that regardless of the degree of polymerization of the galacturonide or the presence of methyl ester groups, the color measured by this procedure is the same. Other uronic acids (e.g. mannuronic) or their derivatives are unlikely to cause interference, since they do not normally occur in pectin extracts. Glucose and fructose interfere slightly only when present in concentrations that are three times that of anhydrouronic acid.

RESULTS

I. Description of the phenomenon of pH induced aggregation

A Spirulina pond growing outdoors at pH 9.5 was observed to be contaminated by Chlorella cells that appeared to be unusual; the cells were found in clusters or aggregates rather than singly. This Chlorella strain was isolated and identified as Chlorella vulgaris Beijerinck var vulgaris fa. vulgaris (see Materials and Methods). Since the aggregates were first observed at pH 9.5, which is known to be optimal for the growth of blue-green, but not for green algae (Merilaenian, 1967; Patrick 1968), it was assumed that the newly isolated strain of Chlorella had a higher pH optimum than the usual strains of Chlorella (grown at pH 6-7).

a. The optimal pH of Chlorella vulgaris Beijerinck

In order to determine whether this strain of Chlorella did have a different pH optimum for growth, it was cultured at several pH's and its growth rate determined. For comparison, Chlorella sorokiniana 711 05 was grown under its own optimal conditions (Table 1).

From the data presented in Table 1 it can be seen that Chlorella vulgaris Beijerinck is more tolerant of alkalinity than is Chlorella sorokiniana. At a pH of 9.5 Chlorella

TABLE 1

THE EFFECT OF pH ON THE GROWTH RATE OF TWO STRAINS OF CHLORELLA

Algae sp.	pH 6.5	pH 8.0	pH 9.5
	doubling time (hr)		
C. sorokiniana 7 11 05	5.5	6.9	No net Growth
C. vulgaris Beijerinck	6.0	5.6	17.3

The Chlorella sp. were grown in a continuous culture apparatus with continuous illumination (as described in Materials and Methods). Each species was grown at its optimal temperature (38°C for C. sorokiniana and 28°C for C. vulgaris Beijerinck). Constant cell concentration was maintained by the continuous adjustment of the optical density of the culture to 90-100 k.u. The medium was N-8 to which 4 g/l sodium bicarbonate were added. The different pH's were achieved by adjusting the CO_2 concentration in the air stream. The volume of the continuous effluent was collected, and the doubling time of the culture was calculated according to that described in Materials and Methods.

vulgaris continues to grow, although more slowly, whereas Chlorella sorokiniana does not grow at all at this pH.

The data shown in Table 1 also indicate that Chlorella vulgaris Beijerinck has a broader pH optimum than Chlorella sorokiniana 7 11 05. The growth rate of Chlorella vulgaris was essentially the same at pH 6.5 and 8.0, whereas the growth of Chlorella sorokiniana is considerably reduced at pH 8.0 relative to pH 6.5. Despite the broad pH optimum, Chlorella vulgaris cannot be considered an alkalophilic alga because although it is tolerant of alkaline conditions, its growth is not enhanced at an alkaline pH.

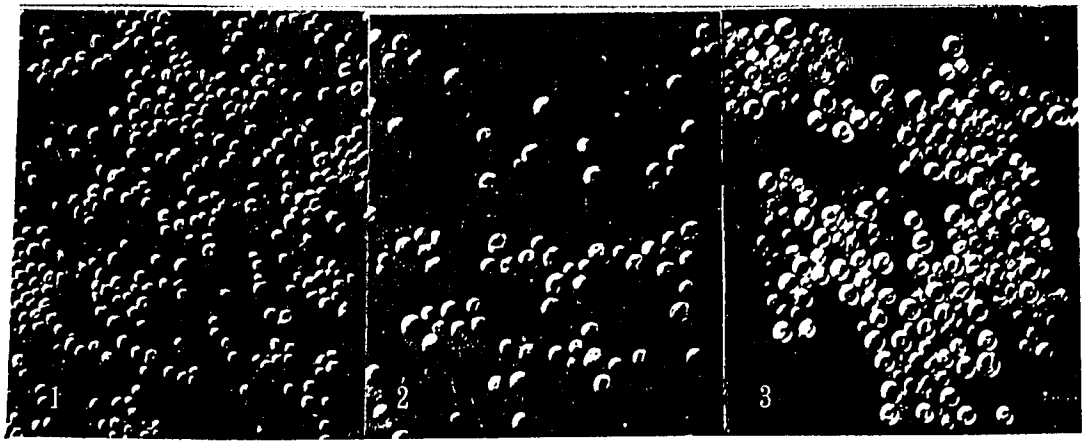
b. The pattern of aggregation

The aggregates first seen in the Spirulina pond were also observed in N-8 medium when the pH was raised to 9.5 (aggregation did not occur at pH 8.5). Figure 2 presents photomicrographs of Chlorella vulgaris cells cultured in N-8 medium that was adjusted to pH 9.5 showing the different stages of aggregation. After a 1 day exposure to alkaline pH, the average cell size increased markedly but the cells still remained solitary (as they were at 0 time or growing at pH 6.3). After 4 days at pH 9.5, the cells were essentially the same size as after 24 hours at the alkaline pH, but they were now in clusters.

Figure 2

PHOTOMICROGRAPHS OF CHLORELLA VULGARIS CULTURED AT pH 6.3 OR 9.5

A culture of Chlorella vulgaris grown asynchronously at pH 6.3 was diluted to 2 x 10^6 cells/ml and the cells cultured in N-8 plus 4 gm/l sodium bicarbonate, aerated with air to achieve pH 9.5, and illuminated continuously as described in Materials and Methods. At daily time intervals, samples were taken and photographed through a Zeiss microscope equipped with Nomarski optics. Plate 1 represents cells grown at pH 6.3, 2 represents cells after 1 day at alkaline pH and 3 after 4 days at the alkaline conditions. The scale represents 20 μ.


1
2
3

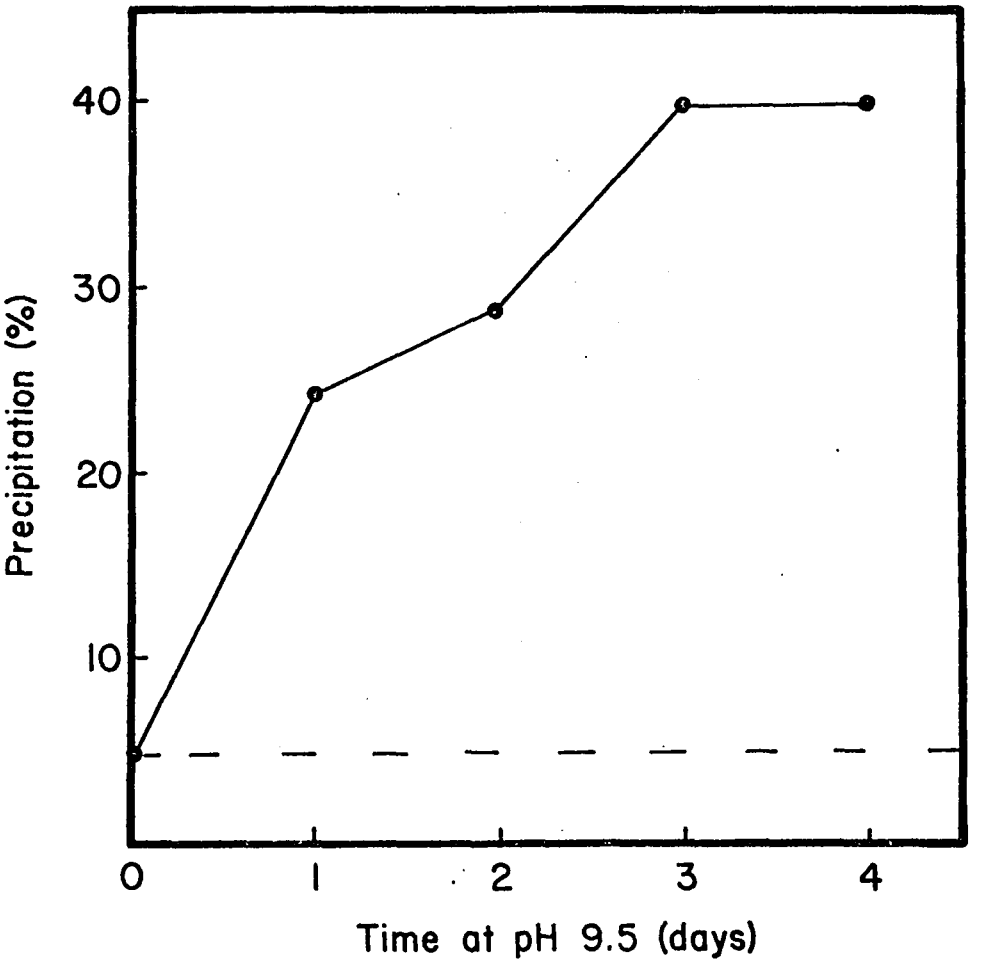
It seemed necessary to somehow quantitate the extent of the aggregation resulting from prolonged exposure to alkaline conditions. When aggregated, the Chlorella cells have the tendency to sediment to the bottom of the tube. This tendency was therefore used as an assay for aggregation. Cultures at different stages of growth were diluted to a constant optical density (80 k.u.), and the tubes left in a vertical position for 60 min at room temperature. Following this sedimentation period, the optical density of the cell suspension in the upper portion of the test tubes was measured. This represents the optical density of the cells which did not sediment. From the difference in the two optical density readings, the percent of precipitation could be calculated. The pattern of aggregation in the culture, when the pH is raised, is indicated by the kinetics of cell precipitation (Fig. 3).

Since there is a change in the mean of the cell size as a function of the duration in alkaline conditions, the optical density measured at 560 nm is not totally reliable due to the potential change in light scatter. But Iler and Warren (1972) stated that: "...In practice light scatter may intensify absorption bands as much as 100-fold.•••There is no simple relationship between wavelength or particle size and intensification as might be assumed from single particle

Figure 3

THE PATTERN OF AGGREGATION OF CHLORELLA VULGARIS CULTURED AT pH 9.5 AS INDICATED BY CELL PRECIPITATION

A culture of Chlorella vulgaris grown asynchronously at pH 6.3 was diluted to 2×10^6 cells/ml and cultured at pH 9.5 (N-8 + 4 gm/l sodium bicarbonate, aerated with air only) and in pH 6.3 as a control. The cultures were illuminated continuously as described previously. At daily time intervals, samples were removed and tested for precipitation as described in Materials and Methods. The dashed lines represent the percent of precipitation of the control cells. Each point is the average of 2 experimental determinations.


40
30
20
10
Precipitation (%)
0
1
2
3
4
Time at pH 9.5 (days)

scattering theory. In general, the intensification is less at shorter wavelengths because the reflectivity is less. The aggregates are not a part of this measurement since they have sedimented out of the suspension. Therefore, it is only the size distribution of the single cells that is important. As a first approximation this general type of information is probably acceptable.

The data presented in Figure 3 show that during the first 24 hours at pH 9.5, about 25% of the algal mass is precipitated. This represents 62% of the total biomass that will ultimately precipitate. A further increase in the extent of cell precipitation continues after the first fast increase. By 72 hours, about 40% of the cells in the culture have precipitated. Only 5% of the cells were precipitated in the control culture at pH 6.3.

A comparison of photomicrographs of control cells with those of cells treated for 24 hours at pH 9.5 (Fig. 2), reveals a substantial increase in cell size (Table 2). One can see that a 7-fold increase in cell volume occurs within 24 hours after raising the pH. There is no further change in cell size beyond 24 hours.

The data represented in the photomicrographs shown in Figure 2, the pattern of aggregation in Figure 3 and the pattern of increase in cell size in Table 2, suggest the

TABLE 2

THE AVERAGE SIZE OF CHLORELLA VULGARIS CELLS CULTURED AT pH 9.5

Days at pH 9.5	Average cell diameter (μ)	Calculated cell volume (μ^3)
0	3.3	19.7
1	6.5	147.1
4	6.2	129.6

Chlorella vulgaris cells were cultured in N-8 plus 4 gm/l sodium bicarbonate at pH 9.5, aerated with air, and continuously illuminated. For every sample at least 200 cells were measured using calibrated eye pieces in a microscope equipped with phase optics. The sizes were confirmed from photographs taken using a Zeiss microscope equipped with Nomarsky optics.

existence of two phases in the process of aggregation. A fast phase occurs during the first 24 hours after the pH is raised and is followed by a slower phase. From the third day on, no net increase in aggregation was observed.

c. Morphological aspects of aggregation

Electron micrographs of cultures maintained for 24 hr at pH 9.5 show mostly single enlarged cells or autospores within a common wall (Fig. 4).

Electron micrographs of cells maintained for 4 days at pH 9.5 reveal clusters of cells that contain numerous groups of autospores. Each group is held within the original mother cell wall, with each autospore having its own cell wall (Fig. 5A,B and C). The space between the autospores and the common mother cell wall is filled with fibrous material. Some groups display a discontinuous original mother cell wall, while in others, accumulation of parallel sheets occurs in the fibrous material within the original mother cell wall (Fig. 5A). Groups of autospores, each within its own mother cell wall, appear connected by incomplete additional common cell walls (Fig. 5A and C).

In control clusters at pH 6.3 (Fig. 6) only cell walls of mother cells containing four autospores were observed. Each has its own cell wall, a structure similar to that de-

Figure 4

ELECTRON MICROGRAPHS OF CHLORELLA VULGARIS CULTURED AT pH 9.5 FOR 24 HOURS

A culture of Chlorella vulgaris grown asynchronously at pH 6.3 was diluted to 2 x 10^6 cells/ml and the cells cultured at alkaline pH. After 24 hours exposure to the alkaline conditions, samples were taken, fixed and prepared for electron microscopy as described in Materials and Methods.

The scale represents 2 μ.

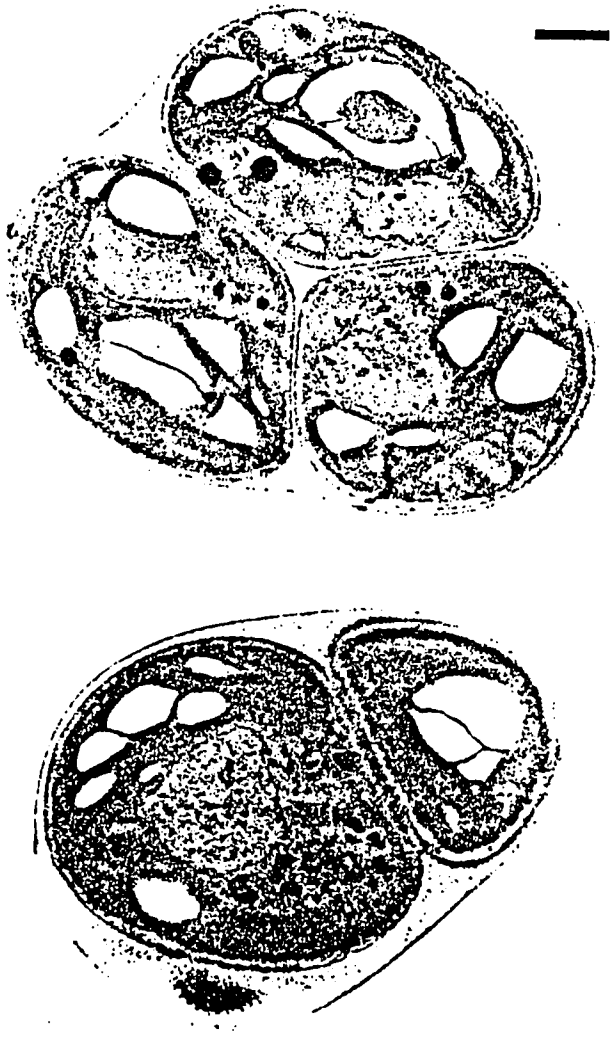


Figure 5

ELECTRON MICROGRAPHS OF CHLORELLA VULGARIS CULTURED AT pH 9.5 FOR 4 DAYS

A culture of Chlorella vulgaris grown asynchronously at pH 6.3 was diluted to 2 x 10^6 cells/ml and the cells cultured at alkaline pH. After 4 days exposure to the alkaline conditions, samples were taken, fixed and prepared for electron microscopy as described in Materials and Methods.

The scale represents 2 μ.

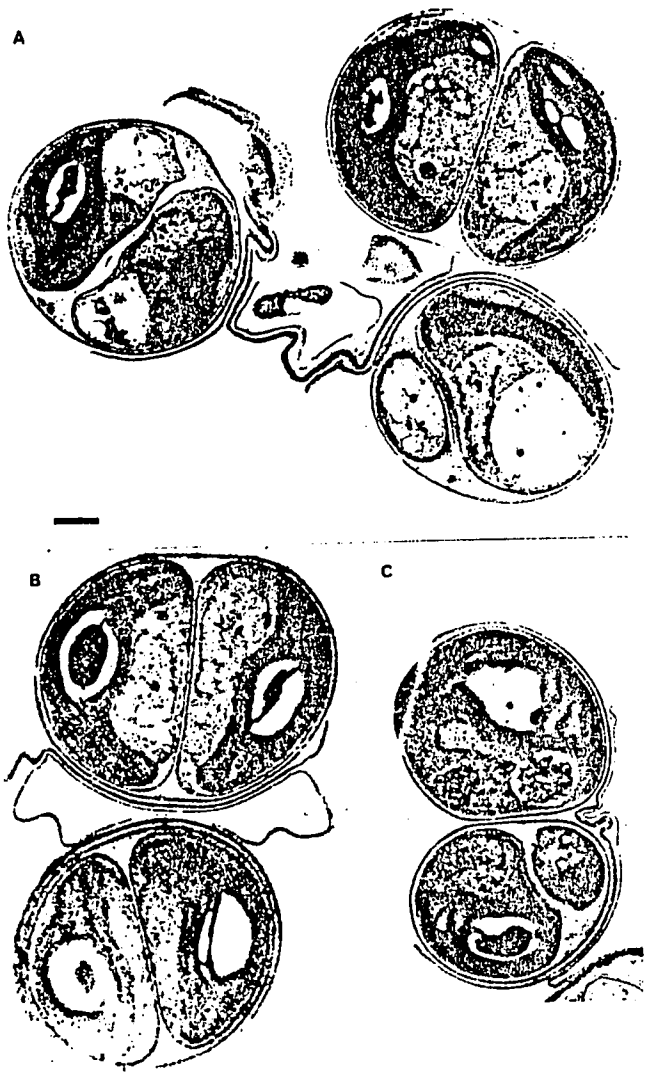
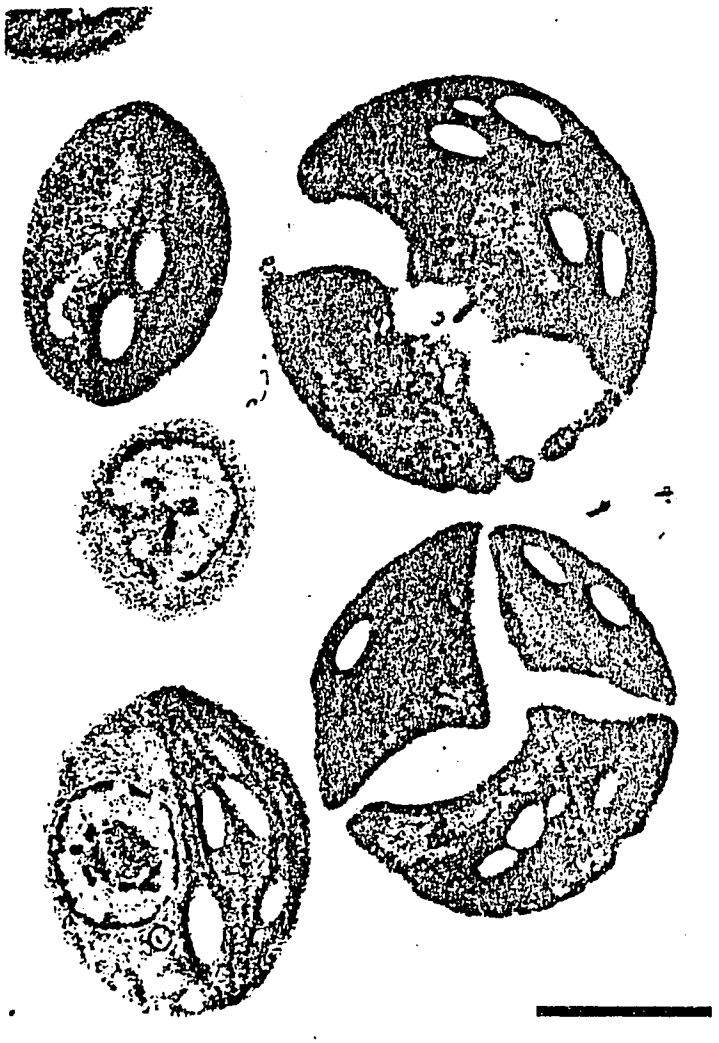

A
B
C

Figure 6

ELECTRON MICROGRAPHS OF CHLORELLA VULGARIS CULTURED AT pH 6.3

A culture of Chlorella vulgaris grown asynchronously at pH 6.3 was diluted to 2 x 10^6 cells/ml and the cells cultured at pH 6.3. After 4 days of growth, samples were taken, fixed and prepared for electron microscopy as described in Materials and Methods.

The scale represents 2 μ.



scribed by Pickett-Heaps for other Chlorella species (1975). No groupings of four autospores clinging to additional cell walls or interconnected by cell wall segments were ever seen in cultures maintained at pH 6.3.

II. The pattern of growth of Chlorella vulgaris at alkaline pH

In order to further understand the phenomenon of aggregate formation, the pattern of growth of Chlorella cells in alkaline conditions, was studied. For this, Chlorella vulgaris cells were grown asynchronously for 4 days at alkaline pH (achieved as described in Materials and Methods) and several growth parameters monitored. As a control, another culture was grown at pH 6.3. The growth parameters that were studied were: cell number, dry weight, optical density and chlorophyll, DNA and protein content (Fig. 7).

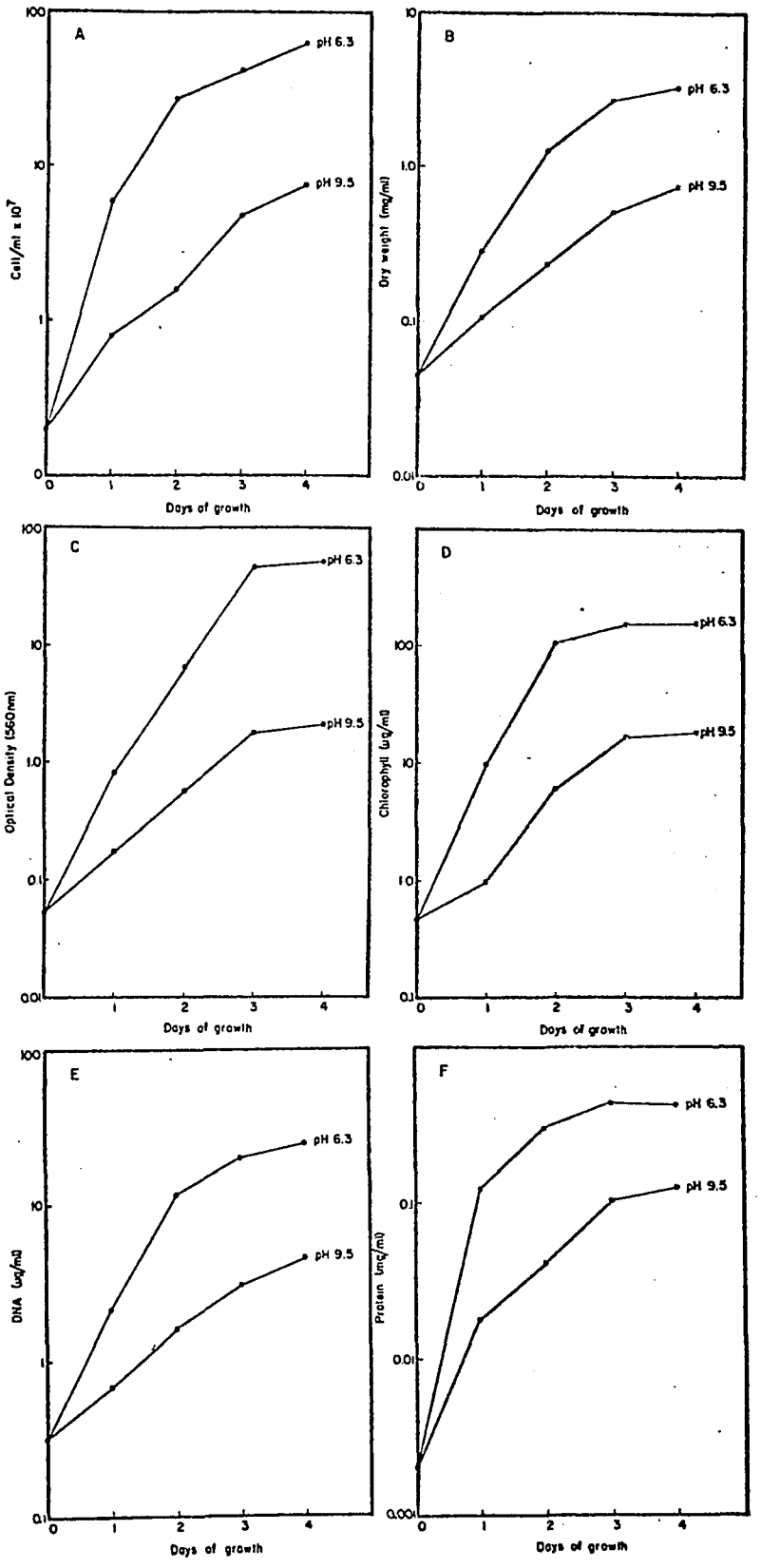
From Fig.7-A it can be seen that the cell number for the control culture (pH 6.3) increases by a factor of 30 during the first 24 hours, but the rate of increase diminishes markedly thereafter. The culture maintained at pH 9.5 increases in cell number from the outset, but this increase in growth rate over a 4 day period is more constant. The culture at the alkaline pH reaches after 4 days a cell concentration of 7.6×10^7 cells/ml as compared to 63.1×10^7 cells/ml for the control culture.

Figure 7

GROWTH AS MONITORED BY CELL NUMBER, DRY WEIGHT, OPTICAL DENSITY, AND CHLOROPHYLL, DNA AND PROTEIN CONTENT, FOR CHLORELLA VULGARIS CULTURED AT pH 6.3 AND 9.5

A culture of Chlorella vulgaris was grown asynchronously at pH 6.3 diluted to 2 x 10^6 cells/ml and cultured at pH 9.5 or 6.3 as described in Materials and Methods. At daily time intervals samples were removed and their cell number, dry weight, optical density (560 nm) and chlorophyll, DNA and protein content determined as described in Materials and Methods.

Each point is the average of two experimental determinations (in each experiment duplicate samples were used). The experiment was done before in another growth system with essentially the same results.


A
pH 6.3
pH 9.5
Cell/ml x 10⁷
Days of growth
B
pH 6.3
pH 9.5
Dry weight (mg/ml)
Days of growth
C
pH 6.3
pH 9.5
Optical Density (560nm)
Days of growth
D
pH 6.3
pH 9.5
Chlorophyll (ug/ml)
Days of growth
E
pH 6.3
pH 9.5
DNA (ug/ml)
Days of growth
F
pH 6.3
pH 9.5
Protein (mg/ml)
Days of growth

The changes in dry weight (d.w.) of the cultures grown at high and low pH are presented in Figure 7-B. The pattern of dry weight increase is generally similar to that for cell number. After 24 hr the dry weight of the control (pH 6.3) is 2.8 times higher than that of the cultures grown at pH 9.5. After 4 days this difference increases to a factor of 4.5. An interesting pattern is revealed by combining Figures 7-A & B into a table expressing the data as the number of cells/dry weight (Table 3). From Table 3 it can be seen that thereare generallymore cells/mg d.w. in low pH than in high pH, which indicates an increase in cellular dry matter at the alkaline pH.

Fig. 7-C describes the changes in the cell density (as measured by O.D. 560 nm) of Chlorella vulgaris cultures grown at pH 6.3 and 9.5. The general pattern of Figure 7-C is similar to that of Figures 7-A & B. However, in the O.D. pattern of both cultures (pH 6.3 and 9.5) there is no additional increase from day 3 to 4. This is not observed for the cell number or dry weight measurements. As previously mentioned, optical density is affected by both cell size and cell number and can be used as a parameter of growth only in combination with other parameters.

TABLE 3

THE EFFECT OF pH ON THE DRY WEIGHT YIELD OF *CHLORELLA VULGARIS* CELLS

Days of Growth	pH 6.3	pH 9.5
0	3.1	3.1
1	27.0	4.8
2	25.4	6.1
3	16.4	6.7
4	15.4	6.6

The experiment was the same as described for Figure 7. The results are expressed as the number of cells per mg d.w. x 10^{-7} and were calculated from results given in Figure 7 -A and B.

Another growth parameter is chlorophyll content (Fig. 7-D). In general, the pattern of chlorophyll content resembles the patterns for the other growth parameters. The significant feature of the pattern for this parameter is at the beginning of the curve, after 24 hr, there is a 10-fold difference between pH 6.3 and 9.5. This may indicate that either chlorophyll synthesis or breakdown is more sensitive to a change in pH of the medium. This difference decreases after 4 days but is still greater than that of the other parameters. The changes in chlorophyll content per ml are not sufficiently diagnostic since there is a difference in the number of cells and in the dry weight per ml of the two cultures. Table 4 puts these data in perspective by describing the changes in chlorophyll content on a per mg dry weight and per cell basis. The pattern of chlorophyll content per dry weight and per cell are generally similar. There is an increase in the content of chlorophyll per cell at the high pH compared with the low pH. The increase in chlorophyll content eventually levels off at 7.42 μg/cell x 10^{-7} in pH 9.5 and 3.87 μg/cell in pH 6.3 (control). The content of chlorophyll on a dry weight basis is higher in the low pH compared with the high pH. The difference is greatest after 24 hr, representing the immediate and specific alteration of

TABLE 4

THE EFFECT OF CULTURE pH ON CHLOROPHYLL CONTENT IN *CHLORELLA VULGARIS* CULTURED AT pH 6.3 AND 9.5

Days of Growth	pH 6.3		pH 9.5	
	µg Chlorophyll Per mg d.w.	Cell x 10^{-7}	µg Chlorophyll Per mg d.w.	Cell x 10^{-7}
0	12.67	5.56	12.67	5.56
1	37.24	7.59	9.34	3.96
2	79.67	2.88	27.72	4.69
3	54.17	3.24	35.42	7.57
4	49.68	3.87	28.93	7.42

The experiment was the same as was described for Figure 7. The results were calculated from data given in Figure 7-A, B and C.

chlorophyll metabolism by an alkaline environment. The difference in the patterns of chlorophyll content on a dry weight basis versus a per cell basis reflects the difference, mentioned before, between the changes in dry weight and cell number.

Figure7-E describes the change in DNA content of Chlorella vulgaris cultures grown at pH 6.3 and 9.5. The pattern of change in DNA content in the culture reflects the other parameters of growth. More interesting is the pattern of cellular DNA content as presented in Table 5. From Table 5 it can be seen that the DNA content of Chlorella cells grown at the alkaline pH is 3 fold higher than is the DNA content of control cells grown at pH 6.3. This suggests the existence of more than one nucleus per cell in the alkaline pH grown cells. In agreement with DNA content, the cellular dry weight is also higher for the cells grown at high pH than it is for cells grown at low pH. This observation supports the idea that the increase in cell size at pH 9.5 is not simply the result of some anomalous cell expansion phenomenon, but rather reflects an alteration of a more fundamental cellular process.

The pattern of protein content is generally similar to that of cell number and DNA and chlorophyll content (Fig7-F).

TABLE 5

CELLULAR DNA CONTENT, AND CELLULAR DRY WEIGHT OF *CHLORELLA VULGARIS* CULTURED AT pH 6.3 AND 9.5 FOR 4 DAYS

	pH 6.3	pH 9.5
DNA/cell ($\mu g \times 10^{-7}$)	0.42	1.13
d.w./cell ($mg \times 10^{-7}$)	0.05	0.1

The experiment was the same as described for Figure 7. The results were calculated from data given in Figure 7-A, B and E.

TABLE 6

PROTEIN CONTENT OF CHLORELLA VULGARIS
CULTURED AT pH 6.3 AND 9.5

Time of Growth (Days)	pH 6.3		pH 9.5	
	% protein (mg protein/ mg d.w.)	protein/cell (µg protein x 10^{-7}/cell)	% protein (mg protein/ mg d.w.)	protein/cell (µg protein x 10^{-7}/cell)
1	36.0	26.0	22.5	66.6
2	29.7	20.1	25.2	73.6
3	25.0	14.6	28.1	84.1
4	20.1	11.6	27.5	68.9

An asynchronized culture of Chlorella vulgaris was diluted to 2 x 10^6 cells/ml and grown with continuous illumination at low or high pH as described previously. Samples were removed daily and the protein, dry weight and cell number determined. The results were calculated from data given in Figure 7-A, B and F.

The cellular protein content, and percent of protein are presented in Table 6. The percent of protein content decreases significantly from day 1 to 4 in the control culture grown at pH 6.3, however, in culture grown at high pH the percent of protein increases gradually during this time.

The effect of alkaline pH on all of the different growth parameters is summarized in Table 7. In general, the pattern that is revealed is that there is an increase in cellular dry weight and chlorophyll, protein, DNA and polysaccharide content. The increase in cell volume (Table 2) together with the results presented here indicate the formation of enlarged cells containing greater amounts of many cell constituents as a result of exposure to the alkaline pH.

When described on per DNA basis the pattern that is revealed is different. There is a decrease in dry weight and chlorophyll content in the alkaline grown cells and a significant increase in protein content. However, in PAS content the same amount is found per DNA basis in pH 6.3 and 9.5.

TABLE 7

A SUMMARY OF THE DIFFERENCES BETWEEN CHLORELLA VULGARIS CULTURED FOR 4 DAYS AT pH 6.3 AND 9.5

Parameter Measured	µg/cell x 10^{-7}			µg/µg DNA		
	pH 6.3	pH 9.5	ratio 9.5/6.3	pH 6.3	pH 9.5	ratio 9.5/6.3
Dry weight	50	100	2.0	119	88.4	0.7
Chlorophyll	3.87	7.42	1.9	9.21	6.56	0.7
Protein	11.60	68.90	5.9	27.6	60.9	2.2
DNA	0.42	1.13	2.7	-	-	-
Polysaccharides*	0.34	0.98	2.9	0.80	0.86	1.0

Chlorella vulgaris was cultured at pH 6.3 and 9.5 for 4 days as described previously. The data presented in this table were derived from the data presented in Figure 7.

* The units for polysaccharides are relative amounts of dye per cell or per µg DNA.

III. Changes in cell wall metabolism induced by the alkaline pH

a. Changes in polysaccharide content and composition

The observation that there was increase in cell size was reminiscent of the reports by Higashiyama (1967 I, II) in which giant cells were induced by the addition of many different sugars to the medium. In his report, the giant cells gave a positive reaction with Ruthenium Red, resulting, according to his interpretation, from an increase in the pectin content of the cells. The electron micrographs of the aggregated cells (Fig. 5) also indicated some changes in cell wall structure.

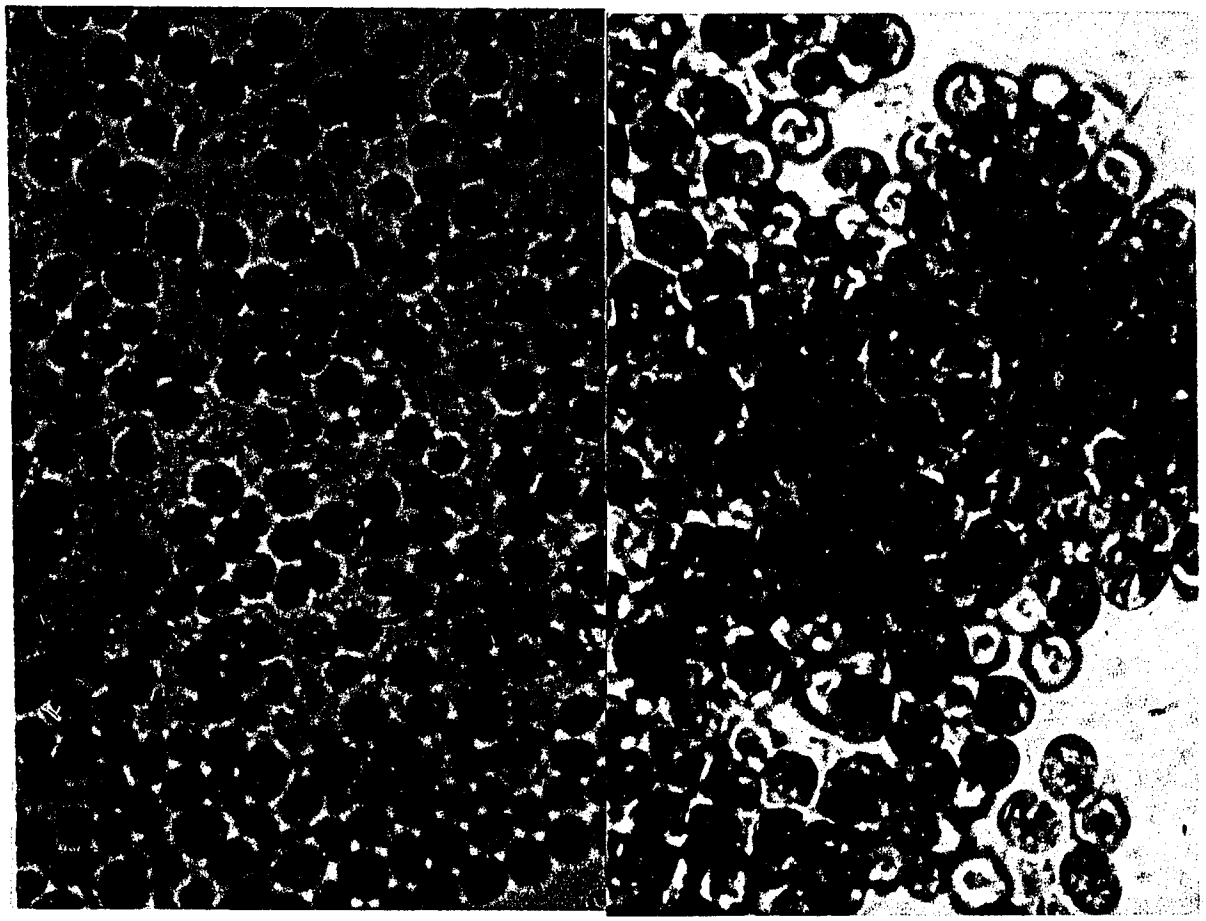
For preliminary experiments, Ruthenium Red was used in order to detect general changes in cell wall composition. From the photomicrographs in Figure 8 it can be seen that cells growing for 4 days at pH 9.5 gave a positive reaction with Ruthenium Red (red color) as compared to Chlorella cells grown at pH 6.3 (stayed green).

Positive results (pink color) were also observed using the histochemical method according to Gee et al. (1959) which is based on the reaction of carbomethoxyls of pectinic acids with hydroxylamine in aqueous-alcoholic solutions. The extent of formation of pectin hydroxamic acid is determined by the development of pale, pink color due to the ferric-

Figure 8

PHOTOMICROGRAPHS OF CHLORELLA VULGARIS CULTURED AT pH 6.3 OR 9.5 AND STAINED WITH RUTHENIUM RED

A culture of Chlorella vulgaris was grown for 4 days asynchronously at pH 6.3 or 9.5 as described in Materials and Methods. Samples were removed from the cultures, stained with Ruthenium Red and photographed through a Zeiss microscope. The photograph on the left represents cells grown at pH 6.3, and the photograph on the right, cells grown at pH 9.5.



pectin hydroxamate complex. This can indicate the degree of esterification of pectic substances. Cells grown at pH 9.5 gave a positive color reaction, but the color development was insufficient to measure or photograph. These results could only be used to support the results of Ruthenium Red staining and prompted the extraction and determination of pectin.

The total pectin content and the distribution into the different pectin fractions is given in Table 8. The total pectic substances of aggregated cells was twice that of the control cells (grown at pH 6.3). The main increase (2-fold) is due to an increase in the water soluble pectin fraction (4-fold) considered to be pectinic acid. In the EDTA soluble fraction there is a slight decrease for the aggregated cells. This fraction contains calcium pectate. The insoluble fraction, containing the protopectin (Rouse and Atkins, 1955), totally disappeared.

Another method of staining polysaccharides is the periodic acid Schiff reagent (PAS). This reagent was used to stain cells from the different stages of growth at pH 6.3 and 9.5. Accordingly, the cells were fixed onto slides, stained with PAS and the amount of dye that reacted with the polysaccharides was monitored microspectrophotometrically as

TABLE 8

PECTIN CONTENT OF CHLORELLA VULGARIS
CULTURED AT pH 6.3 OR 9.5

	WSP	VSP	ISP	TPS
pH 6.3	3.05	2.69	1.45	7.19
pH 9.5	13.2	1.96	0	15.16

Legend: WSP - water soluble pectin
VSP - EDTA soluble pectin
ISP - Insoluble pectin
TPS - Total pectic substances

A synchronized culture of Chlorella vulgaris was grown for 4 days at pH 6.3 or 9.5. Cells were harvested and separated to the different fractions as described in Materials and Methods. Pectin was determined in the carbazole reaction (Dische, 1947; Bitter and Comb and McCready, 1952). Results are expressed in μg/mg dry weight.

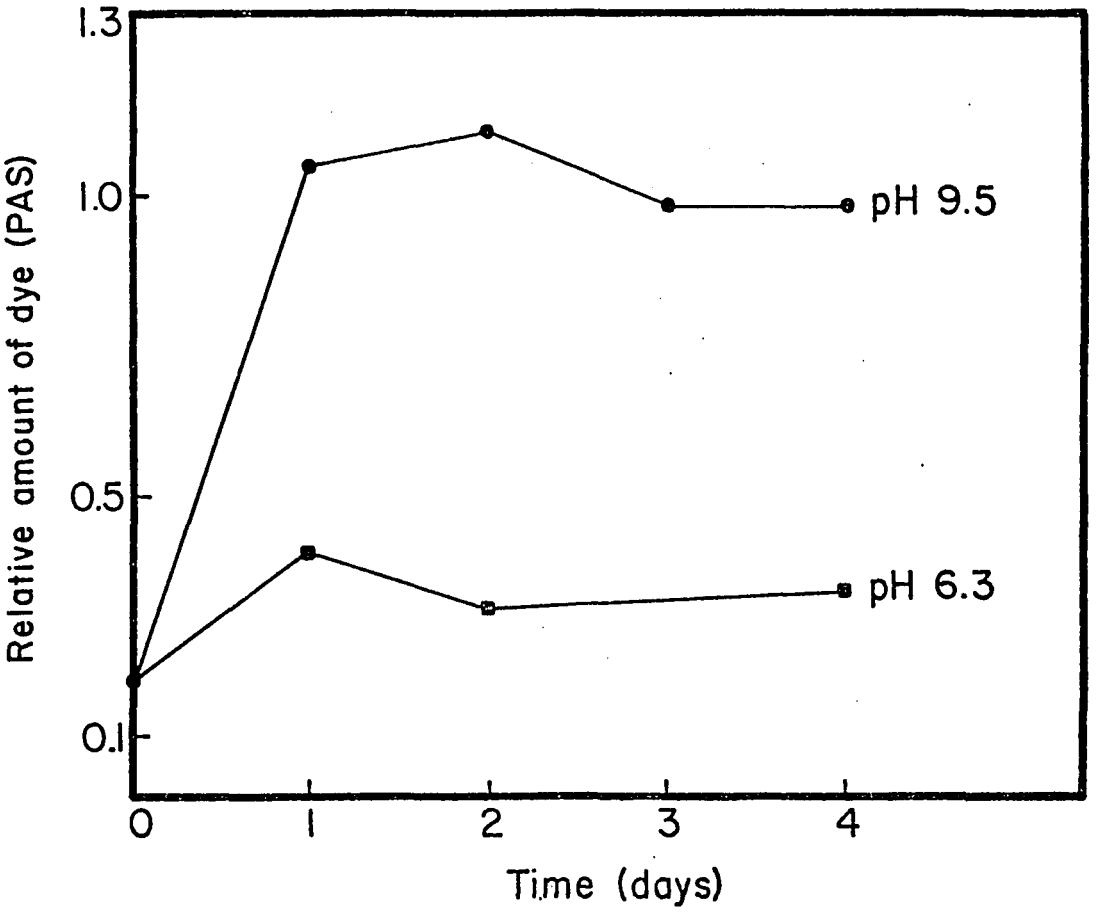
Each number is the average of two experimental determinations.

Figure 9

POLYSACCHARIDE CONTENT AS MEASURED BY PAS STAINING OF CHLORELLA VULGARIS CULTURED AT pH 6.3 AND 9.5

The experiment was the same as described for Figure 7. At daily time intervals, samples were removed, fixed onto slides and their PAS staining measured according to the description given in Materials and Methods.

Each point is the average of two experimental determinations. At least 20 cells were analyzed in each determination.


1.3
1.0
0.5
0.1
Relative amount of dye (PAS)
pH 9.5
pH 6.3
0
1
2
3
4
Time (days)

described in Materials and Methods. As can be seen from Figure 9, in the control cells there were only slight fluctuations with time. On the other hand, the cells grown at pH 9.5 had a significant increase in PAS staining during the first 24 hr (from 0.186 to 1.059) and no change thereafter. Thus, major changes in polysaccharide metabolism occur within 24 hours after the transfer to high pH.

In order to further identify the specific polysaccharides that are effected by the PAS staining, an additional method was used. Accordingly, cells in different stages of the cell cycle were treated with different enzyme solutions (after being fixed onto slides) and stained for PAS thereafter. The results of the various enzyme treatments are represented in Table 9. Obviously, the combined methods (enzyme treatment with PAS staining) does not give precise results. Differences of up to 20% occur, resulting perhaps from the relative long duration of the fixed cells in the enzyme solutions. Pectinase did not affect cells of the low pH treatment, but caused significant change in the cells of the alkaline pH treatment. The main effect seems to be at the 7th hour of the cycle in which both pectinase and cellulase caused decrease in PAS staining (68% and 59% respectively). The difference between enzyme effect in pH 6.3 and 9.5 might indicate differences in cell wall composition. The results do not point to a

TABLE 9

EFFECT OF TREATMENT WITH PROTEASE, AMYLASE, CELLULASE AND PECTINASE ON PAS STAINING OF CHLORELLA VULGARIS GROWN IN pH 6.3 OR 9.5 AT DIFFERENT TIMES OF THE CYCLE

		Control		Protease	Amylase	Cellulase	Pectinase
	Hours of the Cycle	amount of dye	% of change	% of change	% of change	% of change	% of change
pH 6.3	3	.258	0.	+5.0	-10.	+6.	-6.
	7	.701	0.	-7.6	+ 6.3	+6.	-9.
	24	.120	0.	---	+ 6.7	+27.	-4.
pH 9.5	3	.331	0.	---	---	-5.0	+20.1
	7	.462	0.	+9.9	-27.1	-59.	-68.
	24	.752	0.	- .9	-30.9	-42.	-45.

Legend: Chlorella vulgaris culture was synchronized as described in Materials and Methods and transferred after dilution at 0 time to low or to alkaline pH. Samples of cells were taken at different times during the cycle and fixed onto slides. After fixation, the slides were incubated for 16 hrs in the respective enzymes solution or in the buffer as a control. Following enzyme treatment the slides were washed with water, air dried and stained for PAS as described. The stained cells were read in a microspectrophotometer at 550nm, and the relative amount of dye was calculated as described in Materials and Methods. Each value is the average of 3 experimental determinations. At least 20 cells were analyzed in each case.

specific polysaccharide that is specifically effected by the PAS staining.

b. Changes in cellulase activity

The changes in cell wall structure that were revealed by the electron micrographs together with the significant changes in polysaccharide content and composition as shown by the PAS staining (Fig. 9) and pectin content (Table 8) point to a change in cell wall metabolism as one of the first events occurring after the pH is raised. Since there are obviously changes in cell wall metabolism and since the major structural component of the cell wall is cellulose it seemed logical to monitor cellulase activity during the alkaline treatment.

To find the pH optimum of cellulase activity, assays were performed at different pH values (Appendix E, Fig. 22). The optimal pH was found to be 6.3 for both control and aggregated cells. It is interesting to note here that this optimal pH is different from that found for cellulase activity in Trichoderma (King and Vessal, 1969) (the most studied cellulase). Verification of cellulase activity was obtained using both the viscometric assay and the glucostat assay with both alpha-cellulose and carboxymethyl-cellulose (Appendix F, Tables 24, 25, 26).

Figure 10

THE EFFECT OF THE EXPOSURE OF CHLORELLA VULGARIS TO ALKALINE pH ON CELLULASE ACTIVITY

A culture of Chlorella vulgaris was grown asynchronously at pH 6.3 with continuous illumination as described previously. Prior to the experiment the culture was diluted to 2.5×10^7 cells/ml and the pH adjusted to 9.5 as described. At various time intervals after the pH was raised, aliquots were removed from the culture, the cells broken in a Braun Homogenizer, and cellulase activity determined as described in Materials and Methods.

Each point is the average of three experimental determinations.

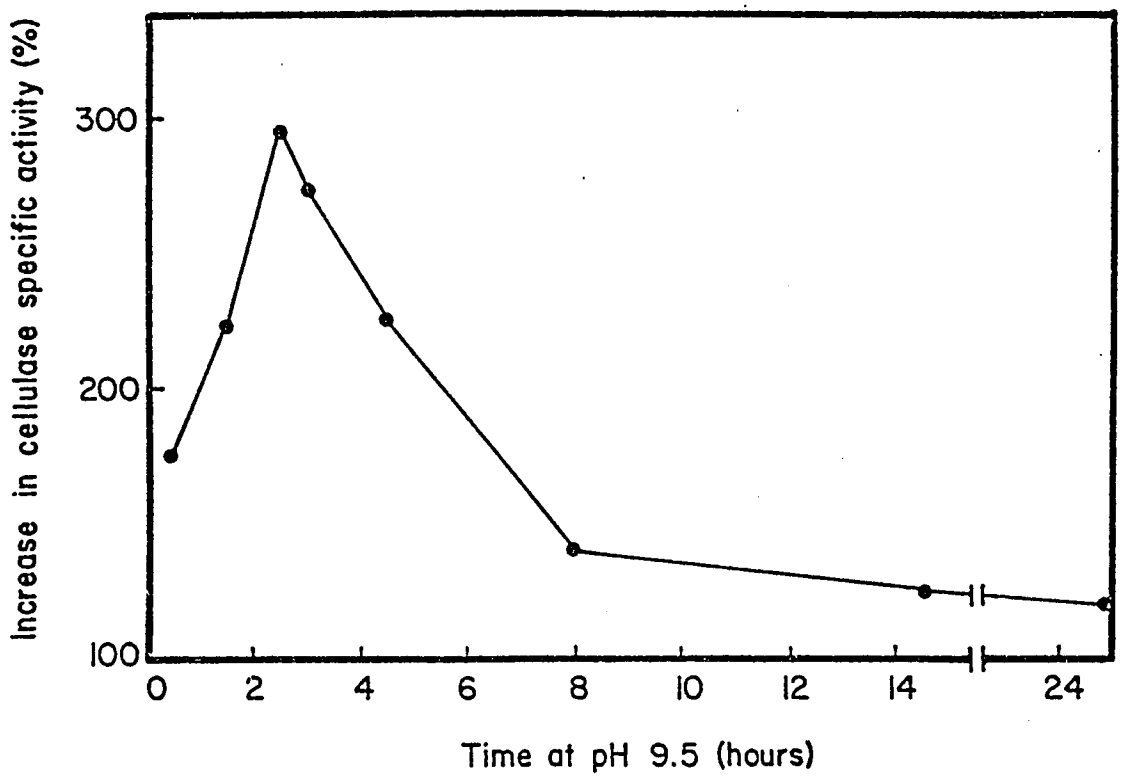

Increase in cellulase specific activity (%)
300
200
100
0
2
4
6
8
10
12
14
24
Time at pH 9.5 (hours)

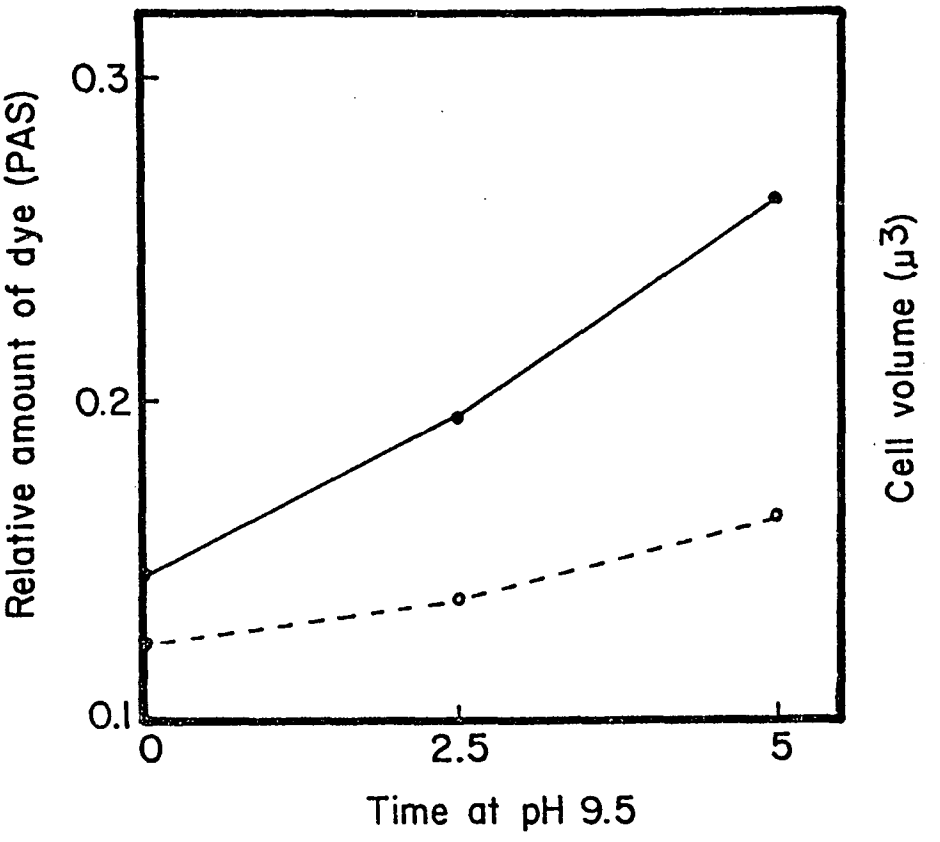
Figure 11

THE EFFECT OF EXPOSURE OF CHLORELLA VULGARIS TO ALKALINE pH ON THE AMOUNT OF POLYSACCHARIDES AND CELL VOLUME

The experimental protocol was identical to that of Figure 15. At different time intervals, samples were removed, fixed onto slides and their PAS staining and cell volume determined as described in Materials and Methods.

The solid line represents PAS staining, the dashed line represents cell volume.

Each point is the average of two experimental determinations. At least 20 cells were analyzed in each determination.


0.3
0.2
0.1
Relative amount of dye (PAS)
Cell volume (μ3)
0
2.5
5
Time at pH 9.5

The effect of exposure of Chlorella vulgaris to alkaline pH on cellulase activity was studied (Fig. 10). As can be seen, there is a peak in cellulase-specific activity 2.5 hours after the exposure to the alkaline pH, followed by a leveling off at a value higher than the starting point. Does this rapid increase in cellulase activity correlate with a change in cell wall polysaccharides? To answer this question, cells at different times, following exposure to the alkaline pH, were stained with PAS. Figure 11 shows that in contrast to cellulase specific activity the PAS staining increased only slightly during the first 5 hours at the alkaline pH. Changes in cellulase activity seems to precede changes in cell wall polysaccharides, a point that seems significant and which will be discussed later.

In order to acertain whether the increase in cellulase activity is a specific result of exposure to alkaline pH and not due to an overall increase in many enzyme activities, Malic dehydrogenase was chosen to be such a control (Table 10). From these data it would appear that the alkaline pH does not result in a stimulation of malic dehydrogenase's activity.

Is the increase in cellulase activity a result of new synthesis of the enzyme or is it the activation of an inactive form of the enzyme? In order to answer this question,

TABLE 10

THE EFFECT OF THE ALKALINE pH ON MALIC DEHYDROGENASE SPECIFIC ACTIVITY IN CELLS OF CHLORELLA VULGARIS

Malic Dehydrogenase Specific Activity	Hours After Transfer to High pH		
	0	2.5	5
(μ moles/min/mg protein)	77.65	74.56	62.85

The experimental protocol was identical to that of Figure 15. Aliquots were removed from the culture at different times after the exposure to the alkaline pH, the cells broken in a Braun Homogenizer and measured for malic dehydrogenase activity as described in Materials and Methods. Each value is the average of 2 experimental determinations.

cells were treated with cycloheximide for 2 hours and then exposed to the alkaline pH for 2.5 hours (the peak in enzyme activity). From the results presented in Table 11, it can be seen that, following cycloheximide treatment there is no inhibition of enzyme activity. On the contrary, cycloheximide treatment results in a 62% increase in cellulase activity in the alkaline pH after 2.5 hr of growth, and a 36% increase at the low pH. This stimulation by cycloheximide of cellulase activity at the low pH brings its level up to that stimulated by alkaline treatment alone. These results, the stimulation of cellulase specific activity following cycloheximide treatment, raises a question about the ability of cycloheximide to inhibit protein synthesis in Chlorella. The inhibitory effect of cycloheximide on protein synthesis in Chlorella has been previously demonstrated (Thinh and Griffith, 1971), and has been confirmed here from experiments performed with synchronized cultures (Table 16). In this system cycloheximide caused a 42% inhibition of protein content at the low pH and only a 10% at the high pH.

In order to avoid a possible specific effect of cycloheximide on cellulase activity another protein synthesis inhibitor, puromycin, was used (Table 11). Results from this experiment, although different from cycloheximide experiment,

also does not support the idea that the increase in cellulase activity is due to de novo synthesis. However, the fact that protein content after the inhibition treatment was decreased might be due to other effects than protein synthesis inhibition. In addition, it might be that the bulk protein synthesis is more sensitive to cycloheximide than is cellulase synthesis as was observed for other enzymes (Vassef et al., 1973).

The involvement of photosynthetically derived energy in the stimulation of cellulase activity was probed using the electron transport inhibitor DCMU and darkness. From results presented in Table 13, it can be seen that both DCMU and darkness prevented totally the increase in cellulase activity when transferred to alkaline pH.

TABLE 11

THE EFFECT OF CYCLOHEXIMIDE ON pH-INDUCED CELLULASE ACTIVITY IN CHLORELLA VULGARIS

	0 Hours				2.5 Hours							
					Low pH				High pH			
	Protein		Cellulase Activity		Protein		Cellulase Activity		Protein		Cellulase Activity	
Treatment	µg/ml	%	µg/ml	S.A.	µg/ml	%	µg/ml	S.A.	µg/ml	%	µg/ml	%
Control	265	100	1.6	6.4	385	145.2	2.5	6.4	350	132	6.1	17.4
Cycloheximide	280	100	2.3	8.2	290	103.5	8.7	30	315	112.5	8.9	28.2

The experimental protocol was the same as described for Figure 15 except that the cells were treated for 2 hours with cycloheximide (35 x 10 µM), collected by centrifugation, preparation and assay were performed as described in Materials and Methods. Cellulase specific activity (S.A.) is expressed as µg reducing sugars per mg protein. Each value is the average of 3 experimental determinations.

TABLE 12

THE EFFECT OF PUROMYCIN ON pH-INDUCED CELLULASE SPECIFIC ACTIVITY IN CHLORELLA VULGARIS

	Time of Exposure to pH 9.5 (Hours)		
	0	2.5	
Treatment		pH 6.3	pH 9.5
Control	7.0	6.4	11
Puromycin	7.0	6.2	10.8

The experimental protocol was identical to that for Table 11 with the exception that puromycin (at a final concentration of 0.5 x mM) was used instead of cycloheximide. Cellulase specific activity is expressed as μg reducing sugars/mg protein. Each value is the average of two experimental determinations.

TABLE 13

THE EFFECT OF DARKNESS OR DCMU ON THE pH-INDUCED INCREASE IN CELLULASE SPECIFIC ACTIVITY IN CHLORELLA VULGARIS

Treatment	Time of Exposure to pH 9.5 (Hours)		
	0	2	5
Control	5.16	10.41	6.2
Darkness	5.17	4.02	3.74
DCMU	5.15	---	4.93

The experimental protocol was identical to that of Table 11 with the exception that DCMU (10 μM) was used instead of Cycloheximide. In addition, another culture was exposed to darkness immediately at the beginning of the experiment. Enzyme preparation and assay were performed as described in Materials and Methods. Cellulase specific activity is expressed in μg reducing sugar per mg protein. Each value is the average of 2 experimental determinations.

IV. The pattern of growth of Chlorella vulgaris during the cell cycle

Since the main effect of transferring to alkaline pH seemed to occur within the first 24 hours, it seemed necessary to study the effect of alkaline pH on the different stages of the cell cycle.

a. Normal pH (6.3)

A Chlorella vulgaris culture was synchronized as described in Materials and Methods in a dark and light regime followed by dilution of the culture to a certain cell concentration (DLD). Several parameters of the cellular growth were measured in order to ensure that the newly isolated strain of Chlorella vulgaris could be synchronized as previously described (Lorenzen and Hesse, 1974). Cell number, cell volume (Fig.12-A), protein, DNA and chlorophyll content (Fig.12-B) were monitored throughout the cell cycle.

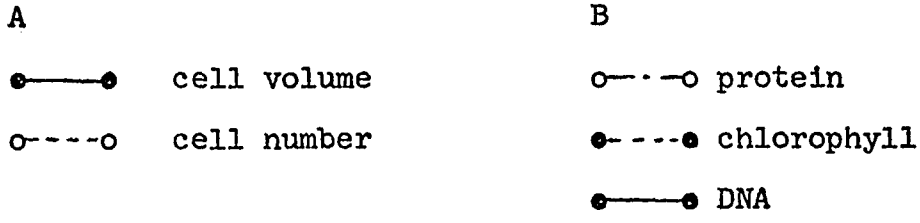
As is known for Chlorella, the increase in cell number (autospore release) occurs during the dark period (between 12-24 hours). Cell volume increases from 0-12 hours in the cycle and then returns to its original value following autospore release (Fig.12-A). The main increase in DNA content commences at 8 hours, with a small increase after 4 hours probably representing chloroplastic DNA (Fig.12-B) (Iwamura, 1970). The increase in chlorophyll content starts at the

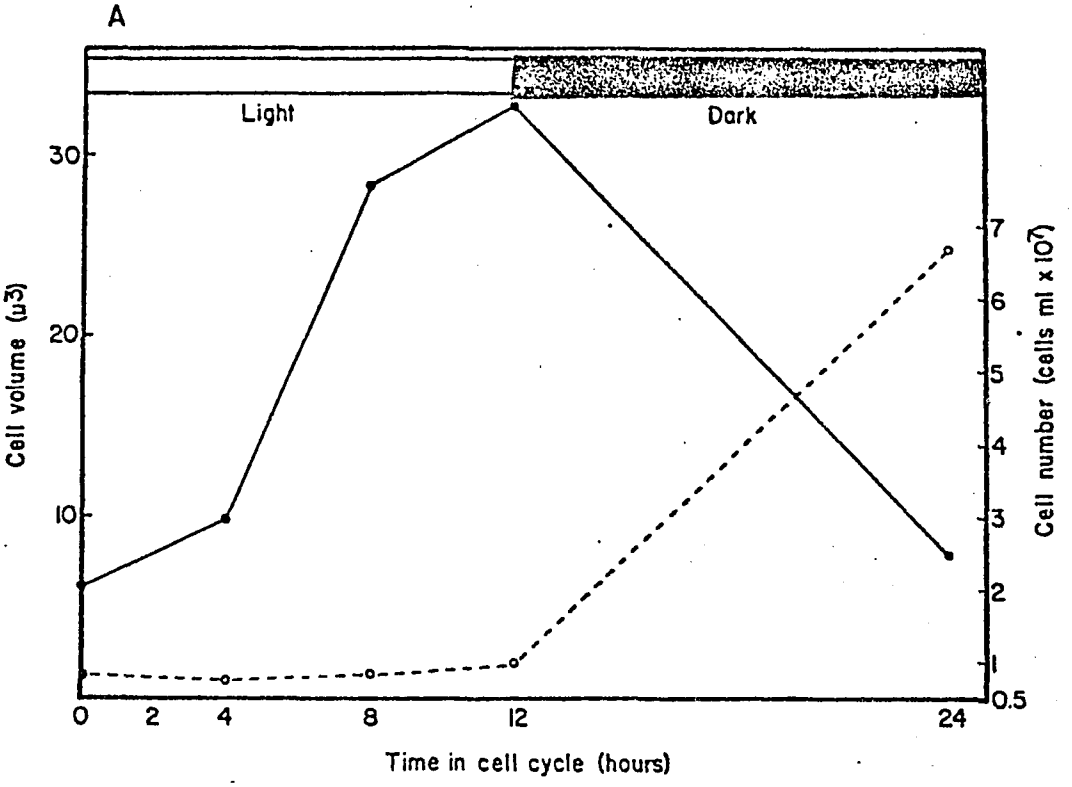
Figure 12

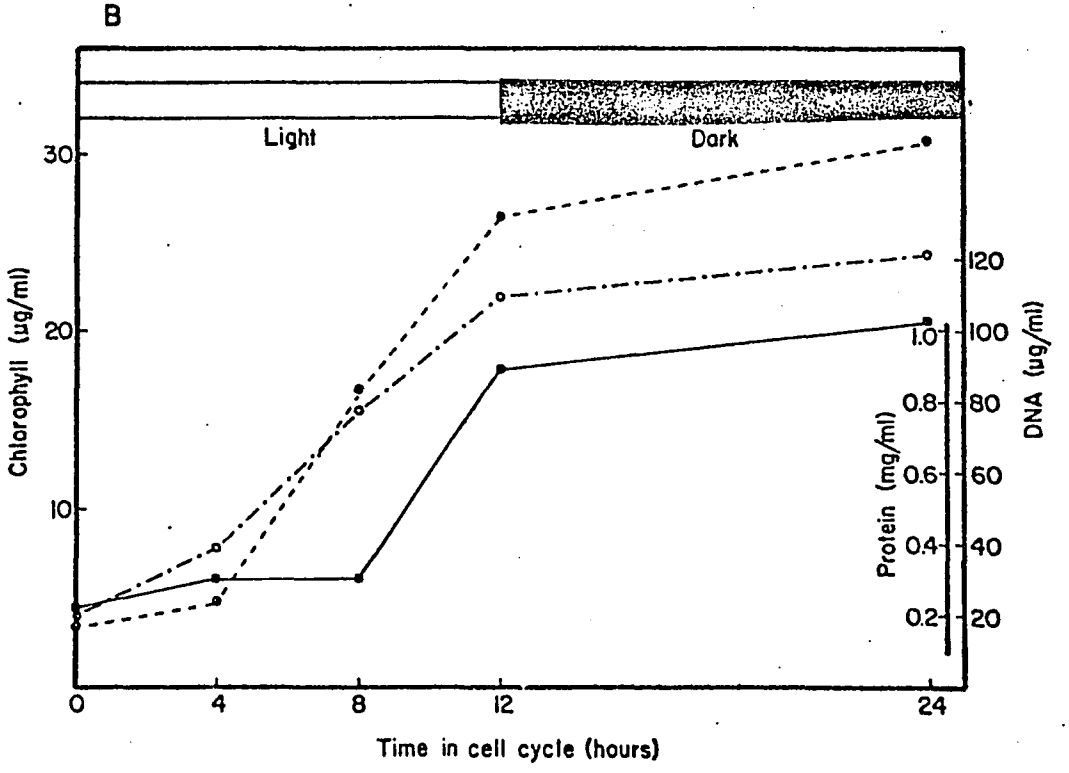
CHANGES IN CELL NUMBER, CELL VOLUME AND PROTEIN, DNA AND CHLOROPHYLL CONTENT DURING THE CELL CYCLE OF CHLORELLA VULGARIS

A culture of Chlorella vulgaris was synchronized for three cycles as described in Materials and Methods. At the onset of the fourth cycle the culture was diluted with N-8 medium to give a final concentration of 7×10^6 cells/ml. At selected times into the cell cycle, samples were removed from the culture and the cell number, and protein, DNA and chlorophyll determined as described in Materials and Methods. In addition, the cells were stained with PAS as described and the volume of the cells calculated from measurement of diameter of cells.

Each point is the average of two experimental determinations. For cell volume determination at least 20 cells were analyzed in each determination.




A
Light
Dark
Cell volume (μ3)
Cell number (cells ml x 10⁷)
30
20
10
7
6
5
4
3
2
1
0.5
0
2
4
8
12
24
Time in cell cycle (hours)


B
Light
Dark
Chlorophyll (ug/ml)
Protein (mg/ml)
DNA (μg/ml)
30
20
10
1.0
0.8
0.6
0.4
0.2
120
100
80
60
40
20
0
4
8
12
24
Time in cell cycle (hours)

beginning of the cycle and continues into the dark period (12% of the chlorophyll is synthesized during the dark period). Protein synthesis starts at the beginning of the cycle and continues throughout the cycle.

b. The effect of alkaline pH

Transferring the culture to alkaline pH at 0 time (immediately after dilution) causes almost immediate inhibition of chlorophyll synthesis (Fig.13-A). An inhibition of 54% was observed at the end of the cycle (Table 14). Transfer at the 4th or 8th hour into the cycle to pH 9.5 causes a gradual inhibition of chlorophyll content, however transfer at the 12th hour does not result in any further change in chlorophyll content despite the fact that some chlorophyll synthesis occurs during the dark period.

The pattern of protein content for Chlorella vulgaris cells, when transferred at different times of the cycle to alkaline pH, is similar to that of chlorophyll (Fig. 13-B). Although there is some protein synthesis in the dark, transfer to pH 9.5 at the 12th hour causes no inhibition (Table 14), demonstrating that a "point of no return," exists for synthesis of both chlorophyll and protein.

The ultimate parameter for measuring the point of no return phenomenon in a cell cycle is cell division or, in the case of Chlorella, autospore release and the division number. Thus, aliquots of a synchronized culture of

Figure 13

THE EFFECT OF THE STAGE IN THE CELL CYCLE ON THE EXPRESSION OF THE ALKALINE pH TREATMENT WITH RESPECT TO CHLOROPHYLL, PROTEIN AND DNA CONTENT OF CHLORELLA VULGARIS

A culture of Chlorella vulgaris was synchronized as described for Figure 12. At various times into the cell cycle 4 gm/l sodium bicarbonate were added to a particular culture to achieve the alkaline pH. The transfer to alkaline pH is indicated by arrows in the figure. The solid line represents the control culture that was maintained at pH 6.3 throughout the experiment. For each culture, at different time intervals during the cell cycle, aliquots were removed and their chlorophyll, protein and DNA content determined as described in Materials and Methods.

Each point is the average of two experimental determinations.

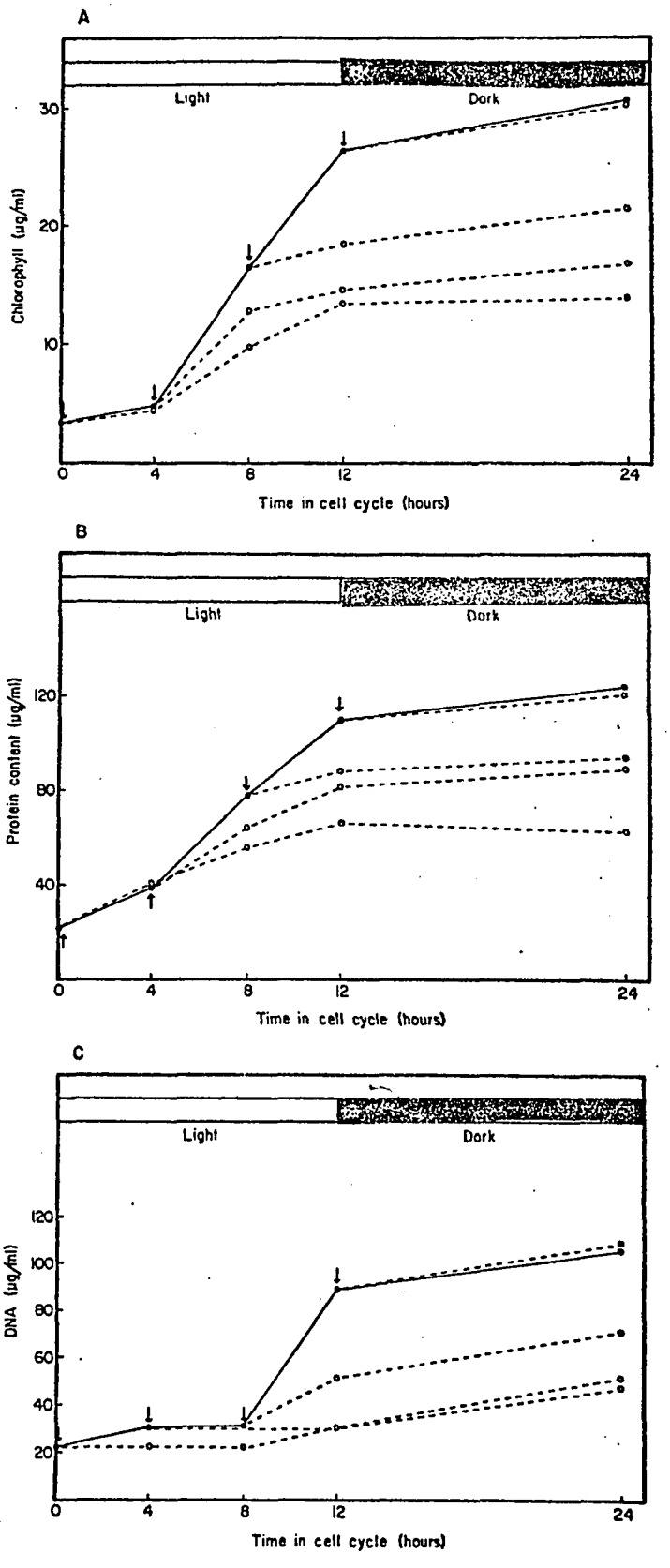

A
Light
Dark
30
20
10
Chlorophyll (µg/ml)
0
4
8
12
24
Time in cell cycle (hours)
B
Light
Dark
120
80
40
Protein content (µg/ml)
0
4
8
12
24
Time in cell cycle (hours)
C
Light
Dark
120
100
80
60
40
20
DNA (µg/ml)
0
4
8
12
24
Time in cell cycle (hours)

TABLE 14

CELL NUMBER, PROTEIN AND CHLOROPHYLL CONTENT OF CHLORELLA VULGARIS CELLS TRANSFERRED AT DIFFERENT TIMES OF THE CELL CYCLE TO ALKALINE pH

Hours in pH 6.3 before Transferring to Alkaline pH	Hours at pH 9.5	Cell/ml x 10^7	No. of Autospores per Cell (Division Number)	Protein				Chlorophyll			
				µg/ml	Percent Inhibition	µg/µg DNA	Percent Inhibition	µg/ml	Percent Inhibition	µg/µg DNA	Percent Inhibition
0	24	1.0	0	63	50	1.3	80.9	14	54	0.29	83.0
4	20	1.1	0.1	89	38	1.9	72.1	17	45	0.36	78.9
8	16	4.0	4.0	94	24	5.2	23.6	21	32	1.17	31.8
12	12	5.8	5.8	121	2.5	6.4	5.9	31	0	1.65	3.0
24 (control)	0	5.8	5.8	124	0	6.8	0	31	0	1.70	0

The experimental protocol was identical to that of Figure 13. The cell number, protein, and chlorophyll content were measured at the end of the cell cycle (24 hours after dilution). The data for protein and chlorophyll content are those of Figure 13.

Chlorella were transferred at different times during the cell cycle to alkaline pH, and cell number was determined at the end of the cycle (after 24 hours). From results presented in Table 14 and Fig. 14 it can be seen that a point of no return exists with respect to cell number and there is inverse correlation between cell number and cell volume as a function of time of alkalinity treatment. From Table 14 it can also be seen that when a culture was transferred at 0 time of the cycle to alkaline pH there was no increase in cell number at the end of the cycle (after 24 hours). However, the decrease in cell volume that occurs after 24 hours in a normal culture grown at pH 6.3 was prevented (Fig.14). Transferring the culture at the 4th hour into the cycle to pH 9.5 also resulted in no increase in cell number after 24 hr (Table 14), but caused a further increase in cell volume (Fig. 14). Transfer of the culture at the 8th hour to pH 9.5 resulted in a 4 fold increase in cell number as compared to a division number of 5.8 for a normal culture (Table 14). The average cell volume was higher than that of the control culture grown at pH 6.3, but lower than a culture transferred to high pH at the 4th hour into the cycle (Fig. 14).

The effect of alkaline pH on the DNA content of Chlorella cells is in general similar to the pattern described

Figure 14

THE EFFECT OF THE STAGE IN THE CELL CYCLE ON THE EXPRESSION OF THE ALKALINE pH TREATMENT WITH RESPECT TO CELL VOLUME OF CHLORELLA VULGARIS

The experimental protocol was the same as described for Figure 19. The arrows indicate the times into the cell cycle in which a particular culture was transferred to alkaline pH. The solid line represents the control culture which was maintained at pH 6.3 throughout the experiment. At different time intervals during the cell cycle, aliquots were removed, the diameter of the cells determined and the volume calculated. Each point is the average of two experiments. At least 20 cells were analyzed in each determination.

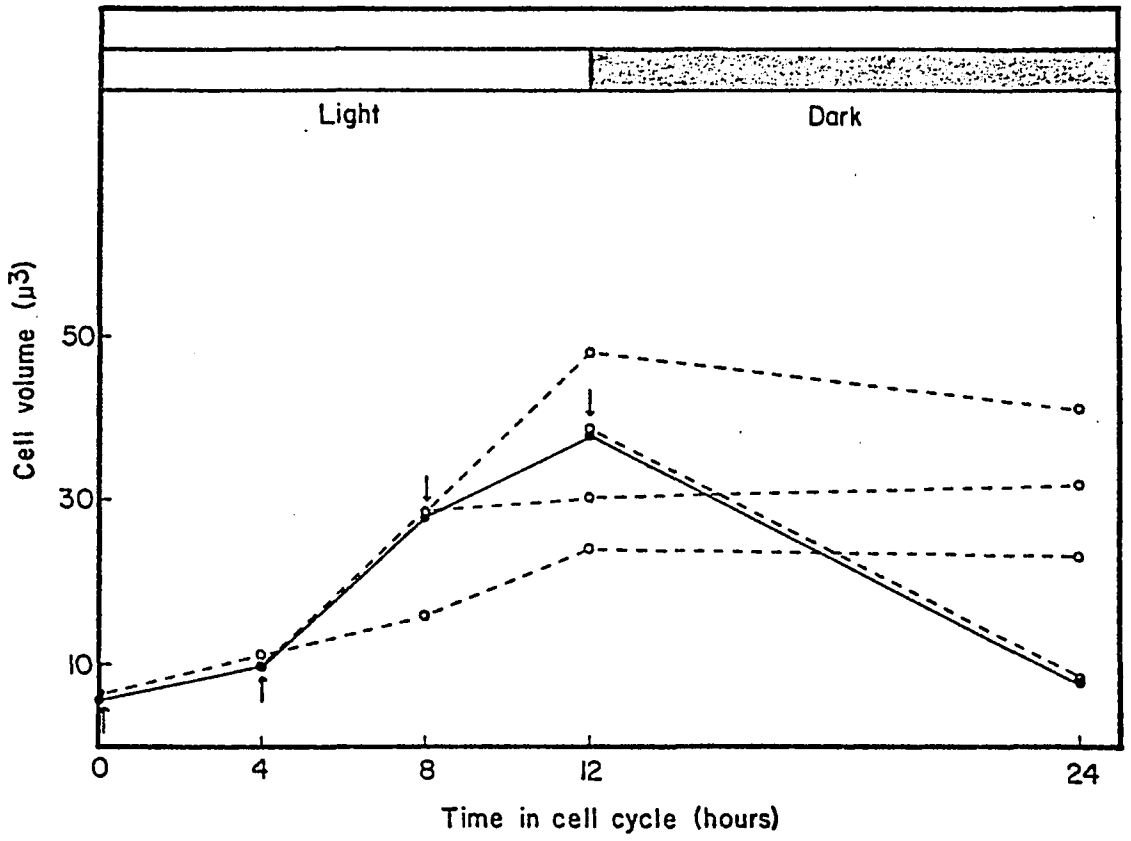

Light
Dark
Cell volume (μ3)
50
30
10
0
4
8
12
24
Time in cell cycle (hours)

TABLE 15

CELLULAR DNA CONTENT OF *CHLORELLA VULGARIS* CELLS AS A FUNCTION OF THE TIME IN THE CYCLE AT WHICH THE CULTURE WAS TRANSFERRED TO ALKALINE pH

Time in pH 6.3 Before Transferring to Alkaline pH (Hours)	Time at Alkaline pH (Hours)	µg DNA/Cell x 10^{-7}	x Times More Than Control
0	24	48.	2.63
4	20	46.8	5.7
8	16	17.8	0.97
12	12	18.7	1.02
24 (control)	0	18.2	1.

The experimental protocol was identical to that described for Figures 19-22. The DNA content and cell number were measured at the end of the cycle (24 hours after dilution) using the data shown in Figure 13.

for chlorophyll and protein. Transfer of the culture at 0 time to alkaline pH results in total inhibition of the first increase in DNA (probably chloroplastic DNA), (Fig. 13-C). The cells that are formed after 24 hours of the cycle contain 2.6 times more DNA than a normal cell (Table 15). Transfer to pH 9.5 at the 4th hour into the cycle causes relief of the inhibition of DNA synthesis compared to cells that were transferred to the alkaline pH at 0 time (Fig.13-C). The resultant cells contain 5.7 times as much DNA as a normal cell (Dn) at the end of the cycle (after 24 hr) (Table 15). Cells that were transferred at the 8th hour into the cycle to alkaline conditions contain the same amount of DNA as a normal cell (Table 15), although the total DNA is lower than a normal culture. A culture transferred to pH 9.5 at the 12th hour into the cycle has the same DNA content (per cell and per ml) as one grown normally (pH 6.3).

From analyzing changes in cell number, cell volume and DNA content per cell, it was concluded that the changes caused by transfer to alkaline pH were primarily a result of the inhibition of autospore release. Since autospores could not be released from their mother cell wall, their volume increased as did their chlorophyll, protein and DNA content. This increase actually represents that of autospores

that were counted as one cell but actually represents many cells held together within their original mother cell wall. The difference in cell volume and DNA content is therefore dependent upon the number of autospores that are within a single mother cell wall. The number of autospores depends on the stage in the cell cycle of the cells when transferred to the alkaline conditions.

To what extent is the prevention of autospore release dependent upon protein synthesis? In order to answer this question, Chlorella cells were treated with cycloheximide at different stages of the cycle, after which the culture was transferred to pH 9.5 (Table 16). Table 16 indicates that treatment with cycloheximide at the beginning of the cycle (after 2 hours) did not affect the alkaline pH inhibition of cell number. A cycloheximide treatment given after 7.5 hours of the cycle also did not change the alkaline pH effect with respect to cell number. When treated at the beginning of the cycle, cycloheximide caused greater inhibition of protein content than that caused by the alkaline pH itself. Less inhibition in protein content was effected when the treatment was given after 7.5 hours of the cycle (66% as compared to 21%). Exposure of a culture to alkaline conditions at this part of the cycle caused a smaller inhibition in protein content as compared to a culture

TABLE 16

THE EFFECT OF CYCLOHEXIMIDE ON CELL NUMBER AND PROTEIN CONTENT OF CHLORELLA VULGARIS TRANSFERRED AT DIFFERENT TIMES OF THE CYCLE TO pH 9.5

Hours in pH 6.3 Before Cycloheximide Treatment	Hours in pH 6.3 Before Transferring to Alkaline	Cell Number /mlx10^7	% Inhibition (per ml)	x Times Increase Over Control/Cell
2	24	0.8	66	2.79
2	4.5	0.65	64	4.07
-	4.5	0.7	46	5.36
7.5	24	3.7	21	1.62
7.5	10	3.6	36	1.32
-	10	3.5	33	1.41
24 (control)	24	5.5	0	1.

A synchronized culture of Chlorella vulgaris was prepared using the DLD procedure as described previously, diluted to 6.5×10^6 cells/ml and treated with cycloheximide (final concentration 3.5×10^{-5}M) for 2 hours at either 2 or 7.5 hours into the cell cycle. Following the cycloheximide treatment the cells were separated from the medium containing cycloheximide by centrifugation and washed twice with N-8 media, the cells were then resuspended in N-8 media at either pH 6.3 or pH 9.5. Cell number and protein content were determined at the end of the cell cycle (24 hours after dilution).

that was transferred at the beginning of the cycle. The results suggest, that the prevention of autospore release is not dependent on protein synthesis as far as cycloheximide inhibition is concerned (other possible effects of cycloheximide were previously discussed).

Based on all the results presented thus, it was assumed that the prevention of autospore release is a result of an early change in cell wall metabolism. Due to this assumption, changes in PAS staining and cellulase activity were monitored throughout a normal cell cycle of Chlorella vulgaris (Fig. 15). It can be seen, that there is a peak in PAS staining between 10 and 12 hours of the cycle which is preceded by a peak in cellulase activity at 4 hours into the cycle. However, when transferred to high pH (Fig. 16-A) at the beginning of the cycle (0, or 4 hours) the pattern of PAS staining changes significantly; there is no decrease at the end of the cycle, on the contrary, there is an increase. A slight decrease, but not to the normal level, is observed in PAS content when the transfer to alkaline pH is performed at the 8th or 12th hour into the cycle.

The pattern of cellulase specific activity (Fig. 16-B) also changes. Transfer of the culture to alkaline pH at 0 time or at the 4th hour of the cycle causes 4 fold increase in cellulase specific activity. There is a decrease after

Figure 15

CHANGES IN CELLULASE SPECIFIC ACTIVITY AND POLYSACCHARIDE CONTENT DURING THE CELL CYCLE OF CHLORELLA VULGARIS

A culture of Chlorella vulgaris was synchronized using the DLD method as described in Materials and Methods. At different times into the cell cycle aliquots were removed from the culture and analyzed for polysaccharide by the PAS staining method and for cellulase specific activity as described in Materials and Methods. The solid line represents cellulase specific activity. The dashed line represents PAS staining Each point is the average of two experimental determinations. For PAS determinations at least 20 cells were analyzed in each determination.

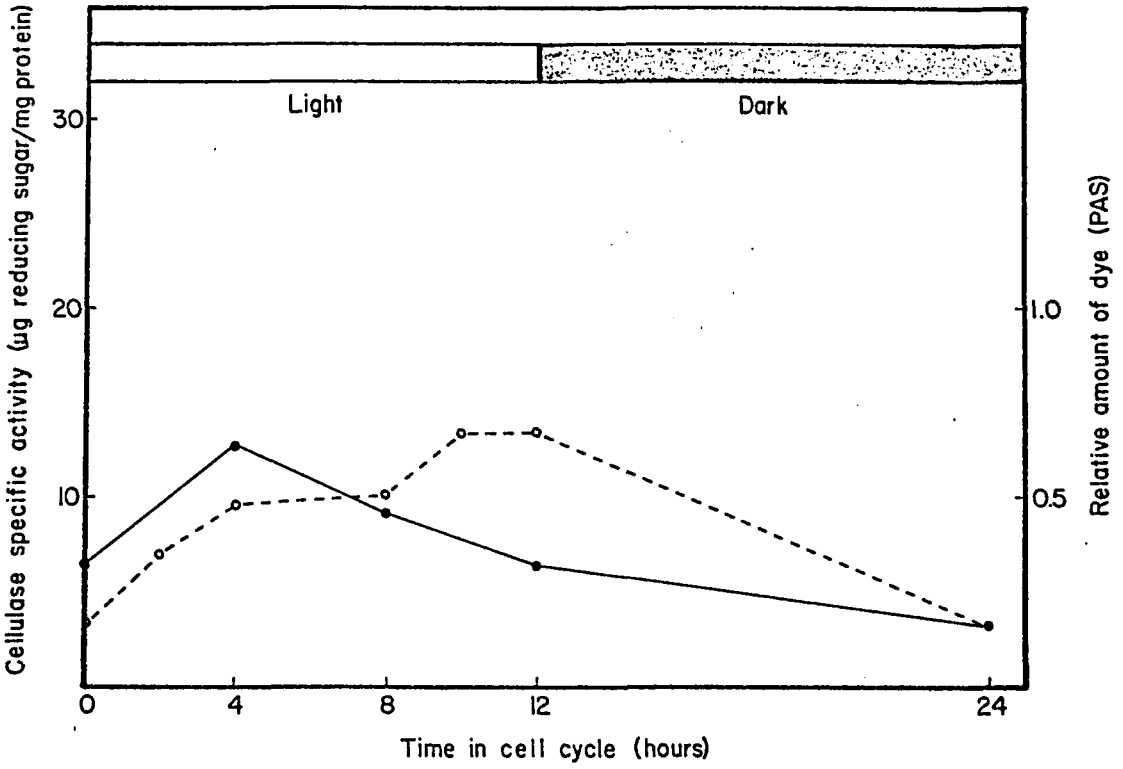
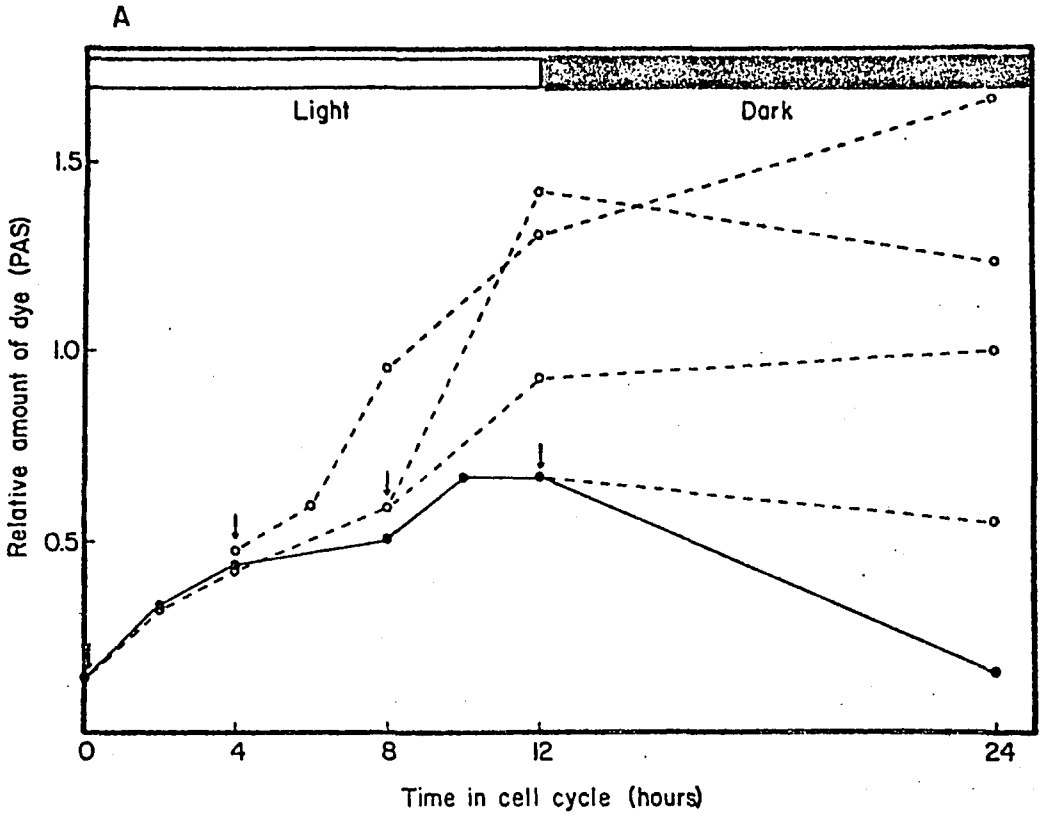

Light
Dark
30
20
10
1.0
0.5
0
4
8
12
24
Cellulase specific activity (ug reducing sugar/mg protein)
Relative amount of dye (PAS)
Time in cell cycle (hours)

Figure 16

THE EFFECT OF THE STAGE IN THE CELL CYCLE ON THE EXPRESSION OF THE ALKALINE pH TREATMENT WITH RESPECT TO POLYSACCHARIDE CONTENT AND CELLULASE SPECIFIC ACTIVITY OF CHLORELLA VULGARIS

The experimental protocol was identical to that described in Figures 13 and 14. The arrows indicate the times into the cell cycle in which a particular culture was transferred to alkaline pH. The solid line represents the control culture which was maintained at pH 6.3 throughout the experiment. At different time intervals during the cell cycle aliquots were removed and analyzed for cellular polysaccharide content using the PAS method and for cellulase activity as described in Materials and Methods.

Each point is the average of two experimental determinations. At least 20 cells were analyzed in each PAS determination.


A
Light
Dark
1.5
1.0
0.5
Relative amount of dye (PAS)
0
4
8
12
24
Time in cell cycle (hours)

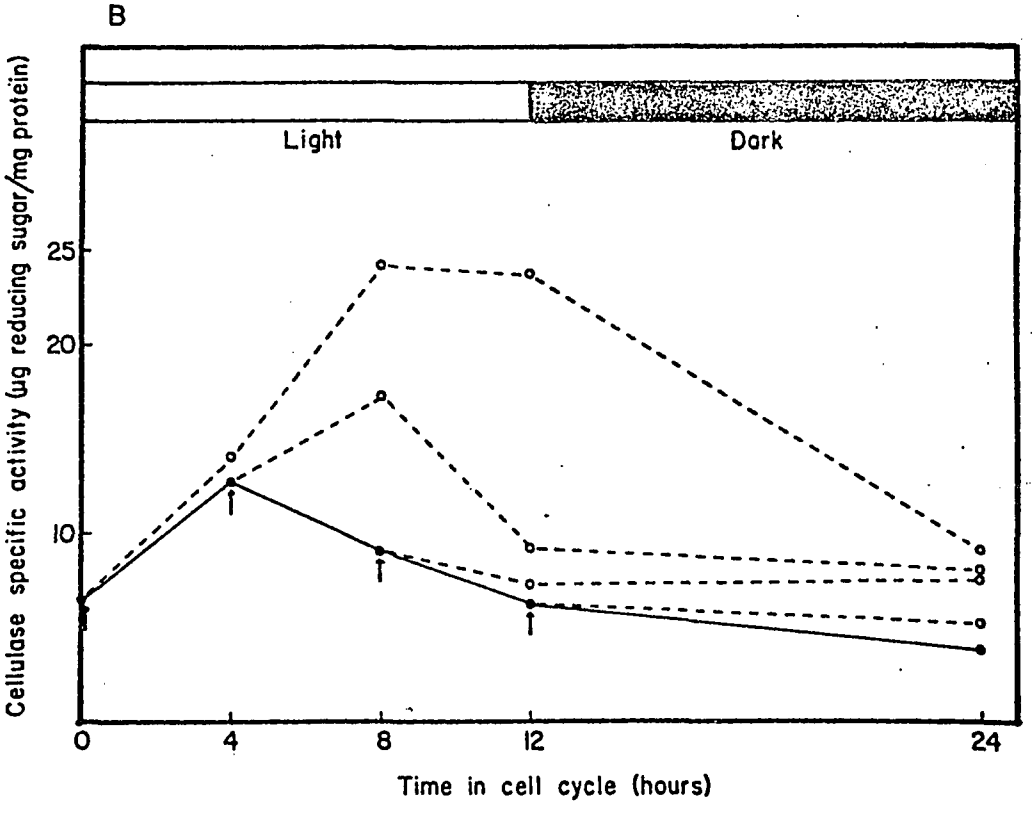
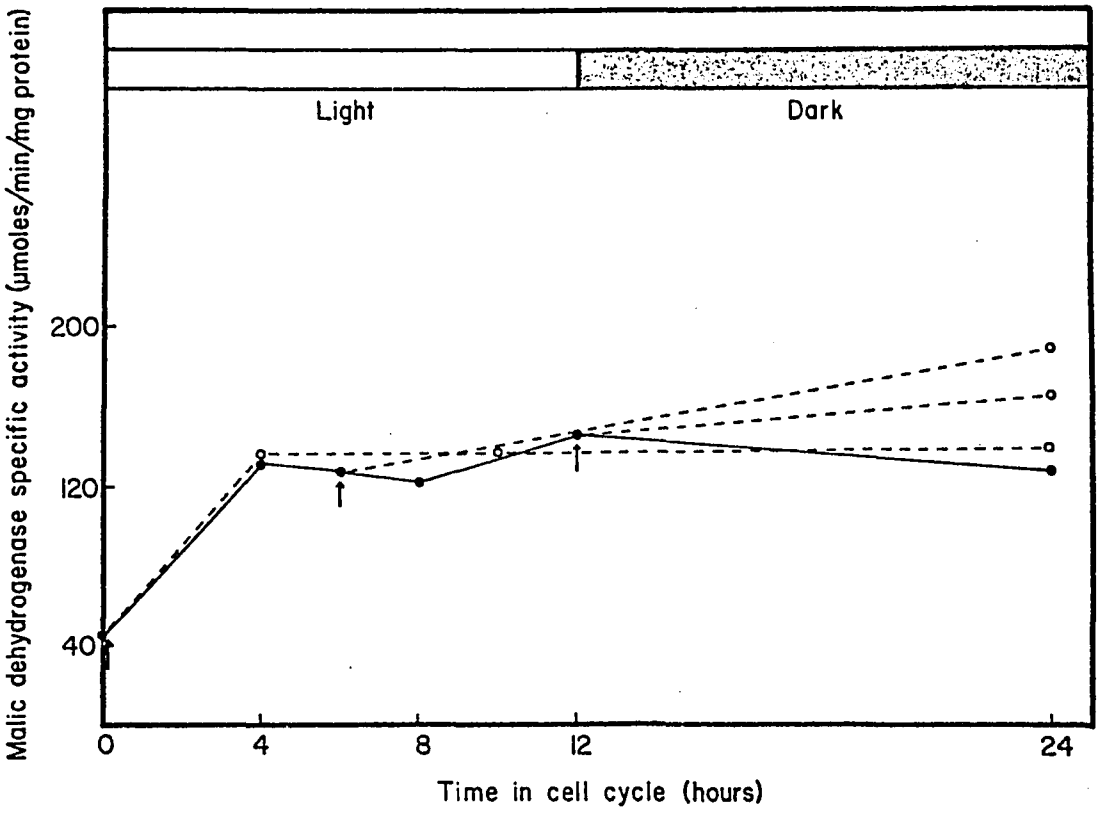

B
Light
Dark
25
20
10
Cellulase specific activity (ug reducing sugar/mg protein)
0
4
8
12
24
Time in cell cycle (hours)

Figure 17

THE EFFECT OF THE STAGE IN THE CELL CYCLE ON THE EXPRESSION OF THE ALKALINE pH TREATMENT WITH RESPECT TO MALIC DEHYDROGENASE SPECIFIC ACTIVITY

The experimental protocol was identical to that of Figures 13-16. The arrows indicate the times into the cell cycle in which a particulate culture was transferred to alkaline pH. The solid line represents the control culture which was maintained at pH 6.3 throughout the experiment. At different time intervals, samples were removed from the culture and were analyzed for malic dehydrogenase specific activity as described in Materials and Methods.

Each point is the average of two experimental determinations.


Light
Dark
Malic dehydrogenase specific activity (μmoles/min/mg protein)
200
120
40
0
4
8
12
24
Time in cell cycle (hours)

24 hours to 2.2 times the level of control. Transfer to alkaline pH at any time after the 4th hour results in a stimulation of cellulase activity but not to the extent of a culture transferred to the alkaline pH at the beginning of the cycle (0 time).

As a control the activity of malic dehydrogenase during a normal cell cycle of Chlorella vulgaris and after transfer to alkaline pH at different stages of the cell cycle was monitored (Fig. 17). The exposure to alkaline pH causes some changes in malic dehydrogenase activity and probably in other enzymes too, however the effect does not seem to be as significant as cellulase specific activity..

c. Recovery experiments

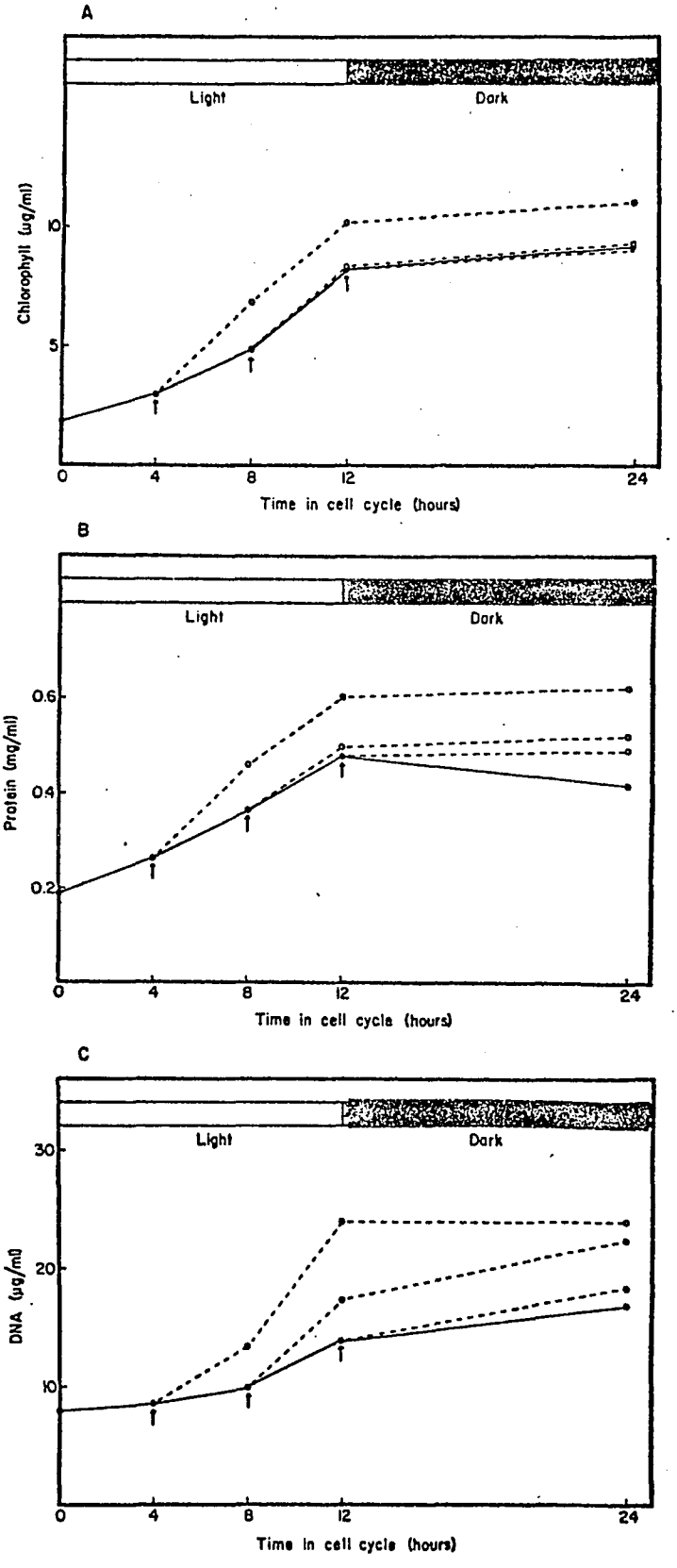
The correlation between autospore release and cell wall metabolism can be shown in still another type of experiment. A synchronized culture was adjusted to pH 9.5 immediately after dilution (0 time). At selected times into the cell cycle aliquots from this culture were adjusted back to low pH ("recovery").

As can be seen in Table 17, transferring the culture at the 4th hour into the cycle back to low pH did not cause any change in cell number as compared to the control culture (grown all the time at the low pH). However, when transferred

Figure 18

THE EFFECT OF TIME INTO THE CELL CYCLE ON THE RECOVERY OF CHLORELLA VULGARIS FROM ALKALINE pH TREATMENT WITH RESPECT TO CHLOROPHYLL, PROTEIN AND DNA CONTENT

Chlorella vulgaris cultures were synchronized using the DLD method as described in Materials and Methods. Immediately after dilution (0 time) 4 gm/l sodium bicarbonate were added and the culture agitated with air alone. At different times into the cell cycle, samples were removed from the culture and readjusted to low pH by the bubbling of CO_2 through the culture. The time of this transfer back to low pH is indicated by arrows. The solid line represents a culture that was maintained at pH 9.5 throughout the cell cycle. At different times during the cell cycle, samples were removed from the various treatments and chlorophyll, protein and DNA content determined as described in Materials and Methods. Each point is the average of 2 experimental determinations.


A
Light
Dark
Chlorophyll (ug/ml)
10
5
0
4
8
12
24
Time in cell cycle (hours)
B
Light
Dark
Protein (mg/ml)
0.6
0.4
0.2
0
4
8
12
24
Time in cell cycle (hours)
C
Light
Dark
DNA (ug/ml)
30
20
10
0
4
8
12
24
Time in cell cycle (hours)

at the 8th hour into the cycle a 50% inhibition in cell number was observed. Transfer of the culture to low pH at the 12th hour resulted in a 2 fold increase in cell number as compared to a culture maintained at alkaline conditions throughout the cycle (in which cell number did not change at all). Microscopic examination revealed no autospore release in the culture that was at high pH for 24 hours. Almost total release of autospores occured in a culture that recovered after the 12th hour. However, the division number in this culture was 2 not 6 as it is in normal culture grown at pH 6.3.

From Fig.18-A it can be seen that transfer back to low pH after 8 hours did not restore the ability of the cells to synthesize chlorophyll. The ability to synthesize protein, however, increased gradually when transferred back to low pH (Fig.18-B) including some improvement after 12 hours at high pH. DNA content also increased gruadually as a result of transfer back to low pH (Fig.18-C).

From Tables 17 and 18 it is obvious that there is a correlation between cellulase specific activity or cellulase activity per cell and the release of autospores. The higher cellulase specific activity is correlated with the lack of release of autospores from the mother cell. When released

TABLE 17

CELL NUMBER OF *CHLORELLA VULGARIS* CULTURED AT ALKALINE pH AND RECOVERED IN LOW pH AT DIFFERENT TIMES OF THE CYCLE

Time at the Cycle when Transferred to Low pH	Time at pH 6.3 (hours)	Cell Nu/ml x 10^7	Autospores/ Mother Cell (Division Number)	DNA/cell ($\mu g \times 10^{-7}$)
0	24	4.62	6.0	5.3
4	20	4.62	6.0	5.2
8	16	2.25	2.9	10.0
12	12	1.5	1.9	12.3
Not Transferred	0	0.77	0	22.0

The experimental protocol was the same as described for Figure 18 except that the cell number was determined at the end of the cycle (24 hours after dilution). Cell number at 0 time was 7.7×10^6 cells/ml.

TABLE 18

THE EFFECT OF TIME INTO THE CELL CYCLE ON THE RECOVERY OF *CHLORELLA VULGARIS* CELLS FROM ALKALINE pH AS EXPRESSED BY CELLULASE ACTIVITY

Time at the Cycle when Transferred to Low pH	Time at pH 6.3 (hours)	Cellulase Specific Activity (μg reducing sugar/mg protein)	Cellulase Activity (μg/cell)	Cellulase Activity (μg/μg DNA x 10^2)
0	24	3.9	0.45	0.08
4	20	4.1	0.54	0.10
8	16	5.5	1.15	0.11
12	12	5.6	1.46	0.11
Not Transferred	0	7.7	3.50	0.15

The experimental protocol is the same as described in Figure 18. At the end of the cycle (24 hours after dilution) cellulase activity and protein content were determined as described in Materials and Methods. Cellulase specific activity at 0 time (immediately following dilution) was 5.4 μg reducing sugars/mg protein. Each value is the average of 2 experimental determinations.

it does not matter how many autospores per cell were released, cellulase specific activity is lowered significantly (from 7.7 to 5.6, and the activity per cell from 3.5 to 1.46). When transferred back to low pH at the 8th hour, there occurred total release of the autospores and an increase in cellulase specific activity and its activity per cell, but the division number is different (2.9 as compared to 6.0 when transferred at 0 time).

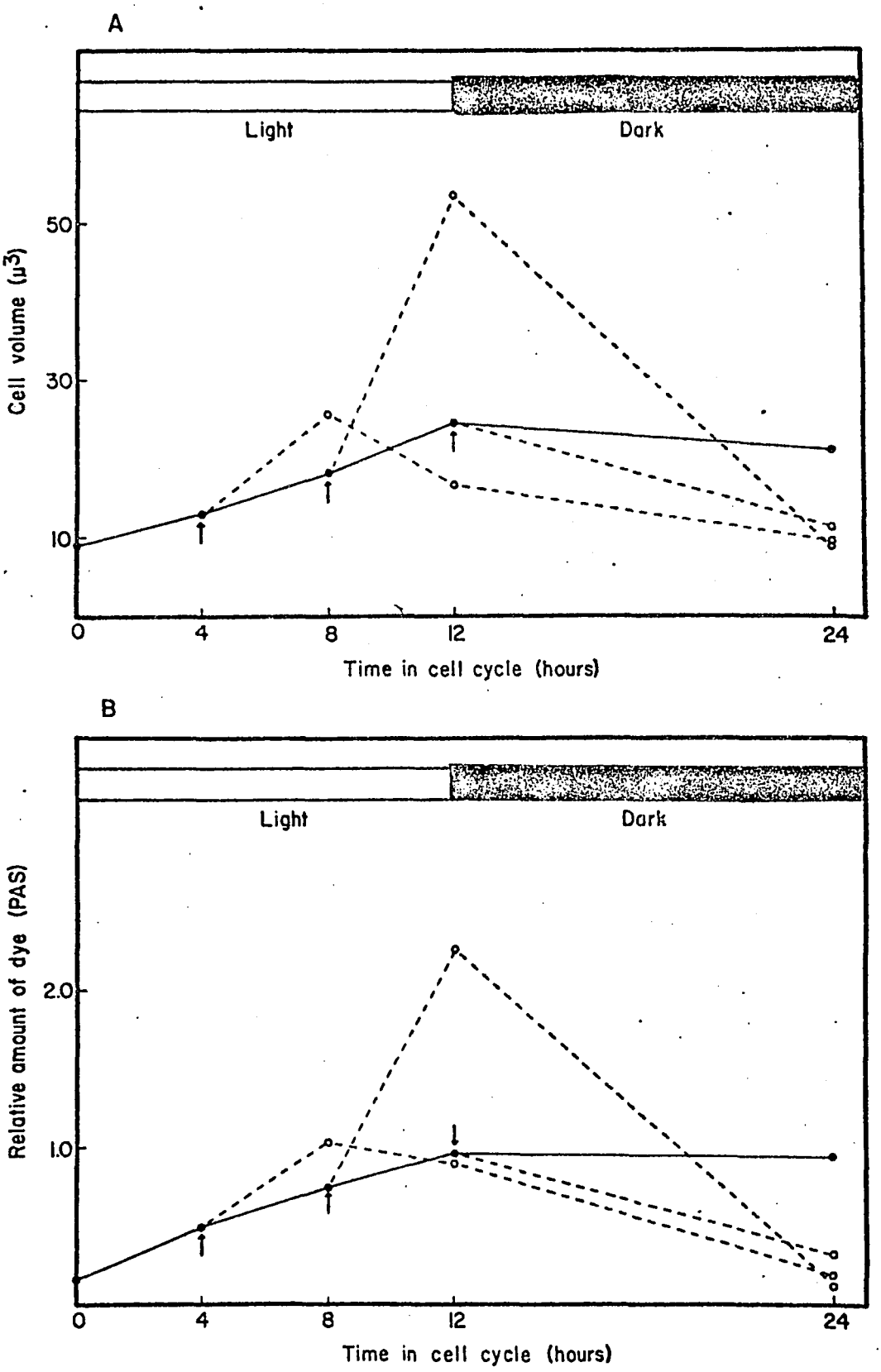
The alkaline pH effect on cell size was previously presented (Fig. 14). When a culture was recovered at low pH changes in cell volume were also observed (Fig.19-A). When transferred back to low pH at the 4th or 8th hour into the cycle a temporary expansion of cell volume was observed. A two fold increase in cell volume was observed after 4 hours, when transferred at the 8th hour into the cycle. However, at the end of the cycle (after 24 hours) the cells reached the size of cells that have never been at alkaline pH. Of interest is the fact that cells transferred at the 12th hour into the cycle back to low pH, end up with an average cell size similar to that of cells that were transferred earlier, although the division number is lower (Table 17). PAS staining of cells that were transferred back to low pH correlates with changes in cell volume (Fig.19-B). A significant increase in PAS staining after transfer back to the low pH occurs

mainly with transfer after 8 hours. All approached the level in PAS staining of a culture that had been constantly at low pH.

Figure 19

THE EFFECT OF TIME INTO THE CELL CYCLE ON THE RECOVERY OF CHLORELLA VULGARIS CELLS FROM ALKALINE pH IN RESPECT TO CELL VOLUME AND POLYSACCHARIDE CONTENT

The experimental protocol is the same as described in Figure 18. The time of transfer of the culture back to CO_2 is indicated by arrows. The solid line represents a culture that was maintained at pH 9.5 throughout the cell cycle. At different times during the cell cycle, samples were taken, their diameter determined and volume calculated.
Each point is the average of two experimental determinations. At least 20 cells were analyzed in each determination.


A
Light
Dark
Cell volume (μ3)
50
30
10
0
4
8
12
24
Time in cell cycle (hours)
B
Light
Dark
Relative amount of dye (PAS)
2.0
1.0
0
4
8
12
24
Time in cell cycle (hours)

V. Localization of cellulase activity

Since many observations suggest general changes in the cell wall, and since no extracellular cellulase activity could be detected, it seemed possible that the changes in the activity of cellulase were localized in the cell wall.

In order to test this idea, the whole homogenate was divided into two main fractions, one including the particulates enriched with cell walls and the other including all soluble materials. Cells at different stages of the cell cycle, grown in low and high pH, were fractionated and the cellulase activity was determined (Table 19).

Results presented in Table 19 suggest that at 0 time most of the activity is associated with the fraction enriched in cell walls. The relative activity associated with this fraction decreases in both low and high pH after 10 and 24 hours into the cell cycle.

Although the different fractions were prepared in the same way for cultures maintained at low or high pH, there is no proof that the fractionation results in the same constituents in each fraction. Thus, it might occur that fractionation of cells exposed to different physiological environments, (e.g. pH 6.3 and 9.5) results in fractions of different composition. Another possibility is, that although the increase in cellulase activity is found in cell wall-enriched particulate

TABLE 19

CELLULASE SPECIFIC ACTIVITY IN DIFFERENT FRACTIONS OF *CHLORELLA VULGARIS* GROWN AT pH 6.3 AND 9.5

Time in the Cycle	pH 6.3				pH 9.5			
	Total	Cell Walls	Super I	Super II	Total	Cell Walls	Super I	Super II
0	10	13.8	3.3	3.3	10.	13.8	3.3	3.3
10	4.2	1.89	12.3	16.4	17.5	13.1	59.	0
24	154	5.3	1.0	0	5.14	2.06	20.	4.2

Cells were synchronized in the DLD method as described in Materials and Methods. Part of the cells were transferred immediately after dilution to the alkaline pH. At different times of the cycle samples were fractionated as described and cellulase activity and protein content of the different fractions was determined. Each value is the average of 2 experimental determinations.

fraction, it is not associated with the cell walls per se. Thus, cellulase activity is soluble and is determined in the supernatant fractions although its source is the particulate fraction.

Malic dehydrogenase activity in the different fractions was measured as a control (Table 20). The results indicate that the main activity of malic dehydrogenase is not associated with the particulate fraction enriched with cell walls.

The effect of increasing the pH of the particulate fraction enriched with cell walls is shown in Table 21. It can be seen, that raising the pH caused a 2.3 fold increase in cellulase activity. It was not proved whether or not the activity is bound to the cell walls.

The possible regulation of cellulase activity in the cell wall by different ions and by various lectins was studied (Tables 22 and 23). It can be seen from Table 22 that calcium has a stimulatory effect on cellulase activity whereas copper and cobalt have a marked inhibitory effect. Table 23 shows that in general lectins have some stimulatory effect on cellulase activity.

TABLE 20

MALIC DEHYDROGENASE SPECIFIC ACTIVITY IN THE DIFFERENT FRACTIONS OF CHLORELLA VULGARIS GROWN IN pH 6.3 AND 9.5

Time in the cycle	pH 6.3				pH 9.5			
	Total Activity	Cell Walls	Super I	Super II	Total Activity	Cell Walls	Super I	Super II
0	156.4	26.1	537.6	169.	156.4	26.1	537.6	169.
10	199.8	25.3	971.0	267.	142.2	49.2	372.3	170.6
24	196.1	42.0	980.8	398.2	148.8	20.5	1109.3	355.5

The experimental protocol is identical to that described for Table 19 except that malic dehydrogenase activity was determined as described in Materials and Methods. Malic dehydrogenase activity is expressed as u moles NADH oxidized/min/mg protein. Each value is the average of 2 experimental determinations.

TABLE 21

THE EFFECT OF ALKALINE pH ON *IN VITRO* CELLULASE ACTIVITY IN THE CELL -WALLS ENRICHED FRACTION OF *CHLORELLA VULGARIS*

Time of Exposure to Alkalinity (Hours)					
0			2.5		
Protein mg/ml	µg sugar/ ml	S.A. (µg sugar/ mg protein)	Protein mg/ml	µg sugar/ ml	S.A. (µg sugar/ mg protein)
0.9	4	4.4	0.8	8.36	10.4

A particulate fraction enriched with cell walls was prepared as described in Materials and Methods from an asynchronized culture of *Chlorella vulgaris* grown in continuous light at pH 6.3. The particulate fraction was suspended in N-8 to which 4 gms/l sodium bicarbonate were added and was shaken for 2.5 hours without a supply of CO_2. Cellulase activity and protein content were measured at 0 time and after 2.5 hours, and the specific activity calculated. Each value is the average of 3 experimental determinations.

TABLE 22

THE EFFECT OF IONS ON THE IN VITRO CELLULASE SPECIFIC ACTIVITY IN CELL WALLS ENRICHED FRACTION FROM CHLORELLA VULGARIS

Type of ion added	Cellulase Specific Activity µg reducing sugars/mg protein	% of Control
Control	4.8	100
$MnCl_2$	4.1	85.4
$MgCl_2$	4.8	100
$CuCl_2$	2.5	52.0
$Co(No_3)_2H_2O$	0.5	10.4
$CaCl_2$	7.4	154.1
$FeCl_3$ $6H_2O$	4.8	100

The particulate fraction enriched with cell walls was prepared from an asynchronized culture of Chlorella vulgaris as described in Materials and Methods. The particulate fraction was suspended in N-8 medium and was used as enzyme preparation. The different ions were added to the reaction mixture to a final concentration of 2mM.

TABLE 23

THE EFFECT OF LECTINS ON *IN VITRO* CELLULASE SPECIFIC ACTIVITY IN CELL WALLS ENRICHED FRACTION OF *CHLORELLA VULGARIS*

Type of Lectin Added	Lectin Concentration (µg/ml)	S.A. (µg reducing sugar/mg protein)	% Change Over Control
Control	0	7.95	0
Castor Bean II	10	11.395	+43.3
"	50	11.13	+40.0
Castor Bean IIA	10	9.80	+23.2
"	50	11.52	+44.9
Garden Pea	10	11.26	+41.6
"	50	10.60	+33.3
Concanavalin A	10	8.74	+9.9
"	50	7.81	-1.8

A particulate fraction enriched with cell walls was prepared from an asynchronized culture of *Chlorella vulgaris* as described in Materials and Methods. The particulate fraction was suspended in N-8 medium and was used as enzyme preparation. The different lectins were added to the reaction mixture from concentrated stock solutions as described in Materials and Methods.

DISCUSSION

This thesis describes pH-induced aggregation in Chlorella vulgaris and postulates some possible explanations for the phenomenon. From the outset, this phenomenon could be examined from several different points of view, but three aspects seemed potentially most rewarding.

a. What is the immediate cause of aggregation? As previously mentioned in the Introduction, the pH effect on algal growth cannot be separated from other effects, primarily those involving carbon nutrition. A question still remains concerning the effect of the pH change on the external environment to which the cell is exposed; this environment might cause the observed aggregation phenomenon.

b. How can this phenomenon be described in morphological terms? Since the initially observed cells in the aggregate appeared different than normal single cells, it was assumed that structural modifications were involved in the aggregation phenomenon. What are these morphological characteristics?

c. What is the cellular mechanism involved in such a change? Alterations in cell structure must be a result of biochemical changes that preceded the structural modifications. Identification of these biochemical changes, then, might supply the clue that could lead to an understanding of the mechanism(s) involved in aggregation.

This thesis does not approach the first question, but instead is directed towards explaining the aggregation response of the cells to the pH change. It is obvious that the change in pH could have some secondary effects which are responsible for the alkaline response. Thus, the increased pH by itself might not be the direct stimulus to aggregation, but rather an indirect one.

As mentioned in the Introduction, increasing the pH alters the carbon nutrition. The distribution of carbon dioxide among carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate ($CO_3^=$) is pH-dependent (Ouellet and Benson, 1952). The amount of bicarbonate increases with a rise in the pH level. In a neutral pH, sufficient amounts of CO_2 are dissolved in the medium, while bicarbonate is the main component in the alkaline medium. The presence of bicarbonate in the medium results in the activation of carbonic anhydrase and has been described by Graham and Whittingham (1968) and Graham and Reed (1971).

A change in carbon nutrition is only one possible effect of alkaline pH. Precipitation of salts, which remove essential elements from the medium and the extent of ionization of the constituents in the medium (including dissolved O_2) might also occur and result in the observed response. It is also reasonable to assume that an additive or synergistic effect might be involved.

The wider optimal pH of the newly isolated Chlorella

vulgaris Beijerinck was shown using a continuous culture technique (Table 1). The necessity of using continuous culture techniques to determine optimal growth conditions (e.g. pH optimum) is not fully understood (Soeder et al., 1964, 1966). A constant growth rate at a certain pH is the decisive factor in determining the optimal conditions for the growth of the population. The ability to survive after a sudden exposure to a certain pH, which resembles tolerance towards a shock rather than growth ability is not the pivotal consideration.

The visible response to the alkaline environment was recorded and analyzed. A schematic illustration of the differences in life cycle of Chlorella vulgaris grown at regular (6.3) and alkaline (9.5) pH is presented in Figure 20. After a 24 hours exposure at pH 9.5, an apparent modification in cell metabolism results in the formation of large cells that contain ripened autospores (Table 2, Figure 4).

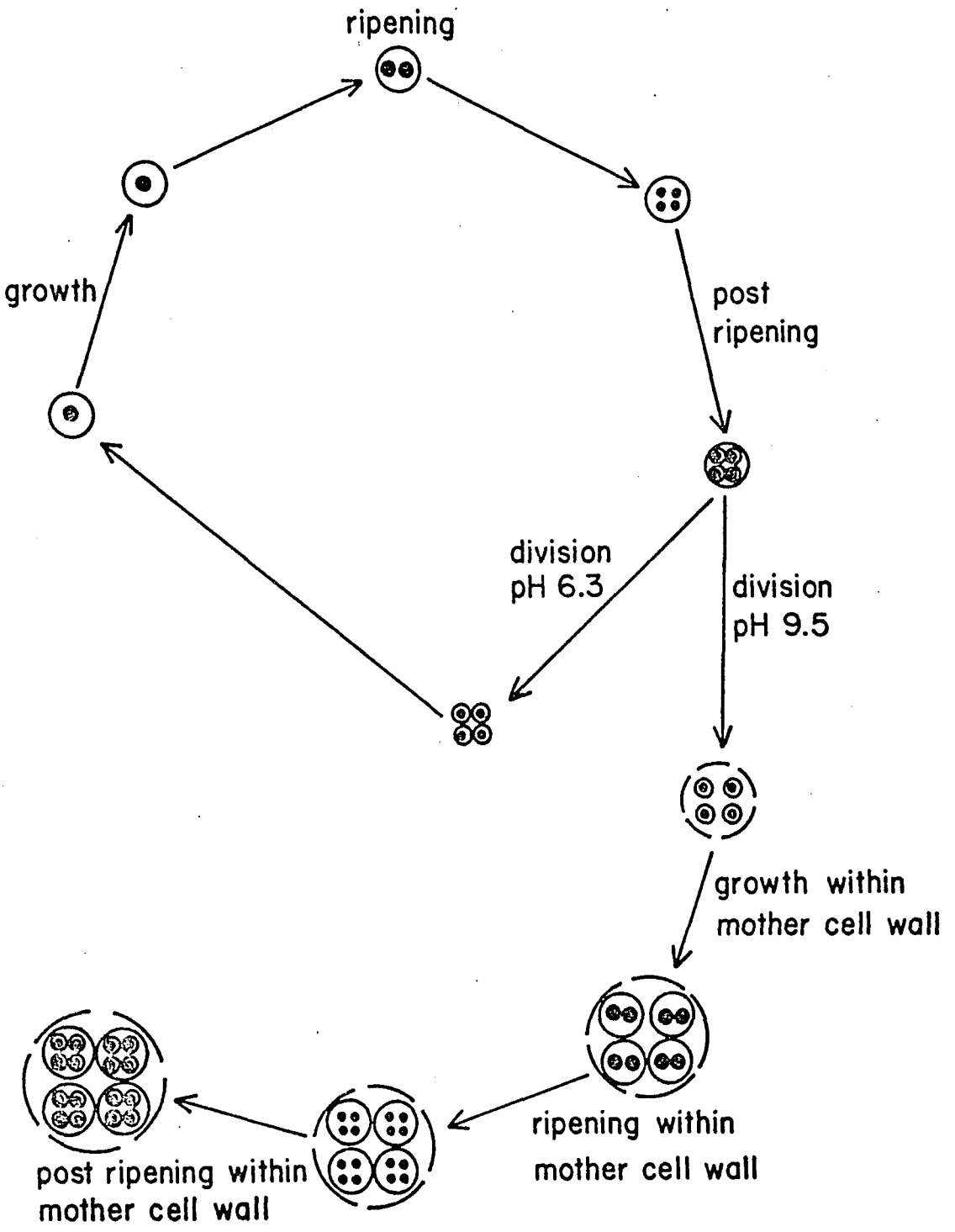
Following the initial stage in which a 7-fold increase in cell volume occurred, autospores growth continues but they remain attached to their mother cell wall as observed in the electron micrographs (Figure 5). Autospores that are grown at pH 6.3 (standard pH conditions) will be released from their mother cell wall when ripened, and will continue to develop as independent cells that eventually develop into mother cells. Figure 12 indicates the accompanying changes in cell volume during the normal cell cycle of Chlorella.

Figure 20

DIAGRAMMATIC REPRESENTATION OF A MODEL FOR GROWTH OF CHLORELLA VULGARIS AT pH 9.5

Schematic illustration of a model suggested for the life cycle of Chlorella vulgaris grown at pH 9.5.

For detailed explanation see text.


ripening
post
ripening
division
pH 6.3
division
pH 9.5
growth within
mother cell wall
ripening within
mother cell wall
post ripening within
mother cell wall
growth

Under alkaline conditions, however, the autospores continue to grow without being released; they disrupt their mother cell wall but remain attached to it (Figure 5). In Figure 14 a decrease in cell volume, when autospores are normally released, is not observed. At pH 9.5 the amount of DNA, protein, chlorophyll and dry weight per cell is higher than that level in the normal cells grown at pH 6.3 (Table 7). The number of autospores that are produced per mother cell (division number) under these conditions depends upon the developmental stage of the cell at the time of exposure to alkaline pH. When a culture is transferred to a basic environment at the beginning of the cycle, only 2 autospores are produced per mother cell. Between 4 to 8 autospores may be produced if the transfer is performed later in the cycle. The autospores within the mother cell will develop into mother cells which divide to form a new generation of autospores. The daughter autospores grow and disrupt their own mother cell wall, and remain attached to the disrupted wall in a manner similar to that observed in the parental generation. After several successive repetitions of this cycle, during which cells grow and divide but remain attached to the fragments of modified mother cell wall, clustered entanglements of interconnected autospores are formed.

At the final stage, still larger aggregates composed of hundreds of cells may be formed; clusters become entangled with or adsorbed to each other forming the big aggregates

seen in Figure 2 and suggested by the pattern of aggregation described in Figure 3. This final process may be a result of a significant change in polysaccharide composition in the cell wall. Indeed, a change in polysaccharide composition is indicated by PAS staining (Figure 9), Ruthenium Red staining (Figure 8) and by pectin determination (Table 8). The pectins that were produced can also be used as flocculants, thus bringing about adsorption of many of these clusters together.

An alternative model describing the aggregation phenomenon has been suggested by Schmidt (1978). According to his model, the pH 9.5 treatment appears to be arresting the cells at the mother cell stage, resulting in the prevention of autospore release. A retention of the original mother cell wall occurs, and pectin is secreted and accumulated on the surface of the mother cell wall. After 4 days under alkaline conditions, aggregation of these mother cells occurs, because of the pectin secretion. According to this alternative, the layers seen in the electron-micrographs are actually secreted pectin and therefore the outer layer observed reflects the newest pectin layer secreted.

This model assumes that the pectin secretion is seen in electron-micrographs as layers. However, the staining technique used in this electron microscopy study does not specifically stain for pectin. In addition, the layer seen in the electron-micrographs represents grandmother and mother cell

wall as were shown by John et al. (1973).

The main difference between the original model and the alternative one is the further development of autospores under alkaline conditions within their mother cell wall. According to the alternate model, autospore development ceases after the first 24 hours. This is not supported by the growth curves (Figure 7) which show an increase in all the growth parameters including DNA, protein and chlorophyll content after the first 48 hours. In addition, calculation of PAS per DNA content would be much higher for aggregated cells if there was no further increase in DNA content. However , Table 7 shows that PAS content on a DNA basis is the same for the control and aggregated cells.

Another idea suggests that there are no alterations of the cell cycle per se, rather there are only changes in cell wall metabolism resulting in cells with modified polysaccharide composition in the cell walls. These accumulated-modified polysaccharides would cause the clumping of the cells.

The results of experiments with synchronized cultures do not support this concept since the process of autospore release is primarily prevented. This process could not have been prevented without significant alterations of successive events of the cell cycle in addition to the biochemical changes.

What is the mechanism involved in the aggregation

phenomenon? To answer this question, the following postulations were considered. The ability of the rigid cell wall in Chlorella to expand is limited. Thus, the increase in cell volume could not have occurred without a commensurate change in cell wall elasticity. In electron micrographs of cells exposed to alkalinity for 24 hours (Figure 4) no change in cell morphology could be detected. However, a significant alteration was observed after 4 days, revealing the existence of a multilayered cell wall. This structure was not apparent in either Chlorella vulgaris Beijerinck grown at pH 6.3 (Figure 6) or in the cell wall of other Chlorella species (Pickett-Heaps, 1975).

The aforementioned modifications are indicated by the differential staining techniques with Ruthenium Red and PAS staining. Both procedures strongly imply changes in the polysaccharide composition and content of the cell wall. In addition, a two-fold increase in the total pectin content was observed for aggregated cells.

Ripening of fruits involves changes in pectic substances, increase in pectic acid, the loss of protopectin and almost no change in the calcium pectate fraction (Salisbury and Ross, 1969). A similar pattern is observed during algal aggregation. The increase in the total pectin content can be ostensibly attributed to an increase in the pectic acid fraction. A slight decrease was found in the calcium pectate fraction and a total disappearance of the protopectin. This

process may indicate a possible softening of the cell wall, in a manner analogous to the process that occurs during fruit ripening.

In a normal culture grown at pH 6.3, the release of autospores from the mother cell wall is a result of the rupture of the rigid cell wall by a mechanical pressure evolving from the ripening autospores. Thus, softening and increasing the elasticity of the cell wall would prevent autospore release since the pressure caused by the ripening autospores will result in the expansion of the cell wall, in lieu of its rupture. The prevention of autospore release after 24 hours under alkaline conditions is primarily a consequence of a change in the plasticity of the cell wall. This change in plasticity allows for the expansion of the mother cell with its developing autospores within. This can be inferred from electron micrographs of cells exposed for 24 hours to an alkaline pH. Despite the increase in the cell wall elasticity, the increasing pressure from inside the wall will ultimately result in the rupture of the cell wall in multiple regions.

The results from PAS staining in which the increase in polysaccharide content was observed after 24 hours and from experiments using synchronized cultures (Figures 9 and 16) confirmed the hypothesis that the change in cell wall metabolism occurred during the first 24 hours at the alkaline pH.

The underlying events that result in the prevention of

autospore release can be viewed from a biochemical perspective. All the above changes in cell wall composition have indicated some significant fluctuations in cell wall metabolism. These biochemical changes, as indicated by the experiments with cellulase activity and PAS staining clearly suggest that a rapid response to the environment occurs. This profile of change in cellulase activity and PAS staining under normal conditions for Chlorella vulgaris was recorded (Figure 15). The increase in hemicellulose has been reported by Takeda and Hirokawa (1978) to increase in proportion to the expansion of the cell surface at the growing phase, while the "rigid wall" remained unchanged during this phase. Rigidity of this "rigid wall" is increased only at the time of autospore formation. These changes in cell wall composition are preliminary preparation for the cell growth and expansion, which coincides with autospore formation, ripening and release.

The increase in cellulase specific activity during the growth phase and its decrease thereafter is further support for the cyclical modification in cell wall rigidity. As an analogy, cellulase activity has been shown to be correlated with fruit softening and ripening (Pesis et al., 1978). The early increase in cellulase specific activity might therefore be correlated with the softening process of the cell wall which enables it to expand when autospores are formed. The subsequent decrease in cellulase activity indicates an

increase in the rigidity of the cell wall, thus allowing for the subsequent autospore release by inherent mechanical pressure. The increase in cellulase activity is followed by a biphasic increase in PAS staining (Figure 15), suggesting that two distinct polysaccharide species are formed during the cell cycle. The relative content of polysaccharides also decreases towards the end of the cycle. When cells are transferred to an alkaline pH, however, a decrease in cellulase activity and polysaccharide content at the end of the cycle is prevented (Figures 16-A and B). It is reasonable to assume that the cell wall does not return to the rigid state. The increase in PAS staining is preceded by an increase in cellulase activity, indicating a softening of the cell wall. This process results in a more elastic cell and permits it to continue to expand during autospore formation.

The increase in the elasticity of the cell wall might be a result of a change in the composition of polysaccharides in the wall, i.e., relative change in pectin content and especially the disappearance of the protopectin fraction. This idea is supported by the histochemical experiments in which cells were subjected to the treatment of various enzymes and then stained with PAS.

The cellulase activity localized in the cell wall might account for the initiation of the pH induced phenomenon. By exposure to an alkaline pH the cellulase in the cell wall is activated. The results have demonstrated that relative to

the soluble fraction, a higher cellulase specific activity is found in the particulate fraction enriched with cell walls (Table 19). Increasing the pH of this fraction resulted in a 2.3-fold increase in cellulase specific activity (Table 21). It should be noted, however, that the particulate fraction is only enriched in cell walls, and does not represent a pure cell wall fraction. The possibility of a release of cellulase from some other particulate material cannot be excluded.

The results support the concept of cellulase localization in the cell wall of bacteria as described by Suzuki et al. (1969). Accordingly, the alkaline pH activates cellulase located in the cell wall which then results in the softening of the cell wall by changing its polysaccharide composition. This process enables the expansion of the wall during autospore development.

The fact that the increase in cellulase activity was neither inhibited by cycloheximide nor by puromycin, but was inhibited by darkness and DCMU supports the idea that the alkaline-induced increase in cellulase activity is not a result of de novo protein synthesis. However, the actual effect of these inhibitors on protein synthesis is discussed later. The control of cellulase activity by inhibitors or activators has been previously documented (Abeles, 1969). The pattern of cellulase activity during the cell cycle of Chlorella might also imply that the decrease in cellulase

activity at the second part of the cycle is due to its inhibition. For example, among the sugars, lactose, maltose and glucose are inhibitors of cellulase activity (Ghose and Kostick, 1969). Cellobiose has been found to function as both an inducer and inhibitor of cellulase activity (Mandels and Reese, 1969). Phenols are known to have an inhibitory role (Mandels and Reese, 1965). The induction of cellulase by various metal ions was studied and cobalt was found to stimulate cellulase activity (Mandels and Reese, 1957). Other investigators (Gascoigne and Gascoigne, 1960) have concluded that mercury, silver, copper, chromium and zinc salts are generally inhibitory whereas manganese, cobalt, magnesium and calcium in the presence of phosphate are stimulatory. The inhibitory and stimulatory effects of the different ions on cellulase activity in the Chlorella vulgaris system are shown in Table 22 and suggest the possibility of ionic regulation of cellulase activity. The sequence of interaction could be triggered by the pH change which then affects the relative availability of ions, influencing cellulase activity. In the system studied, evidence is presented that shows that copper and cobalt are inhibitory and calcium is stimulatory.

Cellulase activity might also be regulated by the available carbon source. Suzuki et al. (1969) have shown this to be the case in bacteria. The pH-induced change in carbon nutrition could be responsible for enhanced cellulase activity in Chlorella. Stress conditions in general, can lead to

increased cellulase production (Bemiller et al., 1969). Recently, Geballe and Galston (1978) suggested that cellulase is a glycoprotein which is controlled by the specific binding of the molecule to lectins (through a sugar moeity). Indeed, the results presented in Table 23 indicate that lectins have some effect on cellulase activity.

A specific binding of lectins to cellulase and its subsequent release might therefore be a mechanism of regulating the activity of the molecule. Alternatively, lectins could bind specifically to the repressive sugar moiety on the cellulase molecule, restoring the enzymatic function. In the latter instance, the effect of different sugars on cellulase activity is not direct but rather indirectly affects lectin availability. While the above-mentioned regulatory factors could provide the mechanisms for cellulase regulation, further investigation is required before a final conclusion can be achieved.

The involvement of cellulase in fruit softening has already been mentioned (Pesis et al., 1978). This softening process correlates with pectin production. It is reasonable to postulate that pectin synthesis requires the end products of cellulase degradation (mainly glucose or some other monosaccharides). The possible biosynthetic pathways for pectin synthesis are discussed in Appendix A III.

The modification in the rigidity of the cell wall due to the sugar composition in the wall introduces a further

possibility. Perhaps changes in the natural properties of the cell wall with alkaline treatment enable an easier digestion to proceed. Protoplast formation in Chlorella was under intensive research for a long time with only limited success (Braun and Aach, 1975). The importance of protoplast formation is primarily for the preparation of cell wall free fractions, that would be suitable for its further investigation.

The practical use of alkaline-induced aggregation in Chlorella: There are some advantages of growing algae on a large scale outdoors at an alkaline pH. The open air cultures are constantly subjected to competition by various microorganisms, bacteria, protozoa and other strains of algae that usually prefer standard pH conditions (6-7) for optimal growth. The ability to maintain a culture at an alkaline pH level, however, will provide the organism with a survival advantage over the potential competitors, and thus helps in maintaining a relatively clean culture. Thus, even though increasing the pH of the medium impedes the growth rate (Figure 7), the ability to grow and survive under outdoor conditions in relatively extreme environments makes alkaline growth advantageous. The importance of development of an alkalophilic algae strain is therefore self-evident.

In large scale biomass production of photoautotrophic unicellular algae, Chlorella is known to be one of the best candidates. The harvesting step, however, is the primary

obstacle. The small size of Chlorella cells does not allow for a harvesting process that is economically feasible. Controlled aggregate formation, however, might permit gross filtration. The enlarged clusters that are formed have the tendency to sediment at the bottom of the pond and could be easily collected without filtration. As demonstrated by the pattern of segregation (Figure 3), approximately 40% of the algal biomass will sediment after 3 days of exposure to an alkaline environment. In addition, since the percent of protein in the aggregate cells (Table 6) is similar to that level found in single cells, then the preharvesting system is suitable for protein consideration. The cells will be grown in medium of pH 6.3 and exhibit a higher growth rate and will be then transferred to an alkaline medium (pH 9.5), which will induce cellular aggregation. After the aggregates are formed and sediment to the bottom of the pond they will be collected by a mechanical means.

The work with synchronized cultures has emphasized the complexity and uniqueness of Chlorella cell cycle. The contribution of this thesis to the study of the cell cycle emphasizes the concept of the so-called "point of no return" in Chlorella. The preparation for cell division occurs during a certain period into the cell cycle after which time changes in the environmental conditions will not affect the division process. By transferring the cultures to an alkaline pH at set points within the cycle, the existence of a "point of no

return" can be shown to occur 8-12 hours into the cycle. Similar results were shown by Pirson et al. (1959) following cold treatment. The existence of a "point of no return" is indicated by changes in the following parameters: chlorophyll, cell number, protein and DNA. The fact that all of these parameters reveal the same pattern of metabolic activity when transferred to alkaline pH is of special interest.

Perhaps some cellular factor is directly affected by the alkaline pH or by the cold treatment (Pirson et al., 1959). This factor, discussed by Mitchison (1970) for Tetrahymena, which is sensitive to the change in the external environment can trigger certain cellular events. The existence of a "point of no return" was demonstrated in Chlorella also by the use of Chloramphenicol and other metabolic inhibitors (Moberg et al., 1968). The cell therefore may have a sensing capacity that informs the various biochemical pathways and directs their operation in a manner consistent with the prevailing external conditions.

According to the postulated model the increase in cellulase activity is not attributed to de novo protein synthesis, but rather to the activation of the enzyme molecule. Since only protein content was measured and not protein synthesis no final conclusion could be drawn regarding the mechanism that accounted for the apparent increase in cellulase activity. The possibility that cycloheximide and other protein synthesis inhibitors used in the study might have side effects can not be excluded. It is quite conceivable that these effects might not be limited to protein synthesis, but extend to protein content. Only experiments that would measure protein synthesis directly could prove this problem. In addition it might be that the bulk protein synthesis is more sensitive to cycloheximide than is the synthesis of cellulase as was observed by Vassef et al. (1973), who found that it requires different concentrations of cycloheximide to completely inhibit the synthesis of different enzymes.

This study has shown that following alkaline treatment the cells increase in size, aggregates are then formed and an increase in pectin and polysaccharide content is observed. Positive proof has not been offered to demonstrate that the enlarged cells are interconnected by pectin molecules. Specific staining for pectin might help resolve this dilemma.

A general increase in enzyme activity resulting from exposure to alkalinity was ruled out by the experiments using malate-dehydrogenase as a control. Since malate-dehydrog-

enase has isozymes and is partially located in the mitochondria other enzymes should be also examined before a final conclusion can be drawn.

The general changes in polysaccharide content are based primarily on PAS staining. There is the possibility that in this technique polysaccharides other than cell wall polysaccharides are stained. The treatment of cells with protease, amylase, pectinase and cellulase can be criticized on a permeability basis. However, the data do tend to support the PAS staining being primarily a wall phenomenon.

Measurements of cellulase activity are based upon the release of reducing sugars. As mentioned in the Materials and Methods this measures only the final cleavage of cellulose and presents actually the combined activity of many enzymes. It is not obvious from the data whether the enzyme activated by the alkaline treatment is the one which primarily cleaves cellulose molecules into their fragments, or whether the enzyme is one of the last in the sequence, directly producing reducing sugars. Identification of the substrate and product of the particular enzyme will help in understanding this issue.

The mechanism suggested above for alkalinity induced aggregation in Chlorella could be examined by a series of additional experiments.

(1) The treatment of cultures of aggregated and control cell with different enzymes (e.g. pectinase, cellulase, prot-

ease, glucosidase) will result in the selective digestion of the cell wall and the release of their respective components. These results would be additional support of changes in cell wall composition. By chromatographic analysis of the released materials, modifications in the cell wall could be detected and identified.

(2) As previously discussed the prevention of autospore release could be achieved if chemical cell wall modifications would result in changes in its plasticity. Thus, the measurement of physical elasticity of the cell walls would supply direct proof of changes in cell wall rigidity.

(3) According to the postulated mechanism the activation of cellulase is one of the first events induced by the alkaline treatment that leads to aggregation. Addition of exogenous sources of cellulase to the cells might induce aggregation if the cellulase molecule could penetrate the cells. Inhibition of cellulase activity by an inhibitory ion (cobalt or copper) could prevent aggregation, The addition of lectins might also induce aggregation by releasing cellulase from inhibition as previously suggested.

(4) The implicated role of cellulase in the inductive phase of aggregation was previously discussed. Pectins were found to increase in aggregated cells. By labelling the cellulose in the cell wall and monitoring the transfer of label among metabolites after the induction of aggregation, one would be able to determine whether the pectin fraction was labelled,

and whether the products of cellulase activity (monosaccharides) were used in the biosynthetic pathway of pectin production. These results would also point specifically which of the pectin's fraction was labelled.

(5) Further purification of the cell wall enriched fraction (using modern techniques) would permit the localization of cellulase within the cell wall structure. From cell wall fraction the specific enzyme (cellulase) can be isolated and identified. Treatment of the cell wall fraction with exogenous cellulase would result in aggregation of the cell wall particles. Treatment with protein synthesis inhibitors will contribute further evidence for the mechanism of cellulase activation.

To support the conclusions presented concerning synchronized cultures the following experiments are suggested:

(1) An alternative method of synchrony by induction could be employed. For example, in lieu of the DLD method used in the above protocols, synchrony could be effected by selection of the cells on the basis of their respective densities, which change during the various stages of the cell cycle.

(2) Elimination of the final dark phase in the DLD regime, resulting in continuous illumination of the culture for an interval of 24 hours.

(3) Examining the effect of the alkaline treatment on other Chlorella strains to determine the universality and species-specificity of the phenomenon under consideration.

(4) Selection of cell wall mutants to explore the structural and/or functional relationship of the wall to the aggregation process.

Appendix A

I. The cell cycle of Chlorella

The effect of light and temperature on the cell cycle

Morimura (1959), who studies the effects of temperature and light intensity upon the life cycle of C. ellipsoidea, showed that at a constant temperature of 16°C the rate of growth increased with increasing light intensity. However, cell division occurred simultaneously at all light intensities (except at the very low light intensity of 0.4 k. lux), irrespective of the difference in the growth rate. The time elapsed from the onset of cell division (L_1 stage) to its completion (Dn stage) was also found to be independent of light intensity. The division number (number of daughter cells/mother cell) was largely dependent on light intensity. It varied from 2 to 4 with increasing light intensities of 1 to 10-25 kilo lux (saturating intensities) for C. ellipsoidea and could be 8 or 16 for high temperature Chlorella strains (Spencer et al., 1961). At a constant saturating light intensity the rate of growth, post-ripening and division were temperature-dependent, both increasing at higher temperature. The division number, however, remained the same at all temperatures.

By comparing the light intensity saturation curve for 'growth', 'ripening' and photosynthesis, Morimura (1959) concluded that these three processes probably involve the same photic system.

The effect of environmental conditions on the "division number"

The number of daughter cells is probably determined by the quantity of nuclear substances which have been formed during the ripening process preceding cell division. Cells grown at a fixed temperature began to divide simultaneously giving rise to different numbers of daughter cells depending on light intensity. Similar "isochromism" of cellular division with different division numbers were also observed when algal cells were subjected, at fixed temperatures, to various environmental conditions, such as the deprivation of phosphate, nitrate or potassium from the medium (Hase et al., 1957).

It seems that the process of cellular division itself is largely independent of the quantity of nuclear substances as well as of the total mass formed previously. It was assumed by Tamiya, that the occurrence of cellular division is initiated by some factor or factors which are formed during the process of ripening. Experimental results show that the formation of such an inducer substance is a light-independent but temperature-dependent process.

Cell division in Chlorella

Chlorella and some other algae present a problem in terms of the comparison of their cell cycles to those of most other cells. The typical cell cycle has a two-fold increase in DNA followed by division into two daughter cells. In Chlorella, there is a four- or eight-fold increase in

DNA followed by 2 or 3 rapid divisions into 4 or 8 daughter cells. There are two ways of regarding the Chlorella interphase. One is to consider it as being equivalent to a single normal cell cycle except that every synthetic event involves a four- or eight-fold increase rather than a two-fold increase. Alternatively, it may be regarded as being equivalent to three successive normal cell cycles except that cytokinesis is dissociated and occurs at the end of the third cycle. This distinction is important for discontinuous events like DNA synthesis or synthesis of a "step enzyme," which occurs once in the normal cell cycle. If we consider the cell cycle of Chlorella in the light of the first alternative, we would expect to observe a single period of synthesis, but one in which more material is being synthesized. If, however, the second point of view is the correct one, we would expect to observe three separate periods of synthesis, each with a two-fold increase. Which of these two possibilities is correct is at the present time unknown. The significance of this issue lies in the fact that it will lead to a better understanding of the processes of autospore release and "cell division" in Chlorella. It can also be seen from Figure 1 that "cell division" is an ambiguous term in the Chlorella system. It is used by Tamiya to designate the period of release of daughter cells when the cell number increases. However, there is no doubt that the mother cell contains four separate daughter cells,

each surrounded by a cell membrane for some time before they are released. "Cell division" therefore is the release of pre-existing cells. Exactly when "true" cell division occurs is more obscure. In an electron micrograph shown by Tamiya (1963a, 1963b), there is an indication that it probably starts in the L_2 cells.

The effect of essential nutrients on cell division

It has been demonstrated (Kanazawa and Kanazawa, 1969) that cell division of ripe cells (L_2 and L_3 cells) in the dark was inhibited by cupric ions in the medium (especially at pH 6.3). The mode of sulfur (S) metabolism in Chlorella was shown to undergo change, depending upon whether S-starved cells were supplied with sulfate under photosynthesizing or nonphotosynthesizing conditions (Hase et al., 1960; 1961).

Under photosynthesizing conditions large amount of sulfur were incorporated into protein, and with little going into sulfur-containing deoxypentose polynucleotides (SDN)(Tamiya, 1964), which have been shown to play an important role in the process of cellular division. Both protein and SDN require nitrogen and sulfur sources for their metabolism. Under nonphotosynthesizing conditions the nitrogen required for SDN synthesis is taken from some endogenous N-sourse, while under photosynthesizing conditions this source is preferentially used for protein synthesis, making it unavailable for formation of SDN. Under photosynthesizing conditions, and in the absence of exogenous nitrogen, most of the endogenous

nitrogen, along with any exogenously available sulfur is preferentially used for protein synthesis. This results in an increase in total mass but not in the occurrence of cellular division, because of the lack of SDN. With the provision of exogenous nitrogen (nitrate) the exogenous nitrogen and sulfur sources are used not only for protein synthesis but also in SDN synthesis, which thereby enables the cells to undergo cellular division. Under non-photosynthesizing conditions, and in the presence of an exogenous sulfur source, only a slight amount of protein synthesis occurs while a large percentage of the endogenous nitrogen source can be used for the formation of SDN, thereby enabling the cells to divide. Observations made by Otsuka (1961) indicate that a similar competitive relationship also exists between the synthesis of protein and CoA, which also appears to be an important S-containing substance functioning in the process of algal cell division.

It has been reported that the cell division of green cells is suppressed by the effect of illumination, a phenomenon known as "photoinhibition" of cell division (Sorokin and Krauss, 1959). In the case of Chlorella, this interpretation seems misleading; it is interpreted by Tamiya (1966) as a competition for certain cellular materials between growth and cellular division, the former being a light-requiring process and the latter a light-independent process.

Effects of various metabolic inhibitors on the cell cycle

By the utilization of inhibitors in research on the cell cycle of Chlorella, the sequence of events taking place during the cell cycle can be studied. Treatment with chloramphenicol, known to be an inhibitor of protein synthesis of 70S' ribosomes, completely suppressed the growth of Chlorella, while cell division was unaffected. This indicates that although it did inhibit the protein synthesis of the cytoplasmic organelles, chloramphenicol did not affect cell division (Tamiya et al., 1962). The extremely small daughter cells produced in this case were unable to grow when transferred, after washing, to normal culture medium.

A transition point in the preparatory stages prior to the actual onset of cell division was found by Moberg et al. (1968). They studied the course of growth and cell division in synchronized cultures of Chlorella pyrenoidosa after the addition of chloramphenicol at different times during the cell cycle. However, the interpretation of the effect of chloramphenicol as related to protein synthesis in Chlorella is uncertain.

When puromycin, an inhibitor of protein synthesis in ribosomes (Nathans, 1964), was added at various times during the light period, it inhibited cell division almost completely, while growth was inhibited only 25%.

It was found that when dinitrophenol (DNP) was added at any time in the synchronization cycle it inhibited autospore

formation completely. It was concluded (Moberg et al., 1968) that dinitrophenol inhibits all stages of preparation for cell division, as well as the division process itself. Even with darkness as an "inhibitor," a "point of no return" was also demonstrated in work done by Wanka (1965). Thus, light is necessary up to a certain stage (about 8 hours) if the cells are to divide. After that point has been reached, light is no longer necessary. This indicates that some photochemical process is linked to preparation for division in Chlorella.

II. The effect of environmental conditions on the life cycle of Chlorella

Suboptimal temperatures

Pirson et al. (1959) reported that synchronized cells of Chlorella pyrenoidosa displayed a marked sensitivity toward cold shock (4°C for 2 hours) during a certain stage of the cell cycle. When the cells were exposed to the cold at the beginning or at the end of the light period, the number of daughter cells was somewhat lower than that in the untreated control. When exposed to cold 7 or 8 hours after the beginning of illumination, subsequent cell division was halted completely, and a considerable loss of chlorophyll ensued. Presumably this sensitive stage corresponds to the L_1 stage according to Tamiya and Morimura (1964). They interpreted the results as being due to a bleaching effect of light, occurring at lower temperatures, which is known to

be more harmful to L cells than D cells (Tamiya et al., 1953). In fact, Pirson et al. (1959) observed that when the cells at the sensitive stage were exposed to low-temperature treatment in the dark, they could divide normally, suffering only some decrease in the number of autospores per cell. They remarked that the cells at the sensitive stage showed maximum photosynthetic activity while at the same time their nuclei became unstainable with Feulgen reagent.

Bleaching of Chlorella fusca can also be induced by heating to 40°C for 15 minutes (Lorenzen, 1963), but the most heat-sensitive stage differs from the stage which is most susceptible to cold shock.

In general, the processes involving DNA synthesis and subsequent cell division are more sensitive to sub-optimal temperatures than is the growth phase of the cell cycle.

Osmotic pressure

In Chlorella, sensitivity of photosynthesis to an increase in the osmotic pressure of the medium varies greatly with the developmental stage of the alga. The cells are most sensitive to osmotic shock prior to autospore release. The effect of osmotic stress upon respiration also depends upon the cell stage. The greatest stimulation of respiratory activity is obtained with autospores, whereas respiration of mature autospore mother cells is inhibited by transfer to a more concentrated medium (Wanka, 1965).

Effect of light

Light plays a role in the different stages of the cell cycle of Chlorella. Other metabolic processes are also influenced by light. In the presence of suitable organic substrates, the growth and metabolism of photosynthetic algae are often enhanced by light of appropriate intensity (Senger, 1962). Such light stimulation, which is apparently lacking in Euglena (Cook, 1965), is usually attributable to light-driven transport and photophosphorylation via photosystem I and has been studied in the presence (Lysek and Simonis, 1968) and absence (Tanner, 1969) of CO_2.

The linkage of photoheterotrophy to photosystem I has been demonstrated in certain members of Volvocales, and the quantum requirement of Chlorella for anaerobic glucose uptake was determined by Tanner et al. (1968). Very dim light stimulates heterotrophic growth in Chromulina sp. (Pintner and Provasoli, 1968) and certain Chlorella strains which cannot grow heterotrophically are able to utilize organic substrate in light well below the compensation point of photosynthesis (Karlander and Krauss, 1966). The action spectrum of this light effect is characterized by a major peak at 425 nm and a smaller one around 575 nm. A porphyrin precursor of cytochromes may be the photoreceptor molecule. The action spectrum obtained by Karlander and Krauss (1966) differs clearly from the action spectrum for the induction of cell division and nucleic acid synthesis in Chlorella fusca grown mixotro-

phically on glucose (Senger and Bishop, 1966; Senger and Schoser, 1966). Blue light directs the metabolism of exogenous glucose mainly towards the synthesis of proteins, while carbohydrates are preferentially synthesized from glucose in red light (Laudenbach and Pirson, 1969). A number of freshwater algae have been investigated, after long-term precultivation at different light intensities and subsequent changes in light intensity (Jorgensen, 1969), with respect to the kinetics of their action spectrum curves. The most usual adaptive reaction is the "Chlorella type" which is characterized by an inverse relationship between the light intensity to which the algae are exposed and their chlorophyll content. That is, the light adaptation is mainly accomplished by changes in pigment concentration.

Impairment of photosynthesis and photoautotrophic growth by supra-optimal intensities of visible light is well known from laboratory (Sorokin and Krauss, 1962) and field studies (Goldman et al., 1963). Sensitivity of Chlorella to strong light or a sudden increase in light intensity varies with cellular age in synchronous culture (Sorokin, 1960; Pirson and Kowallik, 1964). Close to the upper threshold of temperature tolerance, Chlorella can be grown only in a light/dark cycle and not in continuous light (Lorenzen, 1963).

The relationship between light intensity and the rate of photosynthesis and photoautotrophic growth shows a rectangular hyperbolic function with an inhibition of growth

occurring at supersaturating light intensities. A similar situation also applied to algal suspensions growing in a light/dark cycle (Sorokin and Krauss, 1962). The shape of the action spectrum curves and the light-growth curves is markedly affected by temperature (Sorokin and Krauss, 1962) and by other factors such as salinity (McCombie, 1960) and nutrient level (Maddux and Jones, 1964).

III. Cell wall of Chlorella

Cell wall biosynthesis

After cytokinesis has produced naked autospores within the mother cell wall, cell wall formation commences outside the autospore plasma membrane with the appearance of small trilaminar plaques. These enlarge while inter-autospore granular material diminishes in quantity, and they eventually fuse to produce a complete trilaminar sheath around each autospore. A microfibrillar, cellulase-digestible layer is deposited between the trilaminar component and the plasma membrane. Meanwhile, the corresponding microfibrillar component of the mother cell wall is digested leaving only its resistant trilaminar component (Atkinson et al., 1972).

The trilaminar component includes sporopollenin, a substance considered to be a polymerized carotenoid on the basis of its resistance to extreme extraction procedures and its infrared absorption spectrum. Two phases of sporopollenin biosynthesis were detected during the cell cycle in synchron-

ous culture (Atkinson et al., 1972): one, synthesized directly, coinciding with the formation of the sporpollenin-containing trilaminar wall component, and the other, which may represent a precursor, occurs 6-8 hours earlier, while the cells are in karyokinesis. Of six strains tested, only Chlorella fusca var vaculator possesses sporopollenin, as does a strain of Scenedesmus and Prototheca.

It was shown in Chlorella (Murakami et al., 1963) that the dictyosome showed signs of increased activity during cell division, but the activity was not associated with any specific process. Further information on the process of cell wall formation in Chlorella during the early stages of cell division, and the behavior of dictyosomes which seems to be closely associated with the formation of new wall was given (Bisalputra et al., 1966). It was demonstrated that there is a close spatial relationship between the dictyosomes and the partition membrane which, itself, may be derived from the fusion of dictyosomal vesicles. Dictyosomes may also participate significantly in the deposition of wall material.

Cellulases

The term "cellulase" refers to a group of enzymes that contribute to the degradation of cellulose to glucose. The susceptibility of cellulose to such degradation is determined by its structural features. Native crystalline cellulose is insoluble and occurs in fibers of densely packed anhydroglucose chains of from 15 to 10,000 glucose units in

$\beta(1\rightarrow4)$ linkage, its density and complexity make it very resistant to hydrolytic enzyme attack without preliminary chemical or mechanical degradation or swelling (Cowling, 1963). The enzymatic mechanism whereby certain microorganisms can quite rapidly and completely degrade cellulose is not yet understood. Reese et al. (1950) proposed that at least two steps are involved: first, a prehydrolytic step wherein anhydroglucose chains are swollen or hydrated, and secondly, hydrolytic cleavage of the newly susceptible polymers, either randomly or endwise. The first step involves an unknown factor designated C_1. The hydrolytic enzymes, $\beta(1\rightarrow4)$ glucanases, were termed C_2. Both factors have been obtained from Trichoderma viride by Mandels and Reese (1964). Li et al. (1965) purified three components from Trichoderma viride C_1, a $\beta(1\rightarrow4)$D glucan 4-glucanohydrolase, and a β-glucosidase. Selby and Maitland (1967) reported the fractionation of three components from Trichoderma viride - a C_1, a carboxymethylcellulase and a cellobiase, and demonstrated the ability of the recombined system to degrade cotton. In both the above cases, the latter two components are an endoglucanase and an exoglucanase respectively. Okada et al. (1968) describe the purification from Trichoderma viride of three endoglucanases having somewhat different properties. Part of the carboxymethylcellulase activity was identical with xylanase (Toda et al., 1970).

The variety of assays employed with cellulases can be

confusing, measuring the three types of cellulases separately or measuring the combined activity of two or even all three groups. For example, weight loss (dissolution) of cellulose (fibers or microcrystalline pieces) is the best measure of C_1 activity. Viscosity loss of cellulose derivatives (CMC for example) measures only the Cx activity, while cellobiase conversion to glucose measures the cellobiase. Measuring reducing sugars produced from cellulose measures the combined effect of all three, although the cellobiase is not needed. Quantitatively the production of glucose from cellulose is similar to the production of glucose from CMC except that the overall activity may be only half as much without cellobiase. The amount of glucose produced from carboxymethylcellulose measures the Cx with some contribution from the cellobiase, but brings in the uncertainty of the variable structure and composition of different batches of CMC.

Cellulases are generally believed to be inducible in most cellulolytic fungi (Reese and Levinson, 1952) and bacteria (King, 1961; Hammerstrom et al., 1955). The production of cellulases in these microorganisms, however, is influenced by the physiological conditions to which microorganisms are exposed, and this is particularly so in bacteria. When the microorganisms are cultured with cellulose as the sole C-source, initial attack on the cellulose must be due to the small amount of cellulolytic enzyme already in the cells. However, if cellulases are really inducible, subsequent

formation of these enzymes must be accelerated by some products of hydrolysis of cellulose or by cellulose itself.

It is known that hydrolysis products of starch, such as maltose, can serve as inducers for amylases (Welker, 1963). In contrast, the formation of cellulase in the cellulolytic bacterium Pseudomonas fluorescens var cellulosa was not induced by cellobiose added at a concentration sufficient to support bacterial proliferation, but marked production of cellulases occurred with the addition of sephorose, 2-O-β glycosyl-D-glucose, which is not a usual cellulolytic product (Markovitz and Klein, 1955). The extent of this inductive formation of extracellular cellulases by sephorose was comparable to that produced in a medium containing cellulose. Similar results have been observed with regard to cellulase production in the cellulolytic fungus, Trichoderma viride (Mandels et al., 1962).

Highly polymerized cellulosic substances also induced marked formation of cellulase in Pseudomonas fluorescens var cellulosa, and more than 90 percent of the enzyme was released into the culture medium (Yamane et al., 1970). The extracellular cellulase seemed to consist of two components. In contrast, cultures grown on 0.5 percent cellobiose or cellooligosaccharides synthesized only a small amount of cellulase while more than 90 percent of the enzyme was in a bound form in the cells. This cellulase consisted of a single component and its electrophoretic mobility differed

from those of the extracellular components. In cultures with a controlled supply of various C-sources, formation of extracellular cellulase was enhanced to an extent comparable with that of cultures containing cellulose or sophorose, but formation of cell-bound cellulase did not increase significantly. A paucity of absorbable cellulolytic products in the culture medium appears to result in enhanced extracellular cellulase formation (Yamane et al., 1970).

Protoplast release by cellulase is inhibited by wounding of the tissue. Relief from this inhibition was effected specifically by mannose, but not other sugars, which led to the idea (Geballe and Galston, 1978) of the synthesis of lectins that are inhibiting cellulase activity. According to this, the cellulase is a glycoprotein that is inhibited by a lectin which binds to the sugar moiety on the enzyme.

Most microbial cellulases usually occur as a typical extracellular enzyme (Pollock, 1962). Most of the cell-bound cellulase (80%) was recovered in the intra-wall fraction. A low cellulase activity was found in cytoplasmic and membrane fractions (Suzuki et al., 1969). The cell-bound cellulase of Pseudonomas fluorescens could not be removed from the cell by several washings, which also supported the idea that most of the cell-bound cellulase must reside in the cell-wall region or on the surface of the cytoplasmic membrane.

Cellulase activities of Pseudomonas fluorescens that

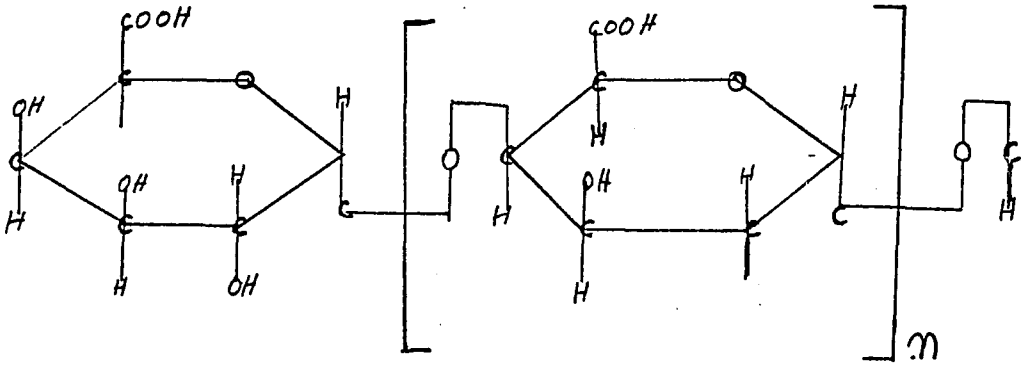
were shown to be located mainly in the extracellular and intra-wall fractions, were attributed to at least three cellulase components, A, B and C (Suzuki et al., 1969). All the extracellulase cellulose preparations from various cultures contained only cellulase components A and B. On the other hand, all three cellulase components were found in the intra-wall cellulase preparations from cultures in which a prominent production of extracellular cellulase (exo-type synthesis) occurred, while only the component C was detected in the intra-wall fraction of a culture in which a low production of cell-bound cellulase was mainly found (endo-type synthesis). It was suggested that the formation of component C was independent of the other two.

Pectic compounds

Three general types of pectin substances have been observed in plants: pectic acid and two derivatives of pectic acid called pectin and protopectin. Pectic substances are found most abundantly in the middle lamella between cell walls, usually in the form of calcium or magnesium salts of pectic acid. However, pectin and protopectin are also present. Pure pectic acid is an unbranched molecule consisting of D-galacturonic acid residues bound together by $\alpha(1\rightarrow 4)$ linkages. On complete hydrolysis, pectic acid releases glacturonic acid molecules. (Galacturonic acid differs from galactose only in carbon 6, which is a carboxylic group (-COOH) rather than carbinol group ($-CH_2OH$).)

Pectic acid is soluble in water and may be precipitated by calcium ions.

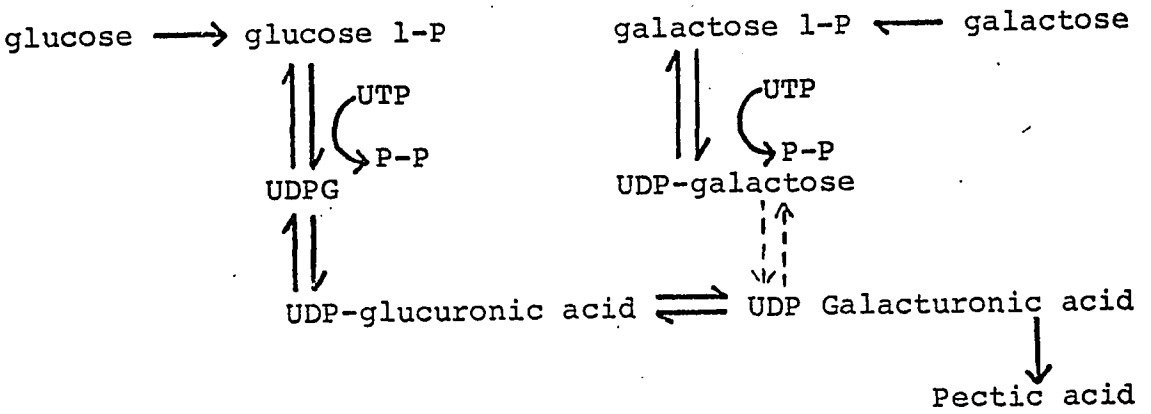
Pectic Acid



Pectin closely resembles pectic acid, the only difference being in the esterification of many of the carboxyl groups with methyl groups. (Pectin will form a colloidal suspension in water that will form a gel upon the addition of small concentrations of alcohol or high concentrations of sugar). The term protopectin is reserved for all insoluble pectin substances. Because of the instability of protopectin, effective isolation of this compound has not been accomplished. As a result, not much is known about the structure and composition of protopectin, although it is thought to be a much larger molecule than either pectic acid or pectin. It is accumulated in large quantities in some fruits (apple and pear). During the ripening of the fruit, protopectin is converted into the more soluble pectic substances pectin and pectic acid.

It is generally felt that the primary pathway for the synthesis of pectic substances is through the mediation of UDPG. This is supported by the fact that both glucose and galactose are good substrates for the synthesis of pectic acid, and the UDPG and UDP-galactose are readily interconverted.

Possible Pathway of Pectic Acid Synthesis



From this pathway, we can see where either glucose or galactose can enter into the synthesis of pectic acid. All of the reactions indicated have been demonstrated in plants, except for the incorporation of galacturonic acid from UDP-galacturonic acid into the pectic acid chain. However, this last step seems to be a logical assumption, particularly in view of the participation of UDPG in the synthesis of other polysaccharides such as starch and cellulose. Methyl groups, which are found in pectic substances esterified to the carboxyl group of the galacturonic acid units, are most likely contributed by methionine through S-adenosyl methionine. The

compound S-adenosyl methionine has been demonstrated to be active in the transfer of methyl groups. Hydrolysis of the $\beta(1 \rightarrow 4)$ linkage of pectic substances is catalyzed by the enzyme pectin polygalacturonase. Enzymatic hydrolyis of the methyl ester bonds of pectin is catalyzed by pectin methyl esterase.

Some of the intercellular polysaccharides of most seaweeds, though different from pectic substances of higher plants, contain carboxyl and/or sulfate groups. In some cryptogamic freshwater plants such as Chara and Nitella, pectic substances are also present, but the extent of their esterification is considerably lower, when compared to that of higher plants in which as much as half of the total carboxyl groups are substituted (Whistler and Smart, 1953). In Zostera marina (Maeda et al., 1966) only 12.4 percent of the total carboxyl groups were substituted or esterified, a value smaller than those found in land plants in general. This fact brought about the suggestion that the pectic substances play some role in ion absorption. Sulfate in an ester form was found to exist in this pectic substance. Gonium cells failed to adhere and produce colonies at the low levels of Ca that supported cell division. The amount of calcium in the medium may be related to the quantity of pectic substances produced in the cell walls of algae that have the capacity to synthesize such materials, and the level of Ca required may be related to whether the species does or does

not produce pectic substances (O'Kelley and Deason, 1962).

IV. "Gigantism" in Chlorella

Chlorella is also known as a heterotrophic alga that can grow in the dark on acetate (Shihara and Krauss, 1965) or glucose (Samejima and Myers, 1958).

Griffiths (1963) reported that a considerable number of very large cells, which he called "giant cells," were found among the normal cells of Chlorella vulgaris in a culture grown in a medium containing 1% glucose for 12 days in the dark. There was no increase in cell number when the giant cells reached a maximum diameter (30 μ). Exposure of these giant cells to light resulted in a decrease in the number of giant cells and a concomitant increase in the number of cells of normal size. Transfer of a growing culture from light to dark with the addition of glucose, temporarily suppressed cell division, which caused Griffiths to conclude that the cells in the light synthesize some substance essential for their division. He stated that the increase in cell volume which accompanied the formation of giant cells in glucose medium was dependent, without an increase in the number of cells, upon an increase in dry weight, and that inhibition of cell division was caused by a condition of heterotrophic growth such as that occurring during glucose utilization in the dark. It was also shown (Rodriguez-Lopez, 1963) that a high temperature (37°C) is more effective for induction of

gigantism than 23°C. The presence of sugars such as glucose, fructose or mannose is essential for the formation of the giant cells resulting from a delay in cell division or sporulation of autospores.

These giant cells are also characterized by a special organization and structure (Rodriguez-Lopez, 1965). Incubation of a synchronous culture of the same strain of Chlorella in a glucose medium in the dark also interferes with the cell's capacity to divide, leading to production of bleached giant cells (Griffiths, 1970). These cells are deficient in certain peptide amino acids when compared to normally grown cells (Thinh and Griffiths, 1975). The delay in the onset of cell division is reduced if L-arginine is introduced in the medium (Thinh and Griffiths, 1974).

These "giant" cells recover the ability to divide when returned to autotrophic conditions, and light induces recovery of cell division and chloroplast development (Thinh and Griffiths, 1972). The division of the giant cells is accompanied by marked pigment synthesis and a consequent recovery of photosynthetic capacity (Thinh and Griffiths, 1970). The contribution of photosynthesis towards recovery is only significant when the reserve of starch has been depleted (Thinh and Griffiths, 1973). Chloramphenicol inhibited chlorophyll synthesis and the development of photosynthetic capacity but did not affect cell division (Thinh and Griffiths, 1971) of recovering "giant" cells. It has been concluded that there is no correlation between cellular division and gigantism.

Based on microscopic observations two stages were distinguished by Higashiyama (1967, I, II) in the sugar induced formation of giant Chlorella: the "giant cell stage" and the subsequent "palmelloid body state." The "giant cell" is much larger in size than the control cell, but other morphological features are the same. The "palmelloid body" is a form composed of many conjoined cells. The algae showed cyclic transformation between "giant cells" and the "palmelloid body." The large amounts of carbohydrate composed of hexoses accumulated in the giant cells, was interpreted as the cause of the increase in the cell volume. Pectic substances were found to be present only in giant Chlorella cells. It was inferred that inductive formation of pectic substances is related to the appearance of a "palmelloid body."

APPENDIX B

FERRICYANIDE METHOD OF PARK AND JOHNSON

(Methods of Enzymology III p. 86.)

Reagents:

Ferricyanide solution: 0.5g of potassium ferricyanide in 1 liter of water (stored in a brown bottle, refrigerated).

Carbonate-cyanide: 5.3g of sodium carbonate and 0.65g of KCN per liter solution (stored refrigerated).

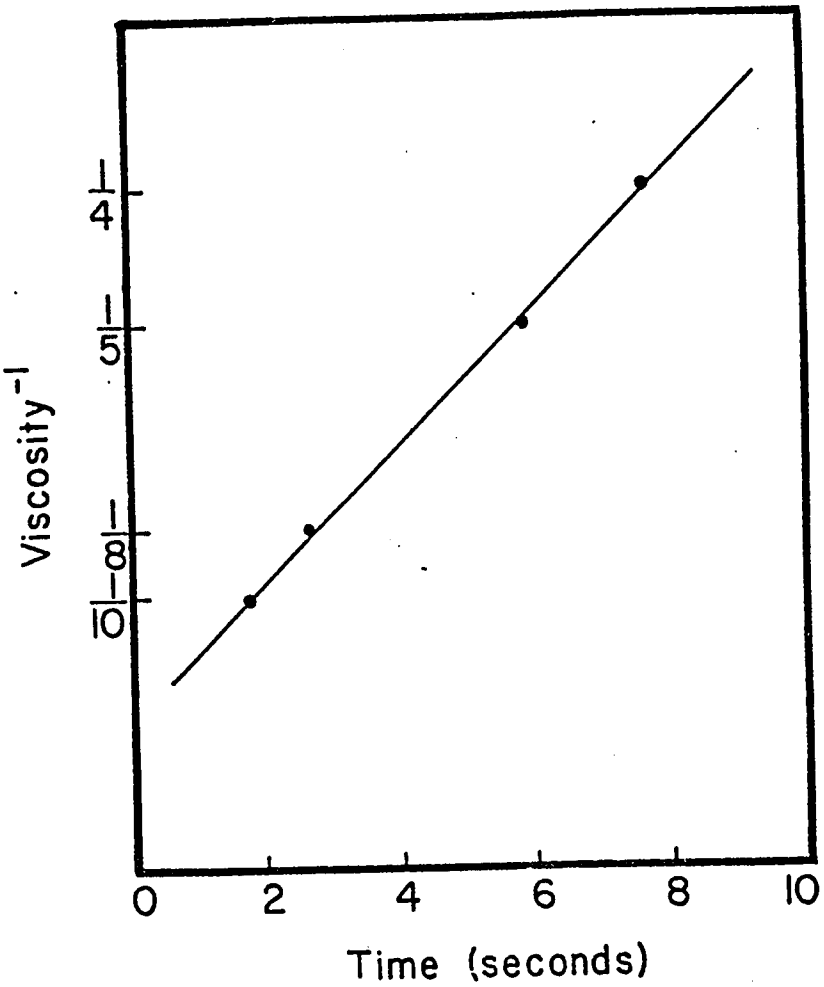
Ferric iron: 1.5g ferric ammonium sulfate and 1g of Duponol (sodium monolausyl sulfate) in 1 liter of 0.05N H_2SO_4.

Procedure: To a 1 ml sample, 1 ml of Ferricyanide and Carbonate-Cyanide were added. After mixing, the tube was heated in boiling water bath for 15 minutes and cooled to room temperature. Five ml of the Ferric iron solution was added, the mixture shaken and the absorbance at 690nm determined after 15 minutes in a Gilford Spectrophotometer.

Figure 21

CHANGES IN VISCOSITY OF C.M. CELLULOSE AS A FUNCTION OF ITS CONCENTRATION

A solution of C.M. cellulose (1.3% w/v) in 0.1 M phosphate buffer pH 6.3 was diluted with the same buffer and its viscosity determined as described in Materials and Methods.


Viscosity^{-1}
1/4
1/5
1/8
1/10
0
2
4
6
8
10
Time (seconds)

APPENDIX D

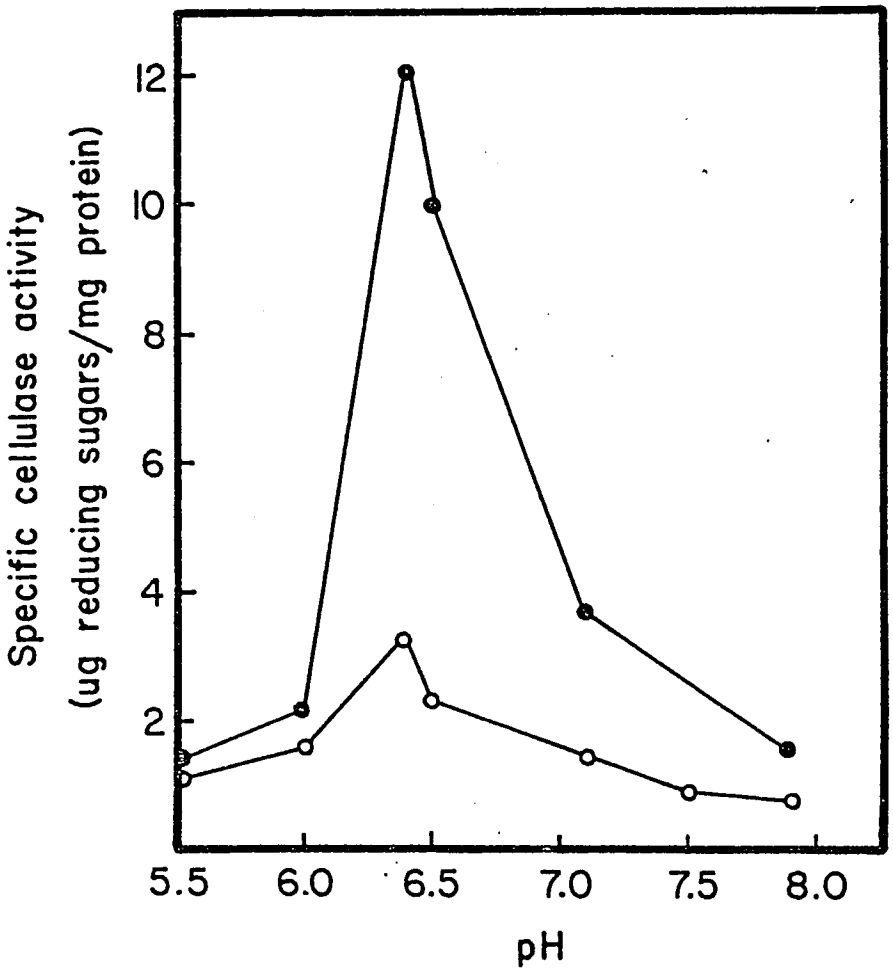
REAGENTS FOR PAS

1. Basic fuchsin-Schiff reagent: 0.5 g basic fuchsin in 100 ml hot (almost boiling) water, cool, 10 ml of 1 N HCl and 1 g of anhydrous sodium metabisufite ($Na_2S_2O_5$) were added. Stood for 24 hours then 1/4 gm activated charcoal was added, shaked and filtered rapidly through filter-paper.
2. Carnoy fixative: 3 volumes of ethanol:1 volume acetic acid.
3. Bleach - 5 ml 1 N HCl, 5 ml 10% Na_2 S_2 O_5 and 90 ml of water.
4. Periodic acid solution: 0.8 g periodic acid in 90 ml of water, 10 ml of 0.2 M Sodium acetate.

Figure 22

THE OPTIMAL pH FOR CELLULASE ACTIVITY IN CHLORELLA VULGARIS

A culture of Chlorella vulgaris was grown asynchronously for 4 days at pH 6.3 and 9.5. Cells from these cultures were harvested, suspended in 0.1 M phosphate buffer at the different pH values, and were broken in Braun Homogenizer. Cellulase specific activity was determined as described in Materials and Methods.


Specific cellulase activity
(ug reducing sugars/mg protein)
12
10
8
6
4
2
5.5
6.0
6.5
7.0
7.5
8.0
pH

APPENDIX F

TABLE 24

CHANGES IN VISCOSITY OF C.M. CELLULOSE AS AFFECTED BY HOMOGENATE PREPARED FROM CHLORELLA VULGARIS GROWN AT pH 6.3 OR 9.5

Time of Incubation (Hours) \ % change over 0 time	pH 6.3	pH 9.5
2.5	5	48
5	38	65

The enzyme solution was prepared as described in Materials and Methods. For the assay mixture 2 ml of enzyme preparation were added to 2 ml of 1.33% C.M. cellulose, in 0.1 M phosphate buffer pH 6.3. Enzyme activity is expressed as the % of change in viscosity per mg protein. Viscosity was measured as the draining time of 0.1 ml sample from 0.2 ml pipette at room temperature.

TABLE 25

COMPARISON OF ALPHA-CELLULOSE VERSUS C.M.-CELLULOSE AS SUBSTRATES FOR MEASUREMENT OF CELLULASE ACTIVITY USING BOTH PARK AND JOHNSON AND GLUCOSTAT FOR PRODUCT DETERMINATION

Substrate	Park and Johnson		Glucostat	
	Activity (µg reducing sugar/ml)	S.A. (µg/mg protein)	Activity (µg reducing sugar/ml)	S.A. (µg/mg protein)
Alpha-Cellulose	8.7	14.5	9.2	15.0
C.M.-Cellulose	13.1	21.9	15.6	25.5

A culture of Chlorella vulgaris was harvested by centrifugation and the cells resuspended in 0.1 M phosphate buffer pH 6.3 and broken using a Braun Homogenizer. For the assay of cellulase activity α-cellulose or C.M.-cellulose were used. The reaction mixture was incubated for 2.5 hours at 37°C and the product determined using either the Park and Johnson or Glucostat determination as described in Materials and Methods.

TABLE 26

A COMPARISON OF CELLULASE ACTIVITY AS MEASURED BY THE PARK AND JOHNSON METHOD AND THE GLUCOSTAT METHOD

Amount of Enzyme Prep. (ml)	Park and Johnson		Glucostat	
	Activity (µg reducing sugar/ml)	S.A. (µg reducing sugar/mg protein)	Activity (µg reducing sugar/ml)	S.A. (µg reducing sugar/mg protein)
1	2.2	11	1.4	7.0
2	9.2	23	8.4	21.

An asynchronized culture of Chlorella vulgaris was harvested and the cells broken in Braun Homogenizer with phosphate buffer 0.1 M pH 6.3. Cellulase activity was assayed using either the Park and Johnson or the Glucostat method as described in Materials and Methods.

APPENDIX G

N-8

KNO_3	1000 mg/l
$CaCl_2$	10 mg/l
$Na_2HPO_4 \cdot 2\ H_2O$	260 mg/l
KH_2PO_4	740 mg/l
$MgSO_4 \cdot 7\ H_2O$	50 mg/l
Fe EDTA	10 mg/l

Trace Elements

$Al_2(SO_4)_3 \cdot 18\ H_2O$	3.577 mg/l
$Mn(Cl_2) \cdot 4\ H_2O$	12.978 mg/l
$CuSO_4 \cdot 5\ H_2O$	1.833 mg/l
$CoSO_4 \cdot 7\ H_2O$	1.833 mg/l
$ZnSO_4 \cdot 7\ H_2O$	3.200 mg/l

pH 6.3

Trace elements were prepared in a stock solution concentrated 1000 times so that 1 ml has to be added to 1 liter solution.

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