HYDROSTATIC PRESSURE IN RELATION TO THE SYNCHRONOUS CULTURE OF ALGAE IN OPEN AND CLOSED SYSTEMS

by

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ABSTRACT

Chlorella ellipsoidea cultured using conventional methods under a 14: $\overline{10}$ light-dark regime in Beijerinck's medium at $25^{\circ} \pm 1^{\circ}$ with 700 ft-c illumination resulted in synchronous division with an n number of 4. Under identical conditions of light intensity, temperature, culture medium, and light-dark regime but in a closed system which was designed to study the effects of hydrostatic pressure on synchronous algae cultures, synchronous cell division did not occur and the n number was 2 or less at 1 atm. The most significant difference in the culture conditions arises because the concentrations of dissolved gases (mainly CO_2 and O_2) remain relatively constant throughout the growth cycle in the open system while the gases vary in concentration continuously during the cycle in the closed system. In the closed system O_2 increases continuously and CO_2 decreases during the light period; the reverse is true in the dark. Analyses of the ratios of dry weight, protein content, and cell size showed increases of approximately 2 in the closed system and 4 in the open system.

The intent of this study was to determine effects of hydrostatic pressure on the physiology and morphology of algae. Therefore it was essential to develop techniques for successful culture in a closed system. Various modifications of culture conditions were made in attempts (only partially successful) to obtain equal growth in the two systems.

The effects of variations in some environmental parameters on the n number, dry weight, protein concentration, and cell size in the open and closed systems were investigated. With varied growing conditions of light intensity (15-700 ft-c), light-dark regimes (14: $\overline{10}$, 16: $\overline{8}$, 18: $\overline{6}$, 6: $\overline{18}$, 8: $\overline{16}$, 10: $\overline{14}$), media (Beijerinck, Burr, Sorokin, Tamiya and Morimura, and Beijerinck's modified with bicarbonate), CO_2 concentration (0-100% saturation), and the addition of organic substances (glycolate, ascorbate, dithiothreitol) the n number and the ratios of increase in the other growth criteria were 4 in the open system but never more than 2 in the closed system.

The n number and ratios of increase in dry weight, protein content and cell size were 4 in the open system at atmospheric O_2 concentration but only 2 or less at high O_2 (50 and 95%) concentration. These results indicate that high O_2 is probably responsible for the descrepancies in the n number and the ratios of increase of the other criteria between the open and closed systems. Photosynthetically evolved O_2 in the closed system during the light period appears to be sufficient to induce this Warburg Effect.

It was hypothesized that the Warburg Effect is responsible for the observed differences in increases in the growth criteria in the open and closed systems. Experiments were carried out to determine if photosynthetically evolved O_2 was inhibiting cell growth. Using the Biological Oxygen Monitor algal samples were exposed to O_2 concentrations varying from 0 to 100% saturation at 25° with 700 ft-c

illumination. On the basis of these results which have indicated a decrease in the relative rates of O_2 evolution at high O_2 concentrations, it was concluded that O_2 evolved in the closed system could be responsible for the inhibition of photosynthesis.

Synchronous growth under pressure has not yet been obtained. However, pressure effects on cell division were investigated. The n number was 4 in cells dividing while exposed to pressures of up to 200 atm, but pressures above 335 atm completely inhibited cell division. A possible mutagenic effect of pressure exposure on algal cells was considered.

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INTRODUCTION

For almost a century it has been known that life flourishes in the depths of the sea. The dredging expedition of the "Talisman" in 1882-1883 revealed that organisms grow and reproduce to depths of at least 6,000 meters, where the hydrostatic pressure exceeds 600 atmospheres (atm). This discovery immediately stimulated study of the effects of hydrostatic pressure on organisms. However, interest in such effects has been sporadic up to the present. In the late 1920's and early 1930's, there was an upsurge of investigations into the physiological action of moderately high hydrostatic pressures of up to 1,000 atm. A number of workers investigated certain effects of hydrostatic pressure on biological materials and at that time it was widely accepted that the biological effect of pressure was, in general, to denature proteins thus killing the organism. This concept has not been experimentally substantiated and much remains to be understood about the process by which pressure acts upon biological systems.

Physically, hydrostatic pressure may be thought of as an external force acting upon the particles of a system in addition to the attractive forces between the particles comprising that system. It is important to realize that hydrostatic pressure is exerted equally in all directions, unlike deformation pressure. Whereas pressures of a few dynes

per square centimeter applied unequally often result in cell deformation and injury, hydrostatic pressure of hundreds of atm may not produce observable effects (24).

No natural biological process is ever subjected to a pressure as great as 10,000 atm. Pressures at the greatest known ocean depths are less than 2,000 atm. At the bottom of the Challenger Deep where organisms live and where the depth is 10,860 meters, the hydrostatic pressure is approximately 1,086 atm. The average depth of the ocean is 3,800 meters representing a pressure level of 380 atm. Pressures of this magnitude and somewhat higher, up to about 1,000 atm, may have a detrimental effect on the physiology of most organisms. Generally, protein and nucleic acid syntheses are retarded within this pressure range. Upon decompression, however, the original rates of synthesis are usually recovered. On the other hand thermal denaturation of some enzymes and proteins is opposed by pressure within this range (24) whereas other proteins are reversibly denatured (21). Still higher pressures, in excess of 1,000 atm generally have irreversible effects on most organisms (24). Consequently, pressures of between one and approximately 1,000 atm are far more significant than much higher pressures in relation to biological reactions. This range therefore, represents what may be termed the physiological pressure range. Pressures used in this study lie within this range. Biological responses of organisms subjected to very high hydrostatic pressures will not be considered here.

Hydrostatic pressure plays an important part in the physiology and ecology of organisms. All metabolic processes occur in a liquid phase and since hydrostatic pressure applies only to fluid systems it is not surprising that pressure exerts a profound influence on biological systems. For convenience, pressure may be considered as an eco-physiological factor for organisms which possess liquid, solid, or gas phase components or any combination of the three. Hydrostatic pressure therefore, determines partly the environment of all organisms including those found in the upper atmosphere (60). Pressure is believed to be of coordinate importance with temperature in affecting the vertical distribution, growth and rate of biological activities of organisms in the sea (64).

Hydrostatic pressure cannot be neglected if adaptative functions which permit life to exist in the deep sea are to be elucidated. Unicellular phytoplankton are the important primary producers of the hydrosphere yet there have been few studies relating hydrostatic pressure to the growth and metabolism of these organisms. Far below the photic zone, at depths as great as 2,500 meters, where the pressure is nearly 250 atm, algae have been found in large numbers (46). In the euphotic zone where algae have primarily been observed, pressures in excess of several atmospheres are never experienced. This fact likely explains the lack of interest in pressure effects on algae. Furthermore, photoautotrophic metabolism is limited to the upper 200 meters of the hydro-

sphere since insufficient light to support photosynthesis is transmitted beyond these depths. Plants surviving at greater depths must live heterotrophically, relying on either organic or inorganic chemical energy sources. It remains uncertain if photoautotrophic organisms can grow and reproduce under pressures exceeding that at which they are normally exposed to in their natural environment.

As a biological tool, hydrostatic pressure is immensely useful. It has been employed in the study of a number of microbial systems. The effects of pressure on protein synthesis and denaturation are well known (24). Such studies have also been conducted on enzymes and the nucleic acids (1,27,28,42). More recently there have been reports on the effects of pressure on bacterial induction, transcription and translation mechanisms (28). For those organisms living below the surfaces of water bodies and indeed, the barophiles of the deepest known ocean trenches, pressure undoubtedly influences the basic metabolism of these organisms. The use of pressure in studies relating to the biology of these organisms at both the organismal and molecular levels can provide much information leading to a clearer understanding of life in the deep sea.

The use of marine and fresh water algae for hydrostatic pressure research has been previously reviewed (59). Some species of unicellular algae are especially suited for this type of research. They are hardy and fairly resistant to contamination by other organisms. They are fast growing

and may be cultured axenically in a defined synthetic medium. Many of these species proliferate asexually and exhibit no complicated differentiation processes. These same features of unicellular algae contribute to their suitability in the establishment of synchronous cultures.

Almost all of the pressure work on plants thus far has been at the organismal level. The fundamental life processes of algae and microorganisms have a great deal in common. At the molecular level, therefore, it may be difficult, perhaps unlikely, to distinguish between the effects of pressure on algae and on bacteria. On the basis of this similarity, it is tempting to speculate that the pressure effects on algae are analogous to those that have been observed for bacteria.

There is some interest in the effects of pressure on the metabolic activities of algae (13,60). Gessner (13) found that 308 hours of compression at 200 atm stimulated population growth in _Chlorella pyrenoidosa_ over atmospheric pressure controls, resulting in almost three-fold increase in cell number after 15 days. There were 30 per cent more cells after a 30 day period of exposure to pressure. At 400 atm for 312 hours there was a decrease in the growth rate of the cells which resulted in 70 per cent fewer cells after 15 days. Sturm reported, as reviewed by Vidaver (60), similar results using _Chlorella_ cultures maintained in an organic medium. There is one study of the effects of pressure on the photosynthetic apparatus and rates of photosynthesis of algae (59).

In that work a number of intriguing questions about pressure effects on algae have been left unresolved. Some of those questions have been reconsidered here in relation with the overall effects of pressure on the physiology and biochemistry of synchronously grown *Chlorella* cells.

In approaching the present problem, *Chlorella* cells were grown synchronously using the techniques of Tamiya and Morimura (55). At certain stages in the life cycle of the algae a number of growth parameters were measured and compared with those of cells grown in pressure vessels in the light at atmospheric pressure and under almost identical growing conditions. The purpose of this study was first, to establish synchronous cultures in the pressure vessels at atmospheric and at higher pressures and secondly, to investigate the effects of pressure on the life cycle, on photosynthesis and respiration, and on the biosynthesis of some macromolecules in such cultures.

Sustained synchronous growth of *Chlorella* within the closed pressure vessels even at atmospheric pressure has not yet been attained. The growing conditions for the cells in the pressure vessels were manipulated in several ways to attempt to provide the optimal environment for growth and division. The cultures remained asynchronous and it was assumed that photosynthesis was somehow inhibited during the illumination periods. This inhibition was presumed to result from the photosynthetic production of oxygen (O_2) by the cells enclosed in the pressure vessels. Retardation of photo-

synthesis by O_2 may retard the biosynthetic activities of the cell (10,11,57). This effect could result in inadequate growth and lead to the absence of subsequent cell division. The inhibition of photosynthesis by high O_2 concentration has been reported by several workers (10,11,57). This phenomenon, first discovered in Chlorella in 1920 is known as the Warburg Effect and appears to be widespread in the Plant Kingdom (58). The Warburg Effect is therefore believed to have prevented adequate growth of Chlorella cells in the pressure vessels and is responsible for the lack of cell division. Cells grown first in the synchronous apparatus during the illumination periods and then transferred to the pressure vessels under aerobic conditions and exposed to pressures of up to 200 atm divided synchronously. It was observed that cells withstood pressures of up to 460 atm for as long as 24 hours with apparent recovery following decompression. The number of survivors decreased, however, as more pressure was applied and/or the duration of pressure exposure increased.

It is hoped that this treatment of culturing algae in a closed pressure vessel and the application of pressure as a tool in investigating algal metabolism will serve to stimulate further research on the effects of pressure on photoautotrophic organisms.

CHAPTER 1

GENERAL EFFECTS OF HYDROSTATIC PRESSURE ON SOME BIOLOGICAL SYSTEMS

In the late 1880's and early 1900's it was reported that yeast fermentation is completely inhibited by a pressure of 600 atm. Later it was shown that bacteria, yeasts and viruses could tolerate pressures above 1,000 atm for periods of several minutes up to a few hours. In those early years of pressure research biologists assumed that deep-sea pressure was of no ecological and physiological significance. Not until the 1940's was it observed that although many microorganisms could tolerate high pressures for a short period, prolonged compression usually caused death. Deep sea bacteria do not reproduce at pressures as high as 1,500 atm (64). Viruses are generally inactivated at pressures exceeding 1,000 atm; enzymes and proteins are either completely or partially denatured (37).

Investigations on the effects of pressure on autotrophic organisms are few. However, the literature indicates some research on the effects of hydrostatic pressure on plants. Oxygen consumption by *Ulva lactuca* decreases under pressure (30). Oxygen evolution also decreases under pressure in *Ulva lobata* (59). The algae *Chlorella* and *Hydrodictyon*, exhibit no adverse pressure effects after exposure to 200 atm for five days (64). Cytoplasmic streaming ceases in *Elodea* at pressures

between 400 and 500 atm, but resumes within minutes upon decompression (9). Guyot (15) observed that in Triticum radicle meristem cytokinesis is usually abnormal when exposed to more than 500 atm at room temperature. Pressures between 500 and 1,000 atm induced permanent pigment mutants in Euglena gracilis (14). Mutations in barley seeds have been induced by pressures of up to 135 atm in combination with high oxygen concentration (3). Vidaver and Lue-Kim (61) reported that certain combinations of pressure exposure and O_2 concentration appeared to stimulate lettuce seed germination while others were inhibitory. The inhibitory effect of pressure in the presence of O_2 on recovery upon decompression was due in part to the toxicity of O_2 at the high concentration which was obtained under pressure.

I. Metabolic Processes And Enzymatic Activities

Many of the pressure effects on the synthesis and inactivation of cellular macromolecules are undoubtedly associated with the energy transforming processes within the cell. Pressure may inactivate proteins and enzymes by affecting the chemical bonds of the molecules and thereby influence the normal functioning of these molecules. Some of these pressure effects may induce changes in the endothermic or exothermic chemical reactions of a cell and affect its overall energy balance.

Molecular volume changes that accompany pressure inactivation of enzymes and proteins can influence the energy relationships of the cell. When hydrogen bonding of any

molecules, including protein or enzyme molecules, is affected by pressure application, the volumes of the molecules change. Under such conditions active catalytic sites on the molecules may become affected such that they no longer function as in their native states. Because enzymes affect the rate but not the equilibrium of chemical reactions, pressure may, by bringing about a change in the molecular volume, alter the catalytic activity of an enzyme as a result of changes in protein configuration. Such a change in the catalytic activity of an enzyme may affect the rate of biological reactions in the cell which could influence the adenosine triphosphate(ATP) turnover rate and hence its pool size in the cell. Indeed, pressure of 2,000 atm has been shown to inhibit magnesium-activated muscle ATPase (38).

The effects of pressure on various enzymes of the tricarboxylic acid cycle and the glycolytic pathway have not been investigated to any great extent. When resting cells of Escherichia coli (E. coli) are exposed to 1,000 atm there is a marked reduction in the rate of malic dehydrogenase activity (36). Succinic dehydrogenase activity decreases with increasing pressure and at 1,000 atm little activity remains. Little is known about the effects of pressure on isolated cell organelles. Hill and Morita (18) attempted to by-pass the problem of membrane permeability in Allomyces macrogynus cells by observing pressure effects on the enzymes of isolated mitochondria. They found that pressures of up to 1,000 atm decreased the succinic dehydrogenase activity

of the isolated mitochondria. A progressive decrease in α-ketoglutarate dehydrogenase activity accompanied increasing pressures and at 1,000 atm no activity could be detected. At 1,000 atm some oxalosuccinic dehydrogenase activity remained but little activity of isocitric dehydrogenase persisted. It was postulated that the pressure-induced death of *Allomyces macrogynus* was caused in part by the blockage of the tricarboxylic acid cycle, depriving the organism of its principal energy producing processes. It was shown by ZoBell and Budge (65) that the nitrate reducing enzyme systems of *Pseudomonas perfectomarinus* were gradually inactivated by pressure of 1,000 atm. With prolonged exposure to 1,000 atm dehydrogenase activity also diminished. Similar studies have not been done on plant dehydrogenases. Perhaps analogous pressure effects would occur in algal dehydrogenase and nitrate reduction systems.

Enzymes are affected by any of the agents of pressure, pH, and temperature which denature proteins. Enzyme synthesis by cells is usually affected by pressure. *E. coli* cells treated with isopropylthiogalactopyranoside to stimulate β-galactosidase synthesis and then exposed to pressures of either 455 or 1,320 atm soon after, are unable to synthesize the enzyme (42). If pressure is applied to cultures actively producing the enzyme, synthesis continues for a brief period and then ceases. Apparently genetic transcription stops and the brief period of enzyme formation is due to the presence of some previously synthesized messenger ribonucleic acid

(mRNA). Landau, (28) using a different strain of E. coli obtained similar results.

Although not pertinent to this study, work on proteins and enzymes has been done at pressures well above 1,000 atm (48,49,50,51). Miyagawa (33,34) reported that pressures of 4,000 or 9,500 atm for five minutes duration completely inactivated the enzyme, Taka-amylase-A. Both inactivation and subsequent recovery of activity were dependent upon the enzyme concentration used, the pH, temperature, and the ionic strength of preparation. Recovery was found to be enhanced by recompression at a somewhat lower pressure. It has been observed that human saliva α-amylase becomes inactivated by pressures above 5,000 atm in an aqueous solution (26). At 9,000 atm loss of activity is complete in five minutes when the temperature is 30° and the pH 6.9. Generally, pressure inactivation of the α-amylase is accelerated in acid media with increasing temperature up to 40°.

The effects of pressures of up to several thousand atmospheres on enzymatic reactions are often reversible. Kettman et al (25) found that the aggregation of modified poly-L-valyl-ribonuclease was inhibited by 150 and 300 atm. Upon decompression to 1 atm, the aggregation rate immediately resumed that of the atmospheric pressure control rate. Despite inactivation of ribonucleodepolymerase by 6,000 atm, upon release to 1 atm, the enzyme recovered its activity (62).

II. Membranes

Pressure application can affect the regulatory function of membranes and influence the kinds and amounts of materials that enter or leave a cell. Many of the observed pressure effects on intact cells may be directly related to the effects of pressure on the protein components of the membrane systems. There is no evidence to indicate what effect pressure might have on lipids in general and on that component of the membrane systems.

Pressure effects on the permeability of plasma membrane of onion inner-epidermal cells have been investigated by Murakami (39). He records plasmolysis and de-plasmolysis times of the cells when placed in various concentration of electrolytes and non-electrolytes in solution during pressure application. Pressures of up to 485 atm increase the plasmolysis times over the atmospheric pressure controls in any of the solutions used.

III. Some General Effects Of Pressure On Macromolecules

1. Proteins

It is not the purpose of this treatment of pressure effects on proteins to review all the available literature. Early work on protein denaturation by pressure was not quantitative enough for theoretical analysis (24,49). Temperature was uncontrolled and the duration of compression was not regulated. In many instances the pH and ionic strength of the medium were not determined. Suzuki et al (49) pointed out that denaturation studies were usually made after

decompression of the protein solution and not during the pressurization period.

Adverse effects of pressure above 1 atm on proteins have been extensively investigated by a number of workers (4,5,6,16,19,20,21,22,23,35). Johnson et al (24) have reviewed the effects of both very high and moderate pressures on various proteins. In a recent review Morita (37) discusses some of the effects of pressure on the proteins of marine microorganisms. More recently the effects of pressures on a number of proteins at pressures above 1,000 atm have been studied by some Japanese workers (35,48,49,50,51).

The volume of a protein molecule is affected by both temperature and pressure. Generally, molecular volume increases when the temperature is raised and decreases when the hydrostatic pressure is increased. As a result of its effects upon molecular volume changes, pressure may play a significant role in many of the physical and chemical properties of the cell. While pressure can change the structure of water itself, (24) it also influences the degree of hydration, ionization, electroconductivity, dipolar interaction, and the chemical reaction rates of molecules in solutions (24).

In _E. coli_, Landau (27) found that the incorporation of ^{14}C-glycine into proteins was stimulated at 265 atm and inhibited at 665 atm at 37°. At this temperature the 400 atm incorporation rate was identical to that of the atmospheric pressure control. He showed further that 265 atm stimulated the incorporation of ^{14}C-leucine into _E. coli_ proteins above

27° but inhibited incorporation at a lower temperature. The incorporation was inhibited by 400 atm when the temperature was less than 37° (27). The author concluded that the pressure-temperature relationships were probably indicative of the effects on a series of different but interrelated biochemical events. Pollard and Weller (42) showed only a negligible incorporation of ^{14}C-valine into proteins of E. coli at 900 atm.

Protein synthesis was completely inhibited in the psychrophile, Vibrio marinus by 1,000 atm (1). At 400 and 500 atm protein synthesis decreased whereas 200 atm initially retarded synthesis but the pre-pressurization rate resumed after about 60 minutes. Pollard and Weller (42) showed that 900 atm delayed the recovery of protein synthesis for only 10 minutes. Landau (27) reported that after protein synthesis was almost completely inhibited for 10 to 15 minutes subsequent to the application of 665 atm of pressure at 37°, the release of pressure resulted in a rapid resumption of amino acid incorporation into the proteins of E. coli. He suggested that the incorporation rate was somewhat greater than that found in non-pressurized cultures.

The thermal denaturation of some proteins and enzymes is opposed by pressures of up to 680 atm (21). Similarly, the inactivation of these macromolecules by alcohol, high pH, and urethane may be retarded by pressures of up to 680 atm provided the temperature is not raised above the optimum. Pressures of from 680 to 750 atm opposed the

rate of precipitation of purified human serum globulin at 65° within a pH range from 5.15 to 6.80 (21). Under similar pressure exposures and at pH 6.0, crystallized egg albumin solutions were not precipitated at room temperature. At 65° however, and at pH 6.0 small amounts of ethyl alcohol increased the precipitation of both proteins at pressures of up to 750 atm.

2. Nucleic Acids

Except for some microorganisms, pressure effects on the synthesis and inactivation of the nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), have not been extensively investigated. No such studies have been reported on algae or higher plants.

ZoBell and Corbet (66) reported that in three strains of *E. coli*, RNA synthesis increased but DNA synthesis decreased as the incubation pressure was increased to 450 atm. At this pressure, however, the total amount of nucleic acid in the cells did not differ from the 1 atm controls. The incubation of *E. coli* at pressures of up to 450 atm appeared to repress the replication of DNA. The repression of DNA synthesis, probably due to the pressure effects on the enzymes of DNA synthesis, may be responsible for the lack of cell division. This phenomenon may also lead to cell death.

The incorporation by *E. coli* of ^{14}C-thymine and ^{14}C-valine into DNA and proteins respectively at room temperature was inhibited at 900 atm whereas the incorporation of labelled uracil into RNA continued at almost the 1 atm

control rate (42). At 675 atm, RNA synthesis was unaffected but DNA and protein synthesis were reduced to one-half that of the 1 atm control. Pollard and Weller (42) concluded that it is essentially those cellular processes associated with genetic transcription and DNA synthesis that are pressure sensitive. Similar results were reported by Landau (27) who investigated the incorporation of ^{14}C-adenine into the nucleic acid fractions of E. coli. The incorporation of ^{14}C-adenine into the nucleic acid fraction of the cells exposed to 265 atm was stimulated at 37°. The incorporation of labelled adenine under 400 atm of pressure equalled that of the controls whereas 665 atm inhibited incorporation. The stimulated rate of incorporation of 265 atm continued for 20 minutes; the inhibition, while marked under 665 atm of pressure, was never complete.

More recently Landau (28) has shown that pressure exerts a marked effect on the translation, transcription and induction processes of E. coli cells. Each phase of induced enzyme synthesis is affected differently depending upon the compression. Transcription continues at 670 atm and is least affected. Translation is completely inhibited at 670 atm and unaffected at 265 atm. Induction, however, is totally inhibited at pressures about 265 atm.

It has been suggested that pressures of up to 2,700 atm promotes the stabilization of the double helix structure of the DNA molecule (17). Weida and Jill (63) using calf-thymus DNA preparations found only small effect of pressures

of up to 1,335 atm upon strand separation of the DNA molecule. An increase in pressure raised the temperature at which strand separation occurs. These authors concluded that the small pressure effects on DNA strand separation appeared to be of little importance in the synthesis of nucleic acids.

It was shown recently that when psychrophilic Vibrio marinus is exposed to 1,000 atm, protein, DNA and RNA syntheses are completely inhibited (1). At 200 atm the synthesis of DNA is unaffected whereas the synthetic rates of both protein and RNA are at first inhibited but resume the 1 atm control rates after an hour. At 500 atm protein synthesis is retarded but DNA and RNA synthetic rates continue normally for a brief period and later decrease with time. The author suggests that the principal effect of pressures ranging from 400 to 600 atm is to lower the rate of protein synthesis which in turn may initiate the decreased rate of both DNA and RNA syntheses.

IV. Mutations

Hydrostatic pressure is probably a mutagenic agent of significance. Pressure might well serve as a specific mutagen because of the highly predictable effects of pressure on proteins, enzymes and nucleic acids (60). Pressure induced mutations, unlike random radiation mutations, could be highly reproducible. It would be of value, however to know if reproducible mutations could be induced by controlling the magnitude and/or duration of pressure exposure. It would be equally valuable to determine if such mutations were

reversible.

Pressure induced mutations have been obtained in some organisms (3,14). Gross (14) has repeatedly obtained pressure induced colour mutants of Euglena gracilis strain Z. Pressure treatments of from 500 to 1,000 atm for a brief period induced stable pigment mutants lacking chlorophyll and having structurally altered carotenoid molecules. Berg et al (3) have used O_2 under pressures of up to 135 atm to induce mutations in barley seeds. The frequency of mutation was found to be dependent upon the water content of the seeds, being higher in dry seeds with water content of 2.1 and 3.1 per cent, than in seeds having a 9.3 per cent water content. Since the solubility of O_2 is proportional to the pressure applied, under the experimental conditions used by the authors the seeds were immersed in water with an O_2 concentration as high as 0.17 M compared with a concentration of 1.25×10^{-3}M O_2 at atmospheric pressure. As suggested by the authors, the mutagenic agent is probably O_2 itself at such high concentration.

When nitrogen mustard gas is used as the mutagen, the frequency of morphological mutants of Neurospora spores is higher than when the gas is used in combination with pressures of up to 600 atm (31). Conversely, pressure along with nitrogen mustard gas appears to induce biochemical mutants more readily than morphological ones. Palmer (37) reported a three-fold increase in the rate of mutation to streptomycin resistance in Serratia marinorubra exposed to 300 atm over the 1 atm controls.

Burns (7) reported that lethal x-ray inactivation of haploid *Saccharomyces cerevisiae* SC 7 was not changed by compression of up to 665 atm applied during and after irradiation. The author inferred that x-ray induced lethal mutation in yeast is not a denaturation process and hence did not involve a change in the configuration of macro-molecules as shown by the lack of pressure effects at 665 atm.

It is clear that hydrostatic pressure does exert a profound influence on the regulation and maintenance of the life processes of cells. Pressure effects on metabolic processes are many and varied. Some effects are observed at only elevated levels (above 1,000 atm), some are reversible whereas others are permanent. Furthermore, the manifestation of pressure effects is dependent upon the duration and/or magnitude of exposure and on such experimental conditions as pH, temperature, and ionic strength of the medium. Although the effects described are principally those that have been observed in bacteria, it is highly likely that similar pressure effects are to be found in algae. The pressure studies reviewed here, therefore, would probably apply in a large part to a consideration of pressure effects on algae, the principal objective of this chapter and the study as a whole.

CHAPTER 2

MATERIALS AND METHODS

General experimental methods and materials used throughout the entire study are described in this chapter. Appropriate specific details are outlined elsewhere when necessary to describe individual experiments.

I. Synchronous Culture

The algae used in this study are *Chlorella ellipsoidea* Gerneck, Indiana University Number 246 (I.U. 246), *Chlorella pyrenoidosa* Chick, I.U. 395 and *Scenedesmus obliquus* (Turp.) Kruger, I.U. 393 obtained from the Culture Collection of Algae at Indiana University (45). Subcultures were maintained on agar slants or in liquid Beijerinck's medium (Table 1) at room temperature under continuous illumination at 900 ft-c. Cells used in the experiments were harvested from both types of sub-cultures.

In preliminary experiments several different media (see appendix 1) were used to culture the algal cells synchronously under a programmed light-dark regime. Beijerinck's medium was found satisfactory for growth and was ordinarily used for the culturing of the cells in the synchronous apparatus (Fig. 1). At first the algal cells were cultured in the pressure vessel (Fig. 2, 3) in Beijerinck's medium and later other media were used, but not before their suitability in supporting adequate cell growth

TABLE I

Beijerinck's Medium (47)

Stock mineral solution	100.00 ml
Buffer A--KH_2PO_4	40.00 ml
Buffer B--K_2HPO_4	60.00 ml
Trace Elements	1.00 ml
Glass-distilled Water	800.00 ml
Stock Mineral Solution	
NH_4NO_3	1.50 g
K_2HPO_4	0.20 g
$MgSO_4.7H_2O$	0.20 g
$CoCl_2$	0.10 g
Glass-distilled Water	1.00 litre
Buffer A	
KH_2PO_4	9.07 g/l
Buffer B	
K_2HPO_4	11.61 g/l
Trace Elements*	
$CuSO_4$	0.15 g
H_3BO_3	1.00 g
EDTA (Sodium salt)	5.00 g
$ZnSo_4.7\ H_2O$	2.20 g
$CaCl_2$	0.50 g
$MnCl_2.4\ H_2O$	0.50 g
$FeSO_4.7\ H_2O$	0.50 g
$CoCl_2.6\ H_2O$	0.15 g
$(NH_4)\ 6MO_7O_{24}.4H_2O$	0.10 g

* The micronutrient solution was prepared by adding each element individually to 100 ml of glass-distilled water heated to 70° or 80°. After each addition of an element the pH of the solution was adjusted to 5.0 with KOH pellet(s) (EDTA precipitates out below pH 2.0). The solution was then cooled to 30° and the pH adjusted to 5.5 by adding more KOH pellets. When the solution was cooled to room temperature the final pH was adjusted to 6.5 by adding 1 N KOH solution.

was determined.

The synchronous culture apparatus consists of a 15 gallon glass aquarium enclosed in a light tight box. Constant water temperature of $25^{\circ} \pm 1^{\circ}$ was maintained by heating and refrigerating units. Continuous stirring of the water was maintained. Illumination of 700 ft-c was provided by two banks of fluorescent lamps placed horizontally on either side of the aquarium. Each light bank had one 20 watt Sylvania Gro-lux lamp in the centre and on either side a 20 watt warm white Westinghouse fluorescent lamp. The light source was connected to a time switch to provide a programmed light-dark regime. The cultures were normally illuminated uninterruptedly for a period of 14 hrs. At the end of this light period the lamps in the light tight box were automatically switched off and the cultures left in complete darkness for 10 hrs. This light-dark regime designated as $14: \overline{10}$ (54) constitutes one complete life cycle and was routinely used to induce synchrony of the cells. In some experiments the $14: \overline{10}$ light-dark regime was changed to correspond to that regime used when the cells were cultured in the pressure vessels.

Pyrex glass culture tubes, 200 x 32 cm were suspended in the water bath from a rack supported on the edges of the aquarium. Each culture tube was fitted with a silicone rubber stopper which had 2 capillary tubes of unequal length and constricted to accommodate cotton plug filters. Each culture tube was aerated by bubbling with

Fig. 1 Constant temperature water bath of the synchronous apparatus. The culture tubes containing algal cells are immersed in the water bath and each tube is bubbled with 5% CO_2-air mixture. Illumination is by the fluorescent tubes on either side of the aquarium.

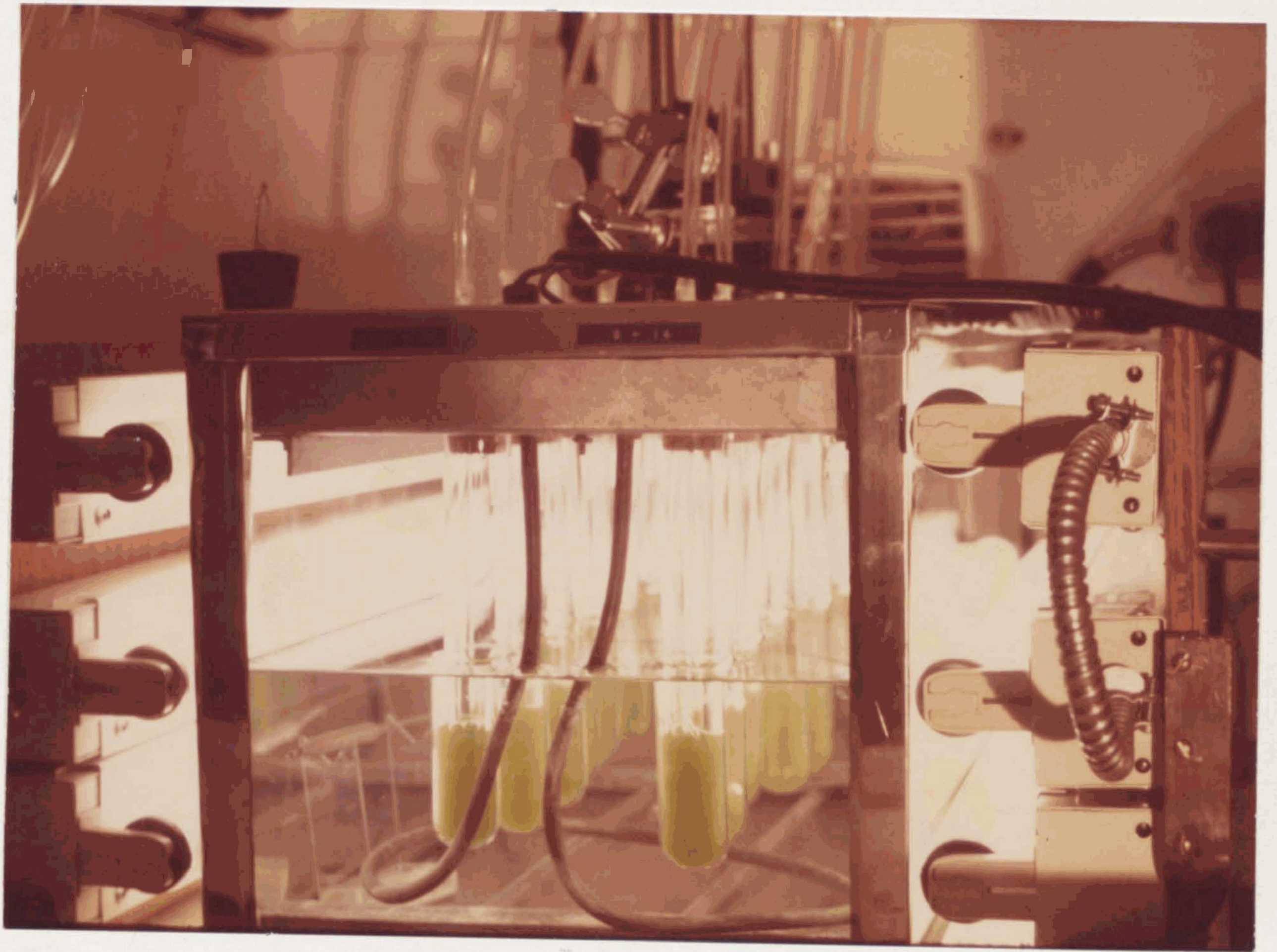

Fig. 1

Fig. 2 Exploded diagram of the pressure vessel assembly including the free piston cylinder. Details of operation are explained in the text.

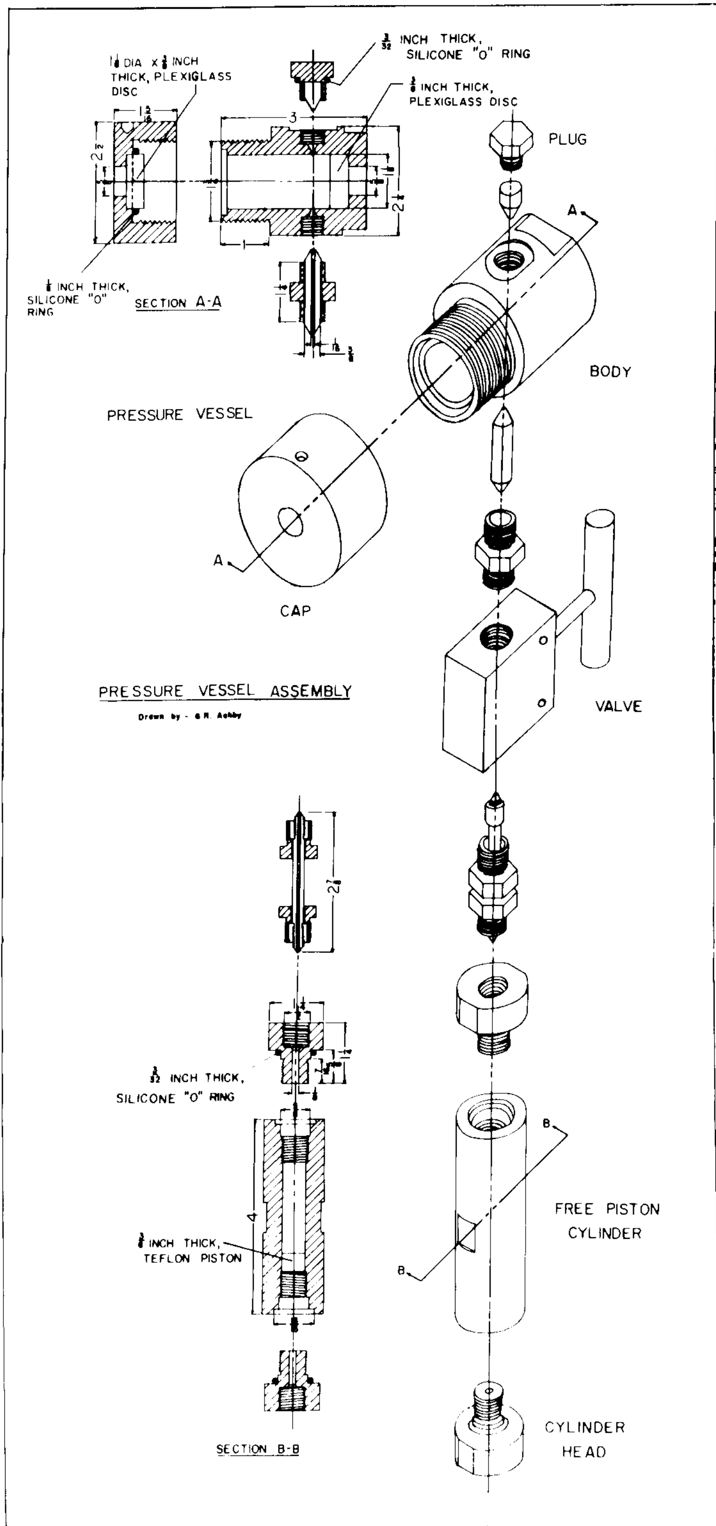

Fig. 2

Fig. 3 Exploded view of the various components of the pressure vessel assembly showing the plug (A), body of vessel (B), cap (C), glass marble (D), needle valve (E), and the free piston cylinder (F).

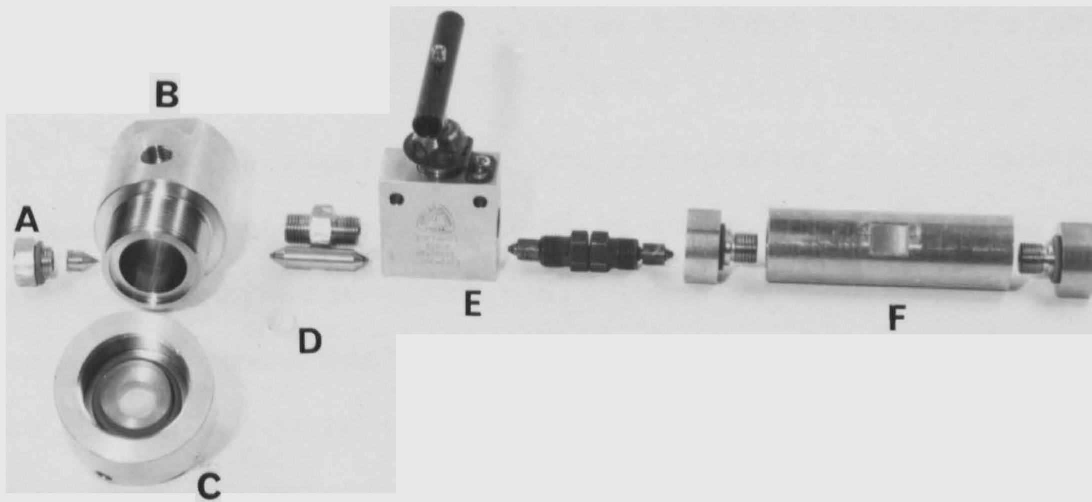

Fig. 3

5 per cent (%) CO_2. The gases passed through a cotton wool filter, were mixed in a water column, and dried by passing through anhydrous $CaSO_4$ (Drie-rite) before entering the algal cultures. The gas line was connected to that capillary tube immersed in the culture and a constant rate of flow maintained by individual valves.

The stock mineral solution, buffers A and B (see Table 1) and the microelements were prepared and kept refrigerated in separate containers. The culture medium was prepared as shown in Table 1. To use in a culture experiment, 25 ml of the medium was poured into a culture tube which was then stoppered and sterilized at 121° for 10 to 15 minutes by autoclaving.

The algal cells were transferred to the culture tubes where they were allowed to grow and increase in numbers in the synchronous apparatus. These preliminary culture operations were not done to establish synchrony but rather to obtain adequate cell suspensions. These cultures provide the starting material for the synchronous cultures. After sufficient growth the liquid algal cultures were first centrifuged at 2,500 rpm for 6 minutes to remove the large cells. The small young D_s cells (52, 55) remaining in the supernatant were used to start the synchronous experiments.

To start a synchronous culture, 5 ml of this liquid culture of small D_s cells at a concentration of approximately 2.5×10^7 cells per ml were aseptically transferred to each tube which was then stoppered and immersed

in the water bath of the synchronous culture apparatus. Each culture tube was then bubbled with the 5% CO_2-air mixture and routinely subjected to the 14: $\overline{10}$ programmed light-dark regime.

Synchrony of the cells is determined by the constancy of the n number (the number of daughter cells released from a single mother cell after one light-dark cycle) (54). The n number is calculated by dividing the final cell number by the initial cell number after one complete life cycle of the alga. If C_f and C_i represent final and initial cell numbers then:

$$\text{n number} = \frac{C_f}{C_i}$$

The n number ordinarily used is four.

II. Pressure Vessel System

The body and cap of the optical pressure vessels used to culture algal cells are made from Atlas 316 stainless steel. With the cap in place the vessel has an internal volume of 28.0 cm^3. The vessels may be used to perform photobiological experiments under hydrostatic pressure exceeding 1,000 atm using light of controlled intensity and quality, constant temperature, and sterile conditions for extended periods of time.

Both the cap and the body of the vessel (see Fig. 2 A-A) have openings to accommodate 1-1/8" diameter x 3/8" thick plexiglass windows. When the cap is in place a straight through light path is obtained. The window fits

tightly in the recessed bottom of the cap whereas the body window fits snugly inside the vessel. The window of the cap protrudes some distance and over this window is fitted a silicone rubber "O" ring, 1-1/8" I.D. x 1-3/8" O.D. x 1/8" thick. The cap with the "O" ring fitted over the protruding window fits with a narrow tolerance into the body of the vessel. The window of the cap with the "O" ring thus forms a seal between the stainless steel bearing surfaces when pressure is applied. A 2 piece stem stainless steel needle valve, (No. 30VM-4071 Autoclave Engineers, Erie, Pennsylvania) attached to the body of the pressure vessel maintains pressure.

To prepare the pressure vessel as a culture chamber for algal cell growth, it is fitted with Beijerinck's medium or any other appropriate medium and closed. The valve and plug (see Fig. 2, 3) are then attached to the body of the vessel. A small glass marble is placed in the chamber of the vessel to keep the cells in suspension when the vessel is shaken. Contamination of the medium in the assembled apparatus was controlled by immersing the apparatus in a water bath at 70° for at least one hour. After the apparatus cooled to room temperature the plug was removed and the vessel inoculated aseptically with algae by means of a sterile syringe fitted with a hypodermic needle. Usually, the cell concentration in the vessel was the same as that in the culture tube at the start of the synchronous experiment in the synchronous apparatus. CO_2 was also injected into the vessel at a concentration calculated on the basis of the amount of

carbon contained in the gas in relation to the amount required by the cells to allow for at least a four fold increase in carbon content at the end of a life cycle. Substances may be added to the vessel or an aliquot of cells withdrawn aseptically by removing and replacing the plug.

Cells were cultured at atmospheric pressure in the vessels prepared as described above. The assembled vessels were then mounted in the temperature-controlled shaker water bath (Fig. 4, 5). The cultures in any of seven vessels (Fig. 6) were illuminated individually through one window with approximately 700 ft-c. For illumination, individual 6 volt, 15 watt microscope illuminator lamps (Wild Herrbrugg Ltd., Switzerland) were mounted on a holder outside the water bath. The lamp holder was fixed in a horizontal position with respect to the windows of the vessels. In this position the lamps were aligned with the windows of the vessels (see Fig. 6). The lamp holder was attached to the shaker device so that the lamp distance from the windows was always constant. At the end of the light period both windows were completely covered for the dark period. After the dark period the cells were removed and assayed.

When applying pressure to the vessel, non-sterile medium forced into the vessel as the pressure is applied could contaminate the culture. To avoid this, the free piston cylinder (see Fig. 2 B-B) was designed. This cylinder is

Fig. 4 The pressure vessel assembly prepared for use. The free piston cylinder and connecting tubing are removed before immersion in the water bath.

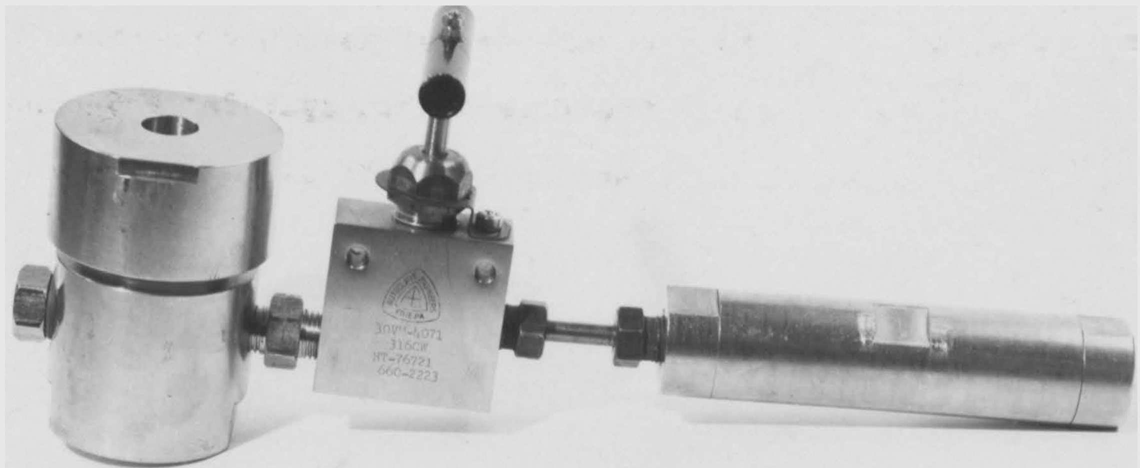

Fig. 4

Fig. 5 Pressure vessels in place in the shaker frame prior to immersion in the water bath. The individual lamps are mounted on a rack which is attached to the frame.

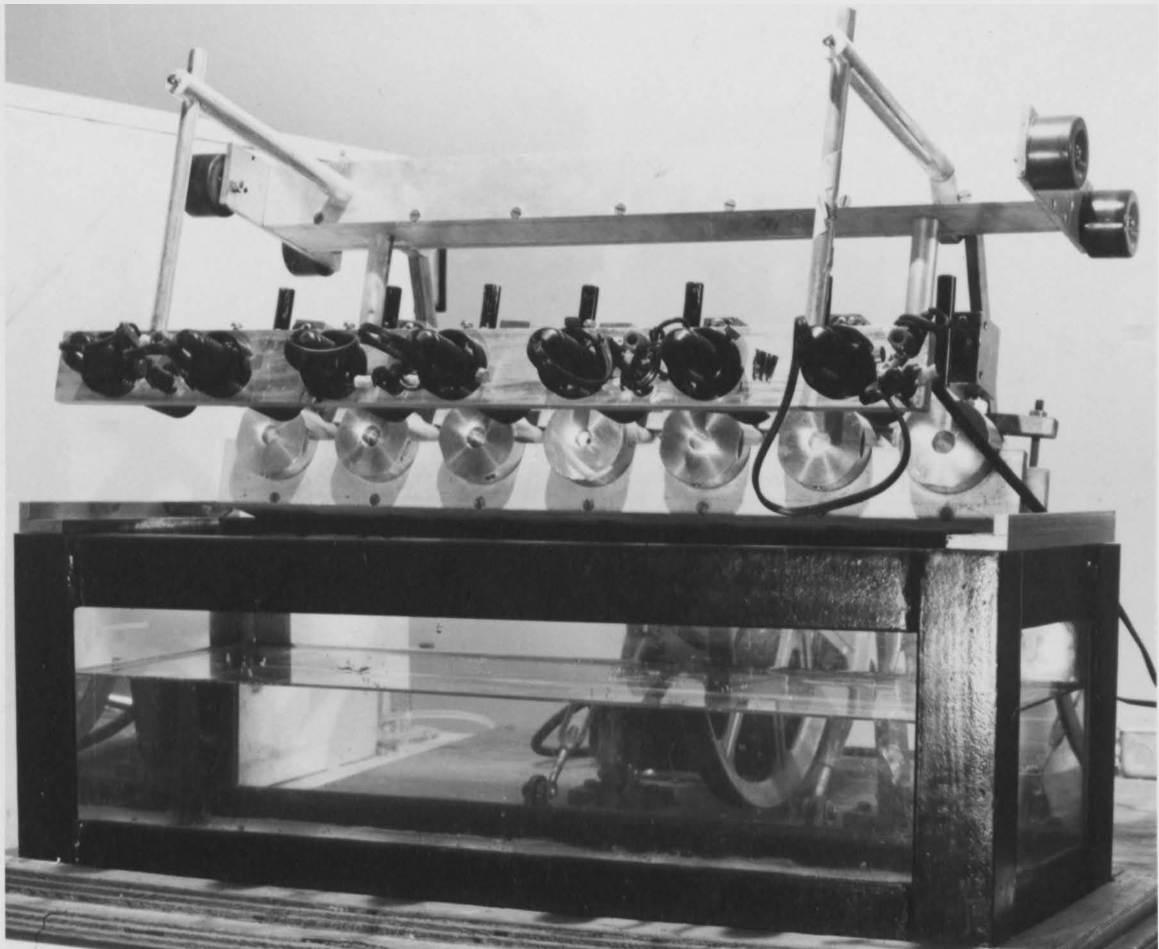

Fig. 5

Fig. 6 Pressure vessels in the water bath showing how each vessel is individually illuminated.

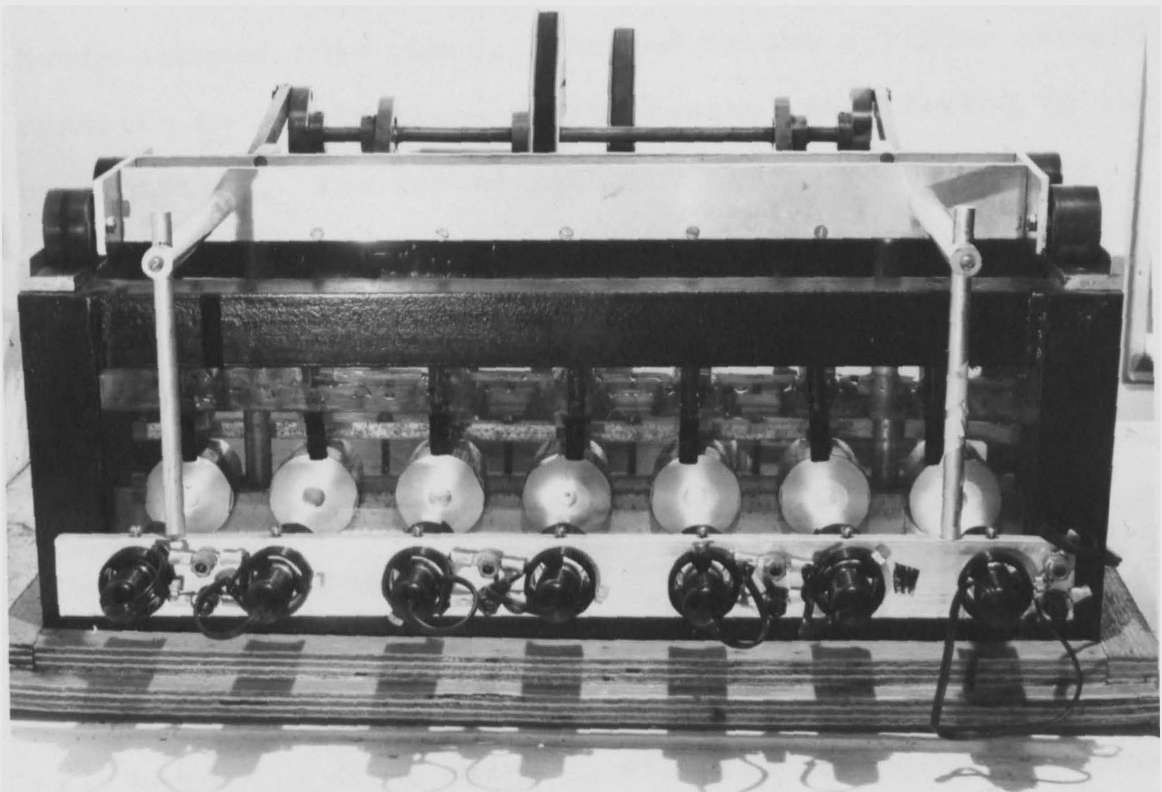

Fig. 6

made of 316 stainless steel. It has an internal volume of 8 cm^3 and contains a close fitting 3/8" diameter teflon piston. An "O" ring is fitted in a grove on the piston to form a seal. The cylinder is closed by cylinder heads on each end. A butt below the threaded portion of the cylinder head accommodates a 3/32" thick silicone rubber 'O" ring which forms a tight seal when the heads are in place. To prepare the cylinder, it was filled with medium and the heads screwed into place. The end of the cylinder assembly opposite to the location of the piston was attached to the needle valve. The vessel with the needle valve and the cylinder attached was then immersed in the 70° water bath for at least one hour. After cooling to room temperature the vessel was inoculated with cells and CO_2 added as before and pressure applied via the free piston cylinder assembly. It was then mounted in the shaker water bath and illuminated after the free piston cylinder was removed.

To apply pressure the free piston cylinder with vessel attached was connected to high pressure tubing leading to a hydraulic press (Fig. 7, 8). The tubing was attached to a stainless steel cylinder fitted with a plunger. The cylinder (Fig. 9 A) is located directly under the plunger (Fig. 9 B) so that pressure may be increased by raising the ram of the press which forces the plunger into the cylinder. When the cylinder is filled with liquid and the plunger is forced into the cylinder hydraulic pressure is transferred through the tubing to the pressure vessel or to the free

piston assembly. The cylinder and tubing are both easily filled by opening a high pressure needle valve with liquid from a reservoir (see Fig. 7 A) situated between the press and the end of the tubing where the pressure vessel is attached for pressure application. In this closed system the experimental fluid may serve as the hydraulic fluid. A pressure gauge indicating from zero to twenty thousand pounds per square inch (psi) or approximately 1,350 atm, is connected to the cylinder of the press but not in the path of the vessel. Once pressure is applied, the valve closed and the free piston cylinder removed the vessel is immersed in the water bath and illuminated as described above.

At the end of a pressure experiment the free piston cylinder which was re-filled with medium, reassembled and heated as before, was re-attached to the vessel and then to the press. To determine whether any pressure loss has occurred during the experimental period, the pressure was raised to the starting value, the needle valve opened and the gauge read.

Growth is determined for the cells grown both in the synchronous apparatus and in the pressure vessels according to several criteria. Initial and final cell concentration and size were measured using a Model B Coulter Counter (Coulter Electronics Inc., Hialeah, Florida). A Coulter Model J automatic cell-size distribution XY plotter is connected to the counter. The Model J Coulter plotter makes an automatic graphical record of cell numbers in the

Fig. 7 Hydraulic press showing the pressure gauge and the separatory funnel (A) used as a medium reservoir. The needle valve (B) placed between the tee (C) and the funnel allows the cylinder (D) and tubing to be completely filled with liquid while applying pressure.

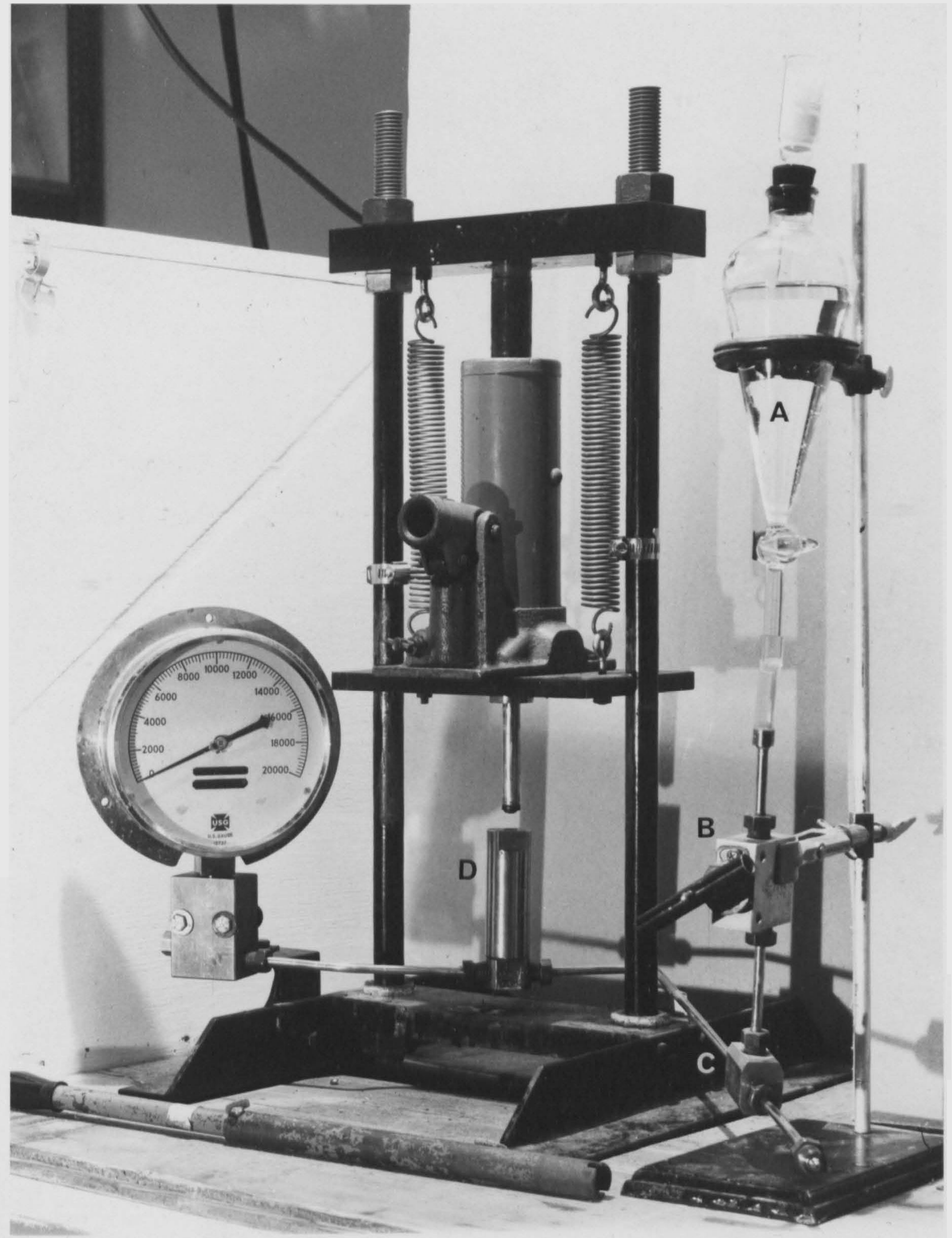

Fig. 7

Fig. 8 The assembled pressure vessel is shown attached directly to the high pressure tubing just prior to pressure application.

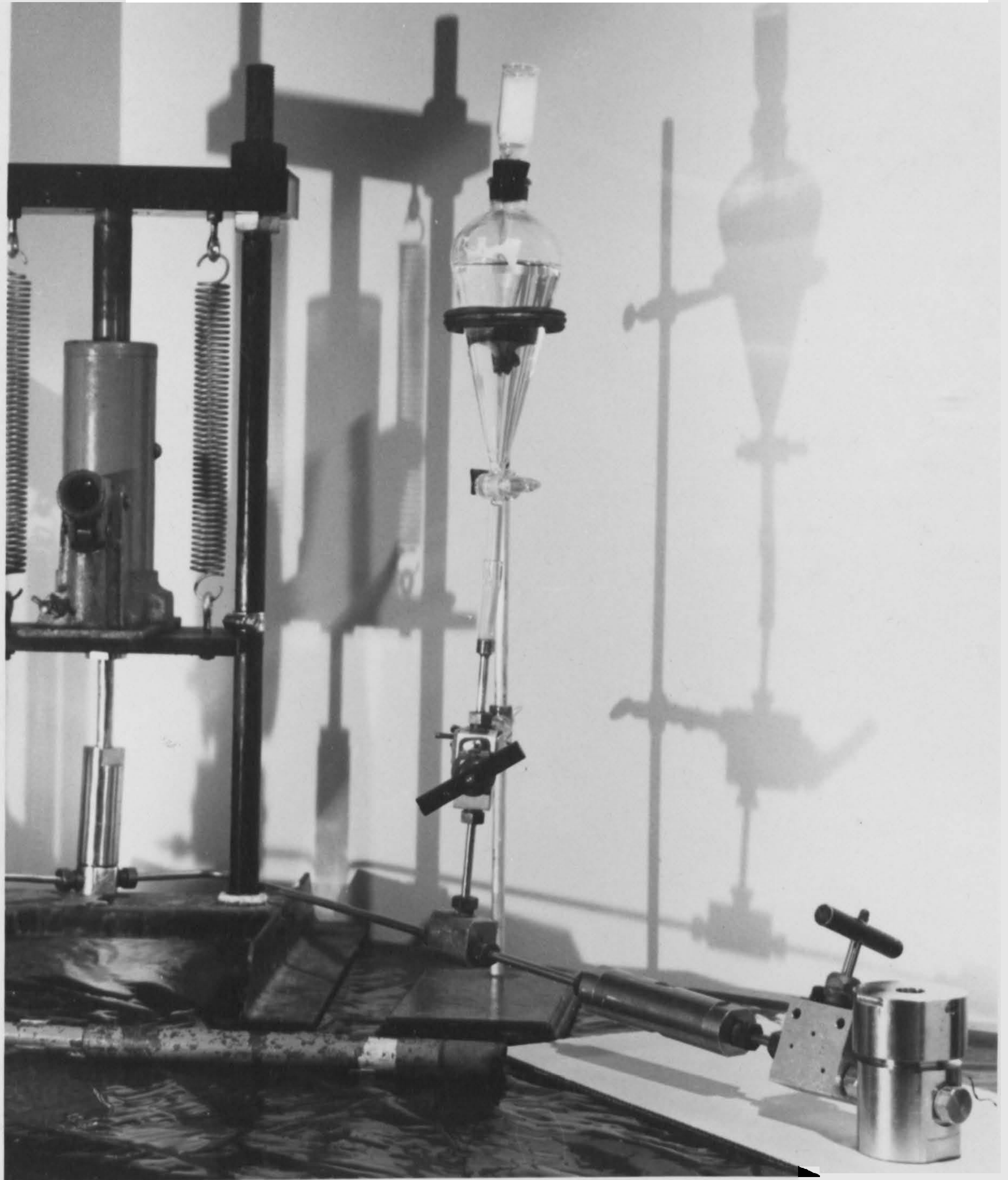

Fig. 8

Fig. 9 The cylinder (A) and the plunger (B) used for pressure application. As the ram of the press is raised the plunger is forced into the liquid filled cylinder thereby transferring hydraulic pressure to the vessel.

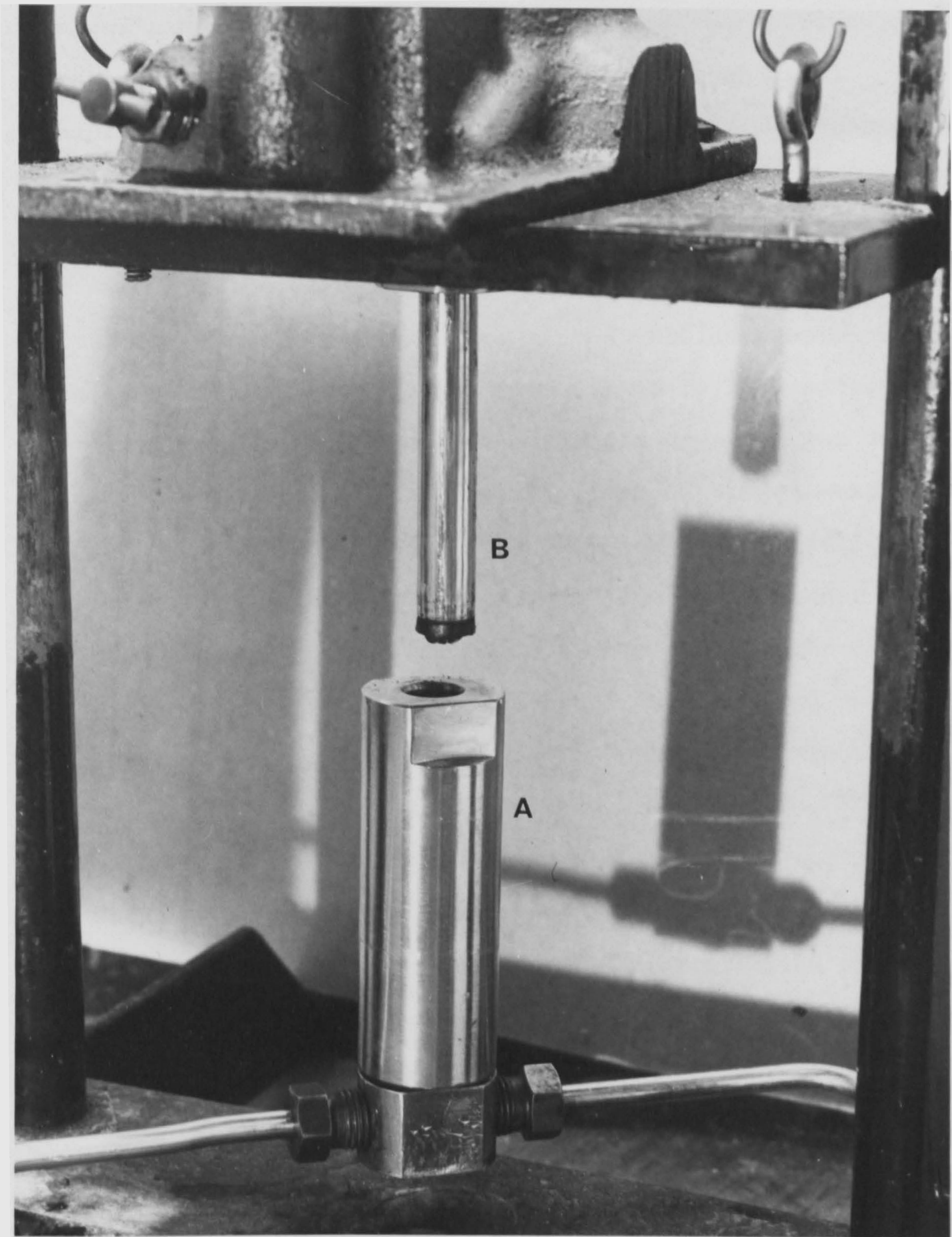

Fig. 9

form of a histogram of cell size distribution. As each sample is counted automatically for a standard time, the volume sampled will vary with the size of the aperture. To measure cell concentrations and sizes, 1 ml of algal suspension was pipetted into 49 ml of a 0.85% saline solution as the electrolyte, and the measurements made using a 50 μ aperture tube.

To compare dry weights of cells initially and after the growth period, 10 ml of the cell suspension were centrifuged at 2,500 to 3,000 rpm for 10 to 15 minutes until the supernatant was clear. The resulting pellet was washed at least three times in deionized glass distilled water and finally resuspended in distilled water. Of this cell suspension, 9 ml were pipetted into a dry pre-weighed aluminum dish and left for 24 hours in an oven at 105°. The dry weight was taken as $DW_c = W_{d+c} - W_d$, where DW_c represented the weight of the cells, W_{d+c} the weight of the dish containing the cells after drying and W_d as the weight of the dry dish. The dry weight of the cells at the end of the growth period divided by the initial dry weight gave a measure of the increase in dry weight. If W_f and W_i represent the dry weight of the cells at the end and beginning of the growth periods respectively, then the ratio of increase in dry weight may be expressed as:

$$\frac{W_f}{W_i}$$

The cells were assayed for proteins by a modified

Lowry method (29). The method is based on the development of colour when the reagents react with a protein sample. This colour is a result of the biuret reaction of protein with copper ion in an alkali solution and the reduction of the phosphomolybdicphosphotungstic reagent by the tryptophan and tyrosine amino acids in the treated protein sample. The method was modified in that the algal cells were heated for 10 minutes in boiling water (to break the cell walls) after the addition of the reagents except for the Folin-Ciocalteu reagent. This reagent was added after the samples had cooled to room temperature. The absorbance of the resulting coloured samples was measured using the Bausch and Lomb Spectronic 20 Spectrophotometer at 750 mμ with the reagent blank as reference. The concentration of proteins in the samples was read in gamma units per ml (γ/ml) by comparison with a bovine serum albumin standard calibration curve. Serum albumin controls of known concentration (50γ/0.2 ml) were routinely included in each experiment. The ratio of increase in protein concentration of the cells after growth over the starting culture was determined from the relationship $\frac{P_f}{P_i}$ where P_f and P_i represent the protein concentration at the end and at the beginning of the growth periods respectively.

III. Biological Oxygen Monitor

Rates of O_2 evolution by *Chlorella* cells in light were determined using a Model 53 Biological Oxygen Monitor, (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio). The instrument was calibrated and a probe test conducted each

day before use. A model SP-J2 Riken Denshi Strip Chart Recorder (Riken Denshi Co., Tokyo, Japan) attached to the Monitor recorded the changes in the O_2 concentration as detected by the electrodes in the probe of the instrument.

The monitor measures the O_2 concentration of the cell suspension in the sample tube by means of a platinum cathode. Changes in O_2 concentration then may be recorded. From changes in O_2 concentration the rates of O_2 evolution by the algal cells were calculated.

In a typical experiment, 3 ml of *Chlorella* cell suspension and the requisite magnetic stirrer were placed into a sample tube. After temperature equilibration to $25^{o} \pm 1^{o}$ and maintained by a Haake Model 5214 Constant Temperature Circulator, (Gebrueder Haake, KG, Berlin, West Germany), the probe was inserted in the sample tube expelling all air bubbles and the cells illuminated at 700 ft-c. Any O_2 evolved photosynthetically was recorded.

Light was completely excluded from the algal cells at the beginning of some experiments. When the cells became anaerobic due to respiration they were illuminated, the change in O_2 concentration recorded and the rate of O_2 evolution calculated. In other experiments known quantities of O_2 were supplied to the cells all in one operation or at various time intervals to study the changes of evolution rates as the cells were illuminated under different O_2 concentrations. The effect of O_2 concentration on O_2 evolution by the cells was then determined by calculating the

rate of evolved O_2 after making corrections for that consumed by the electrode.

Slight modifications of the above methods were used for some experiments. These modifications will be described where appropriate.

CHAPTER 3

SYNCHRONOUS CULTURES IN OPEN AND CLOSED SYSTEMS AND CELL DIVISION UNDER HYDROSTATIC PRESSURE

Synchronous cultures of Chlorella ellipsoidea Gerneck were established using the techniques of Tamiya and Morimura (55). Preliminary experiments indicated that the growth of the alga in the closed pressure vessels never attained that of cultures grown in the open system. In the synchronous apparatus, which represents an open system, the gases are in equilibrium with the atmosphere and therefore their concentrations remain relatively constant throughout the 24 hr cycle. In contrast, the pressure vessels represent a closed system since the gases in the vessels are not in equilibrium with the atmosphere but vary constantly during the course of an experiment. When algal cells are cultured in the closed system O_2 increases continuously in the light and CO_2 decreases; the reverse is true in the dark.

The intent of this study initially was to examine the effects of hydrostatic pressure on the physiology and morphology of algae grown synchronously. Consequently, it was essential that techniques for growing algae synchronously in a closed system be developed. Some such techniques were developed empirically. Variations in the growing conditions were applied to the closed system and where applicable, were also carried out in the open system. Attempts were made to obtain equal growth of the cultures in both systems. Results

were compared for the two systems.

Regardless of changes made in the growing conditions, the discrepancies in growth between the two systems persisted. Yet some of the changes had definite effects on the growth responses in either or both of the systems and are therefore reported here.

I. Variations In Growth Parameters

1. Media

A number of media (see appendix 1) were used to culture the algal cells in both systems (Table II) keeping other growth conditions (see Chapter 2) constant.

In the open system the n number and ratios of increase in dry weight, protein concentration and cell size were almost exactly 4 but only 2 or less in the closed system for Chlorella ellipsoidea and Scenedesmus obliquus. The n number of Chlorella pyrenoidosa in the open system fluctuated markedly and this species was not used for further experiments (Table III). Although Scenedesmus obliquus was readily synchronized in the open system it was not studied in detail in the closed system. All the experiments reported here used Chlorella ellipsoidea as the experimental organism. In the open system synchrony was easily maintained in Beijerincks's medium and it was used routinely in all experiments except as reported here.

2. Gases

Carbon was supplied in both systems as CO_2

TABLE II

A comparison of n number and ratios of increase in growth criteria between the open and closed systems of culturing *Chlorella ellipsoidea* in various media using a 14: 10 regime at 25° and 700 ft-c illumination.

		Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
System	Media	Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	Beijerinck	25.0	99.0	16.6	16.6	4.0	4.0	4.3
	Burr	25.0	98.0	16.6	16.6	3.9	3.9	4.1
	Bicarbonate	25.0	101.0	16.6	16.6	4.0	4.1	4.2
	Sorokin	25.0	99.0	16.6	16.6	4.0	3.9	4.0
	Tamiya	25.0	100.0	16.6	16.6	4.0	4.0	4.1
Closed	Beijerinck	25.0	49.0	16.6	16.6	2.0	2.1	2.3
	Burr	25.0	51.0	16.6	16.6	2.0	2.0	2.1
	Bicarbonate	25.0	52.0	16.6	16.5	2.1	2.0	2.1
	Sorokin	25.0	50.0	16.6	16.6	2.0	1.9	2.0
	Tamiya	25.0	51.0	16.6	16.6	2.0	2.1	2.2

TABLE III

The n numbers for algae grown synchronously under various light-dark regimes, temperature and light intensities.

Algae	Culture Conditions	n number
Chlorella pyrenoidosa (strain 211-8b)	16: $\overline{12}$ (30°, 900 ft-c) 16: $\overline{8}$ ($25-28^{\circ}$, 800 ft-c)	8, 16, 32 * 2, 4, 8, 16 *
Chlorella pyrenoidosa (Emerson's strain)	14: $\overline{10}$ (25°, 700 ft-c)	2, 4, 8, 16 **
Chlorella ellipsoidea	17: $\overline{15}$ (21°, 1,000 ft-c)	4.1, 4.3, 4.9 ***
Chlorella ellipsoidea (strain 211-1b)	14: $\overline{10}$ (25°, 700 ft-c)	4 **
Scenedesmus obliquus	14: $\overline{10}$ (25°, 1,000 ft-c)	45 *
Scenedesmus obliquus	14: $\overline{10}$ (25°, 700 ft-c)	4 **

Source: * Tamiya, H. (54)

** This work

*** Tamiya, H. (53)

or bicarbonate. In the open system 5% CO_2-air mixture was normally bubbled through the cultures and CO_2 gas was injected into the pressure vessels except where otherwise stated. The amount of CO_2 (4-6 ml/vessel) normally added was sufficient to supply carbon for four-fold growth. In some of the closed system experiments the amount of CO_2 added varied from 1 to 10 ml/vessel but other growth conditions were unchanged. When bicarbonate medium was used in the open system a 1% CO_2-air mixture was bubbled into the cultures since HCO_3^- at pH 6.8 is the main carbon source.

In the open system synchrony was maintained over repeated light-dark cycles. The n number was 4. Ratios of increase in dry weight, protein concentration and cell size were four-fold. On the other hand synchronous cultures were not maintained in the closed system. The n number and ratios of increase of the other growth criteria were 2 or less even in bicarbonate medium (Table IV). When Beijerinck's medium was used in the closed system there was inhibition of growth at both low (below 4 ml CO_2/vessel) and high (above 6 ml CO_2/vessel) amounts of CO_2 (Table V).

3. <u>Light-Dark Regime</u>

In the open system variations in the types of media or the carbon available appeared not to affect synchronous growth and do not explain the differences observed between the two systems. Experiments were conducted in which the 14: $\overline{10}$ light-dark regime was changed to 16: $\overline{8}$, 18: $\overline{6}$, 6: $\overline{18}$, 8: $\overline{16}$ and 10: $\overline{14}$ in both systems while keeping the other

growth parameters constant. In these experiments 6 ml of CO_2 was added to each vessel.

Table VI compares the results obtained under these various light-dark regimes. Whereas synchrony persisted in the open system, the ratios of increase in growth criteria remained 2 or less in the closed system. At 6:$\overline{18}$ the n number was 1 and the ratios of increase in the growth criteria were only slightly above 1.

4. Light Intensity

Experiments were conducted using various light intensities (Table VII). Light from individual lamps (see Fig. 6) used to illuminate the algal cultures was regulated by inserting metal discs with circular apertures of various diameters into the light path which furnished light intensities of from 15 to 700 ft-c. At first the light-dark regime, 14: $\overline{10}$, was used with the various light intensities. In some experiments the 14: $\overline{10}$ regime was changed as described in the previous section. In these experiments 700 ft-c was taken as the control since at that intensity in the open system synchrony was maintained. When the light intensity was less than 500 ft-c the n number and ratios of increase in dry weight, protein concentration and cell size were always below 2. At 700 ft-c the n number and ratios of increase in growth criteria were about 2. Regardless of the changes in the light-dark regimes the results in the closed system were the same as before, except at 8: $\overline{16}$ and 6: $\overline{18}$ and with 700 ft-c the n number was 1 and there were

TABLE IV

A comparison of n number and ratios of increase in growth criteria of *Chlorella ellipsoidea* cultured in both systems using bicarbonate and Beijerinck's media. CO_2 was bubbled in the open system but was injected into the vessels. Standard deviation for 30 experiments is shown.

System	Media	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	Beijerinck 5% CO_2-air mixture	25.0±2.0	99.0±1.0	16.1±0.3	16.0±0.5	4.1±0.2	4.0±0.2	4.2±0.4
	Bicarbonate 1% CO_2-air mixture	26.0±3.0	100.0±2.0	16.0±0.2	16.0±0.3	4.0±0.1	4.1±0.1	4.3±0.2
Closed	Beijerinck 4-6 ml CO_2/vessel	25.0±2.0	49.0±2.0	16.0±0.3	15.9±0.5	2.0±0.1	2.1±0.1	2.2±0.2
	Bicarbonate 4-6 ml CO_2/vessel	24.0±3.0	51.0±1.0	16.6±0.3	18.4±0.2	2.1±0.5	2.3±0.1	2.4±0.3

TABLE V

Growth responses of *Chlorella ellipsoidea* in the open and closed systems in Beijerinck's medium. The open system was bubbled with 5% CO_2-air mixture. Various amounts of CO_2 were added to the vessels. Standard deviation for 40 experiments is shown.

System	CO_2	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	5%-air	25.0±2.0	99.0±1.0	16.5±0.3	16.5±0.5	4.1±0.2	4.0±0.2	4.2±0.4
	ml CO_2 per vessel							
Closed	1	25.0±2.0	26.0±3.0	16.1±0.2	20.5±0.4	1.0±0.1	1.1±0.2	1.2±0.2
	2	25.0±2.0	24.0±2.0	16.1±0.2	21.4±0.3	1.0±0.2	1.6±0.2	1.3±0.2
	3	25.0±2.0	27.0±1.0	16.1±0.2	21.5±0.1	1.1±0.1	2.0±0.2	1.9±0.1
	4	25.0±2.0	49.0±3.0	16.1±0.2	18.3±0.2	2.0±0.1	2.1±0.1	2.1±0.2
	5	25.0±2.0	50.0±2.0	16.1±0.2	20.4±0.1	2.0±0.1	2.3±0.2	2.2±0.3
	6	25.0±2.0	51.0±3.0	16.1±0.2	16.3±0.1	2.1±0.1	2.4±0.3	2.4±0.1
	7	25.0±2.0	48.0±1.0	16.1±0.2	17.4±0.2	1.9±0.1	2.1±0.1	2.1±0.1
	8	25.0±2.0	[illegible]9.0±2.0	16.1±0.2	18.6±0.1	1.6±0.1	1.7±0.1	1.9±0.1
	9	25.0±2.0	29.0±1.0	16.1±0.2	18.6±0.2	1.2±0.1	1.5±0.1	1.7±0.2
	10	25.0±2.0	25.0±2.0	16.1±0.2	17.3±0.2	1.0±0.2	1.1±0.2	1.5±0.1

TABLE VI

Growth responses of _Chlorella ellipsoidea_ grown in the two systems in Beijerinck's medium at various light-dark regimes. Other growth conditions were identical for both systems.

System	Light-Dark Regime	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	6: $\overline{18}$	25.0	98.0	16.6	16.6	3.9	3.9	4.0
	8: $\overline{16}$	25.0	99.0	16.6	16.6	4.0	4.0	4.1
	10: $\overline{14}$	25.0	100.0	16.6	16.6	4.0	4.0	4.0
	14: $\overline{10}$	25.0	99.0	16.6	16.6	4.0	4.1	4.1
	16: $\overline{8}$	25.0	98.0	16.6	16.6	3.9	4.2	4.2
	18: $\overline{6}$	25.0	100.0	16.6	16.6	4.0	4.1	4.1
Closed	6: $\overline{18}$	21.0	22.0	16.6	20.3	1.0	1.1	1.3
	8: $\overline{16}$	23.0	46.0	16.6	16.6	2.0	1.6	1.8
	10: $\overline{14}$	25.0	47.0	16.6	16.9	1.9	1.9	2.0
	14: $\overline{10}$	25.0	49.0	16.6	18.2	2.0	2.1	2.3
	16: $\overline{8}$	25.0	47.0	16.6	17.7	1.9	2.0	2.0
	18: $\overline{6}$	24.0	51.0	16.6	18.3	2.1	2.3	2.5

TABLE VII

Growth responses of *Chlorella ellipsoidea* in the open and closed systems under various light intensities. Standard deviation for 10 experiments is shown.

System	Light Intensity ft-c	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	700	25.0±1.0	100.0±2.0	16.6	16.6	4.0±0.1	4.1±0.2	4.3±0.2
Closed	15	24.0±1.0	23.0±2.0	16.6±0.2	16.6±0.1	1.0	1.1±0.1	1.0±0.1
	31	24.0±1.0	24.0±1.0	16.6±0.2	16.6±0.1	1.0	1.1±0.1	1.1±0.1
	62	24.0±1.0	23.0±1.0	16.6±0.2	16.6±0.1	1.0	1.1±0.2	1.2±0.1
	125	24.0±1.0	24.0±1.0	16.6±0.2	18.2±0.1	1.0	1.4±0.2	1.3±0.1
	250	24.0±1.0	25.0±1.0	16.6±0.2	20.3±0.2	1.0	1.5±0.1	1.8±0.2
	500	24.0±1.0	35.0±1.0	16.6±0.2	21.2±0.1	1.5	1.8±0.1	1.9±0.1
	700	24.0±1.0	51.0±2.0	16.6±0.2	16.6±0.2	2.1	2.0±0.1	2.3±0.1

no increases in any of the growth criteria.

5. Nitrate-enriched Medium

Chlorella ellipsoidea cells utilize various elements in the medium. However, this assimilation does not necessarily keep pace with the photosynthetic assimilation of carbon (53). Throughout much of the life cycle the available nitrogen in the medium remains relatively constant, decreasing only slightly during cell growth. The availability of sufficient nitrogen becomes essential just prior to cell division (41,54).

To determine the effects of additional NO_3^-, cells were cultured in Beijerinck's medium with KNO_3 concentrations varying from 3.8×10^{-2} to 1.9×10^{-1} M in both systems (Table VIII). The open system was bubbled with 5% CO_2-air mixture as usual and 6 ml CO_2 was added to each vessel. In some experiments 3.6×10^{-3} M glycolate was also added but appeared to have no effect. In both systems a 14: $\overline{10}$ regime was used at a light intensity of 700 ft-c.

Synchrony in the open system was maintained as usual. In the closed system the n number and ratios of increase in dry weight, protein concentration, and cell size were below 2 at the various nitrate concentrations (Table VIII). Nitrate concentrations greater than those present in Beijerinck's medium did not significantly improve cell growth in the closed system.

TABLE VIII

A comparison between the n number and ratios of increase in the growth criteria of *Chlorella ellipsoidea* in the two systems at various NO_3-concentrations. The open system was bubbled with 5% CO_2-air mixture and 6 ml CO_2 injected into the closed system. Standard deviation for 4 experiments is shown.

System	KNO_3	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
	(mM)	Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	38.0	25.0±1.0	100.0±1.0	16.6	16.5	4.0±0.1	4.2±0.3	4.1±0.1
	95.0	25.0±1.0	101.0±3.0	16.6	16.5	4.0±0.2	4.2±0.2	4.1±0.2
	190.0	25.0±1.0	99.0±1.0	16.6	16.5	3.9±0.1	4.0±0.1	4.0±0.1
Closed	38.0	25.0±2.0	25.0±1.0	16.6	18.2±0.1	1.0	1.2±0.1	1.4±0.1
	95.0	25.0±1.0	25.0±1.0	16.6	19.1±0.1	1.0	1.1±0.1	1.5±0.1
	190.0	25.0±1.0	25.0±2.0	16.6	15.9±0.2	1.0	1.0±0.1	1.1±0.1

6. Glycolate Supplemented Medium

Glycolate may be excreted or assimilated by *Chlorella* cells depending upon the growth conditions of the cells (32). There is evidence that leaves utilize exogenously supplied glycolate to produce sucrose in a pathway involving glycine and serine (10,11). Fogg (12) and Nalewajko et al (40) have shown that the addition of 1 mg/l of glycolate improved growth in light-limited cultures by decreasing the lag period of growth. It was reported by Nalewajko et al (40) that there was rapid equilibration between intracellular and extracellular glycolate suggesting that when photosynthesis begins in fresh medium there must first be a lag while an equilibrium concentration of glycolic acid is established. Only when this equilibrium is achieved are the products of carbon fixation available for growth. Similar results have been reported by Sen and Fogg (43).

Experiments were conducted in which glycolate concentrations varying from 3.6 x 10^{-3} M to 10.7 x 10^{-3} M were supplied to the cells in both systems. Both Beijerinck's and bicarbonate media were used; cell concentrations were between 1.8 x 10^{7} and 2.6 x 10^{7} cells/ml. Light intensity was 700 ft-c in both the open and closed systems. Other growth conditions were unchanged.

The results obtained with *Chlorella ellipsoidea* cells grown in both systems and at various initial cell numbers and glycolate concentrations are shown in

TABLE IX

The n number and ratios of increase in growth criteria of *Chlorella ellipsoidea* grown in both systems in Beijerinck's medium at various concentrations of glycolate and dilute cell concentrations.

System	Glycolate (mM)	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	0.0	18.0	71.0	16.6	16.6	3.9	4.0	3.9
	3.6	19.0	77.0	16.6	16.6	4.0	4.1	4.0
	5.4	21.0	83.0	16.6	16.6	3.9	4.1	4.3
	7.1	20.0	81.0	16.6	16.6	4.1	4.2	4.2
	10.7	20.0	79.0	16.6	16.6	3.9	3.9	4.0
Closed	0.0	18.0	34.0	16.6	18.2	1.9	2.0	2.1
	3.6	19.0	38.0	16.6	16.6/48.0	2.0	1.9	2.2
	5.4	21.0	21.0	16.6	16.6/53.0	1.0	2.1	2.4
	7.1	21.0	21.0	16.6	16.6/45.0	1.0	2.0	2.2
	10.7	20.0	21.0	16.6	16.6/43.0	1.0	1.9	2.0

TABLE X

The n number and ratios of increase in growth criteria of *Chlorella ellipsoidea* grown in both systems in Beijerinck's medium with various concentrations of glycolate

System	Glycolate (mM)	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	0.0	26.0	103.0	16.6	16.6	4.0	4.1	4.3
	3.6	26.0	102.0	16.6	16.6	4.0	4.2	4.3
	5.4	25.0	101.0	16.6	16.6	4.0	4.1	4.1
	7.1	24.0	101.0	16.6	16.6	4.0	4.1	4.2
	10.7	25.0	99.0	16.6	16.6	3.9	3.9	3.9
Closed	0.0	26.0	48.0	16.6	16.6	1.9	2.1	2.3
	3.6	25.0	49.0	16.6	18.2/53.0	1.9	2.1	2.4
	5.4	24.0	25.0	16.6	16.6/45.0	1.1	2.0	2.3
	7.1	23.0	23.0	16.6	16.6/50.0	1.0	2.1	2.5
	10.7	24.0	24.0	16.6	16.6/48.0	1.0	2.1	2.2

Tables IX and X. In the open system regardless of the medium and the glycolate concentrations synchrony was maintained.

Similar results were not obtained in the closed system. Without glycolate the n number and ratios of increase in growth criteria were 2 or below. When the initial cell number varied from 1.8 (Table IX) to 2.6 x 10^7 (Table X) the n number and ratios of increase in growth criteria were 2 or below in the closed system. At both initial cell number and at the various glycolate concentrations used there were cells of 2 different sizes. This result indicates that glycolate stimulated an increase in cell size. Nevertheless, even extending the light period did not result in further cell growth.

II. Examination Of Other Parameters Which May Affect Synchronous Growth In The Closed System

1. Toxicity Of Pressure Vessels

The possibility that toxic substances from the metal pressure vessels might inhibit cell growth was examined by using the vessels as an open system.

Cells were cultured in the open body-half of the pressure vessels in Beijerinck's medium. A 5% CO_2-air mixture was bubbled in the culture and the cells illuminated with 700 ft-c through the window which in this position was in the bottom of the vessel. The 14: $\overline{10}$ light-dark regime was used.

Synchrony was maintained in the open vessel. The n number and ratios of increase in all the growth

criteria were 4.0.

In a different experiment the pressure vessels were assembled completely and the experiment conducted in the usual way except that the valve was removed. Two capilliary tubes were inserted through the opening where the valve was previously attached. One tube carried 5% CO_2-air mixture to the culture, the other functioned as an outlet. In this experiment synchrony was maintained and the results were identical to those found in the open system.

It was now clear that synchronous cultures were maintained in any open system regardless of whether this consisted of the synchronous apparatus or involved the use of the pressure vessels. Sustained synchronous growth was never maintained in the closed vessels. The possibility was examined that the inhibition of photosynthesis by high O_2 concentrations (Warburg Effect) might be interfering with synchronous growth. In the course of a closed system experiment in the light the O_2 concentration does increase continuously.

2. Effects Of Increased O_2 In Cultures Grown In The Open System

To determine whether there was a relation between O_2 produced photosynthetically and trapped in the closed system and the inhibition of cell growth, experiments were conducted using cultures in the open system by subjecting the cultures to high O_2 concentrations. The cultures in the open system were bubbled with 50% CO_2-50% O_2 or 5% CO_2-95% O_2. Controls were supplied with 5% CO_2-air mixture. All

TABLE XI

The effects of high O_2 concentrations on the various growth criteria of synchronously grown *Chlorella ellipsoidea* cells in the open system. Standard deviation for 4 experiments is shown.

CO_2/O_2	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
	Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
5% CO_2-air	25.0±1.0	99.0±2.0	16.2±0.3	16.4±0.2	4.0±0.1	4.1±0.2	4.2±0.1
50% CO_2-50% O_2	25.0±1.0	51.0±1.0	16.6±0.3	16.6±0.4	2.0±0.1	2.1±0.1	2.3±0.3
5% CO_2-95% O_2	24.0±2.0	25.0±1.0	16.6±0.2	16.6±0.1	1.0±0.1	1.1±0.2	1.2±0.2

other growing conditions were identical to a typical open system experiment.

Table XI compares the results obtained for the n number and ratios of increase in growth criteria at the various gas mixtures. When the gas mixture was 5% CO_2-air (21% O_2 in air by volume) synchronous growth was obtained. At 50% CO_2-50% O_2 the n number and ratios of increase in dry weight, protein concentration and cell size were 2 but were only 1 at 5% CO_2-95% O_2. These results demonstrated an inhibitory effect of high O_2 concentration on cell growth and division.

3. The Inhibition Of Photosynthesis By O_2 – The Warburg Effect

Additional confirmatory evidence was provided on the effect of high O_2 concentrations on photosynthesis. Experiments were conducted on the effects of various O_2 concentrations on the relative rate of O_2 evolution by Chlorella ellipsoidea using the Biological Oxygen Monitor. The Monitor was calibrated as described in Chapter 2. In all experiments 5 µl CO_2 was added to the cultures initially. When a steady concentration of O_2 was observed indicating that O_2 evolution had ceased, additional CO_2, usually 5 µl CO_2, was supplied to the cultures.

The relative rates of O_2 evolution were calculated from the slope of the O_2 concentration curves. Determinations began when the cells started to photosynthesize with an initial O_2 concentration of either 0 or 20%. To

achieve zero O_2 concentration the cell suspension was first aspirated. Complete anaerobiosis was achieved by permitting the cells to respire in the dark. In some experiments 10 μl O_2 was injected into the cell sample at intervals to determine the rates of O_2 evolution at various concentrations of added O_2.

Figure 10 shows the results of a typical experiment in which photosynthesis started at 20% O_2. The initial rate at 20% O_2 was arbitrarily designated as 100. It was found that as photosynthetic periods progressed and O_2 concentrations increased, the relative rates of O_2 evolution decreased. When CO_2 was added to the cultures the responses were similar. The relative rate of O_2 evolution decreased by more than one-half at 50% O_2 within an hour and continued to decrease with time.

Relative O_2 evolution rates determined when O_2 was added to the sample tubes (Fig. 11) decreased with increasing O_2 concentrations. The relative rate of O_2 evolution was almost zero when 100 μl O_2 had been added.

The effects of increases in O_2 concentration resulting from photosynthesis on the relative rates of O_2 evolution starting at zero O_2 are shown in Figure 12. The initial rate at zero O_2 was arbitrarily taken as 100. Figure 12 shows that within 20 min the relative rate of O_2 evolution decreased by 50% at only 15% O_2 saturation. Initially, the rate decreased rapidly but the decrease became slower in time.

Fig. 10 The time course of change in the relative rates of O_2 evolution of *Chlorella ellipsoidea* at various O_2 concentrations. Cell concentration was 2.5×10^7 cells/ml illuminated with 700 ft-c. 5 µl of CO_2 was added at the beginning of the experiment and cells allowed to photosynthesize. Numbers in brackets represent percentage of O_2 saturation. The rate of O_2 evolution was calculated starting at 20% O_2 and this value arbitrarily designated as 100.

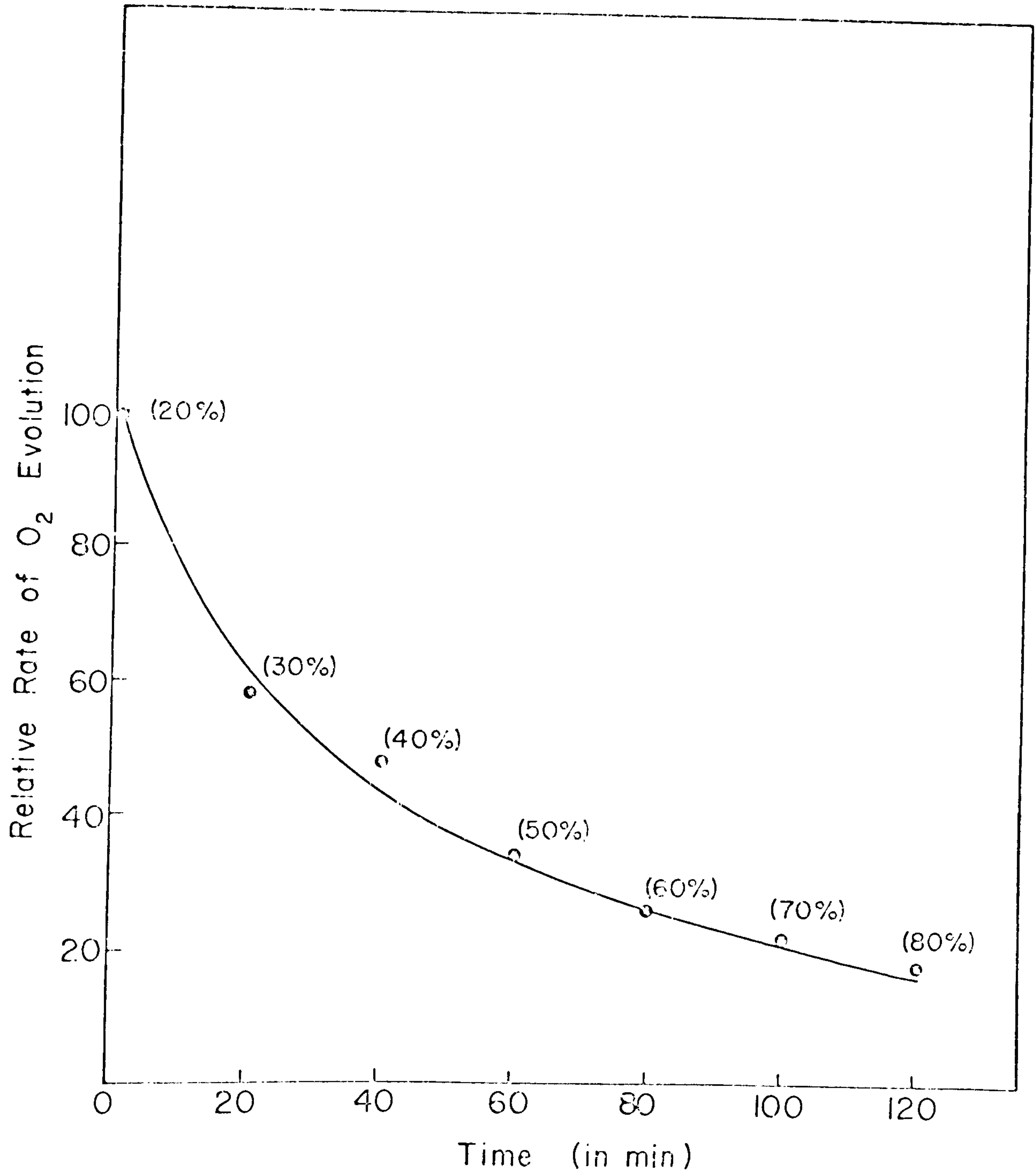

Fig. 10

Fig. 11. The relative rates of O_2 evolution of *Chlorella ellipsoidea* at various O_2 concentrations. Cell concentration was 2.5×10^7 cells/ml illuminated with 700 ft-c. 5 µl CO_2 was added initially and 10 µl O_2 supplied at 10 min intervals. Additional CO_2, usually 5 µl, was supplied to the cells at 30 min intervals. The rates of O_2 evolution were calculated starting at 20% O_2 and this value arbitrarily designated as 100.

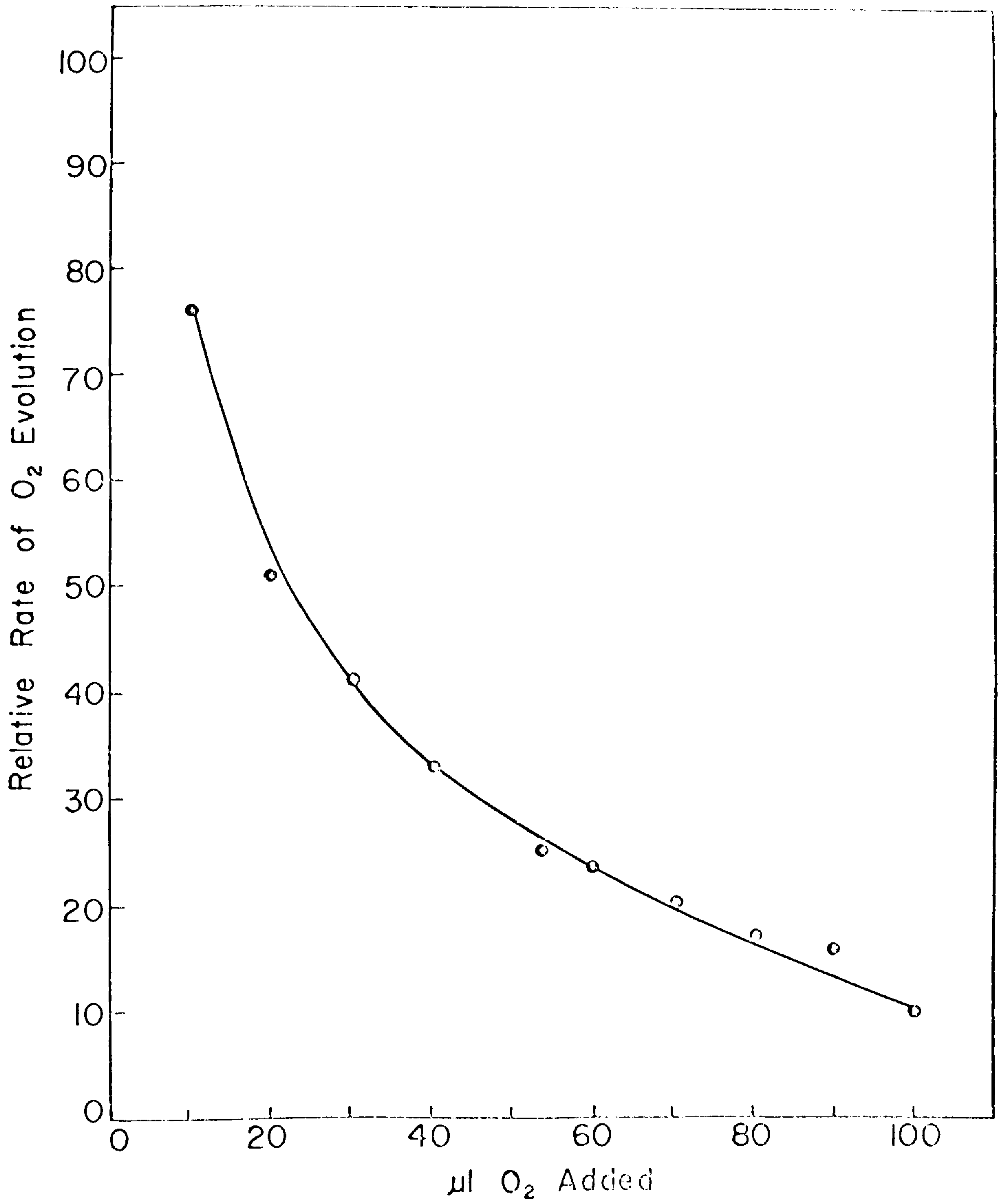

Fig. 11

Fig. 12 The time course of the relative rates of O_2 evolution of *Chlorella ellipsoidea* starting at zero O_2. Cell concentration was 2.5×10^7 cells/ml illuminated at 700 ft-c. 5 μl CO_2 was added initially and cells allowed to photosynthesize. Numbers in brackets represent the percentage of O_2 saturation. The rate at zero O_2 was arbitrarily taken as 100.

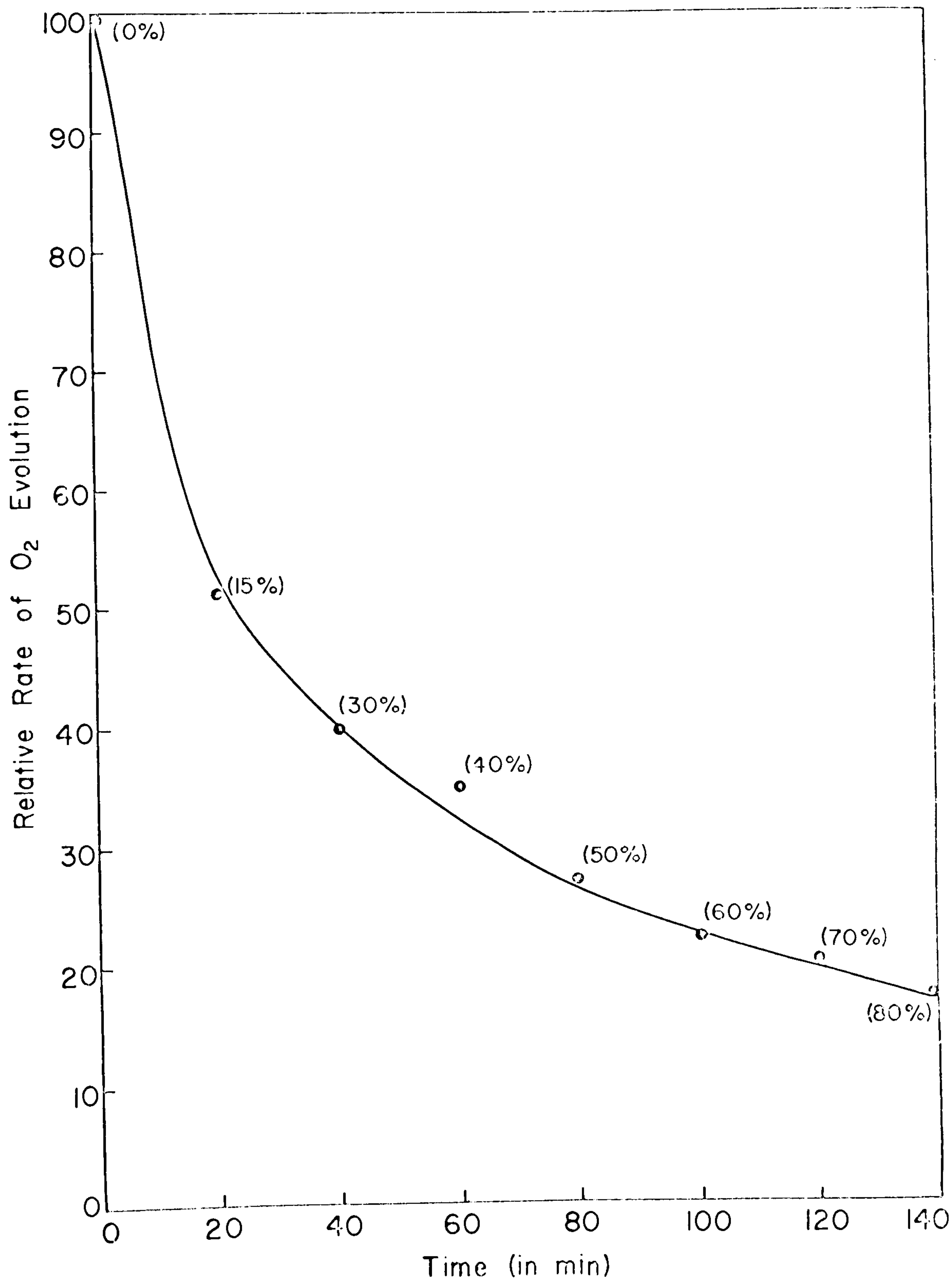

Fig. 12

When the rate of O_2 evolution was plotted against added O_2 (Fig. 13) the rate decreased as added O_2 amounts increased. At 100 µl O_2 the relative rate of O_2 evolution was almost zero. These results were unaffected by adding CO_2 to the sample at 30 min time intervals.

These results indicating the effect of O_2 on photosynthesis and those obtained in the open system when cells were cultured under high O_2 concentrations suggest a strong influence of the Warburg Effect on synchronous growth. It appears highly probable that this effect inhibited cell growth in the closed system.

III. Addition Of Reducing Agents

In a closed system it is extremely difficult to control O_2 concentrations without opening the vessels. Photosynthetic rates could not be measured under pressure in the closed system. Substances could however be added to the cultures to control O_2 concentration or reduce its effect.

Ascorbate and dithiothreitol were added to the medium in the closed system in attempts to control O_2 effects.

1. Ascorbate

These experiments were conducted in the usual way. Ascorbate was added in concentrations of from 7.2×10^{-3} M to 5.4×10^{-1} M.

In the open system synchrony was maintained at all concentrations of ascorbate. The n number remained near 1 and ratios of increase in the growth criteria of cells

Fig. 13. The relative rates of O_2 evolution of *Chlorella ellipsoidea* at various O_2 concentrations. Cell concentration was 2.5×10^7 cells/ml illuminated with 700 ft-c. 5 µl CO_2 was added initially and at 30 min intervals. 10 µl of O_2 was added at 10 min intervals. The rate of O_2 evolution was calculated starting at zero O_2 and this value was arbitrarily set at 100.

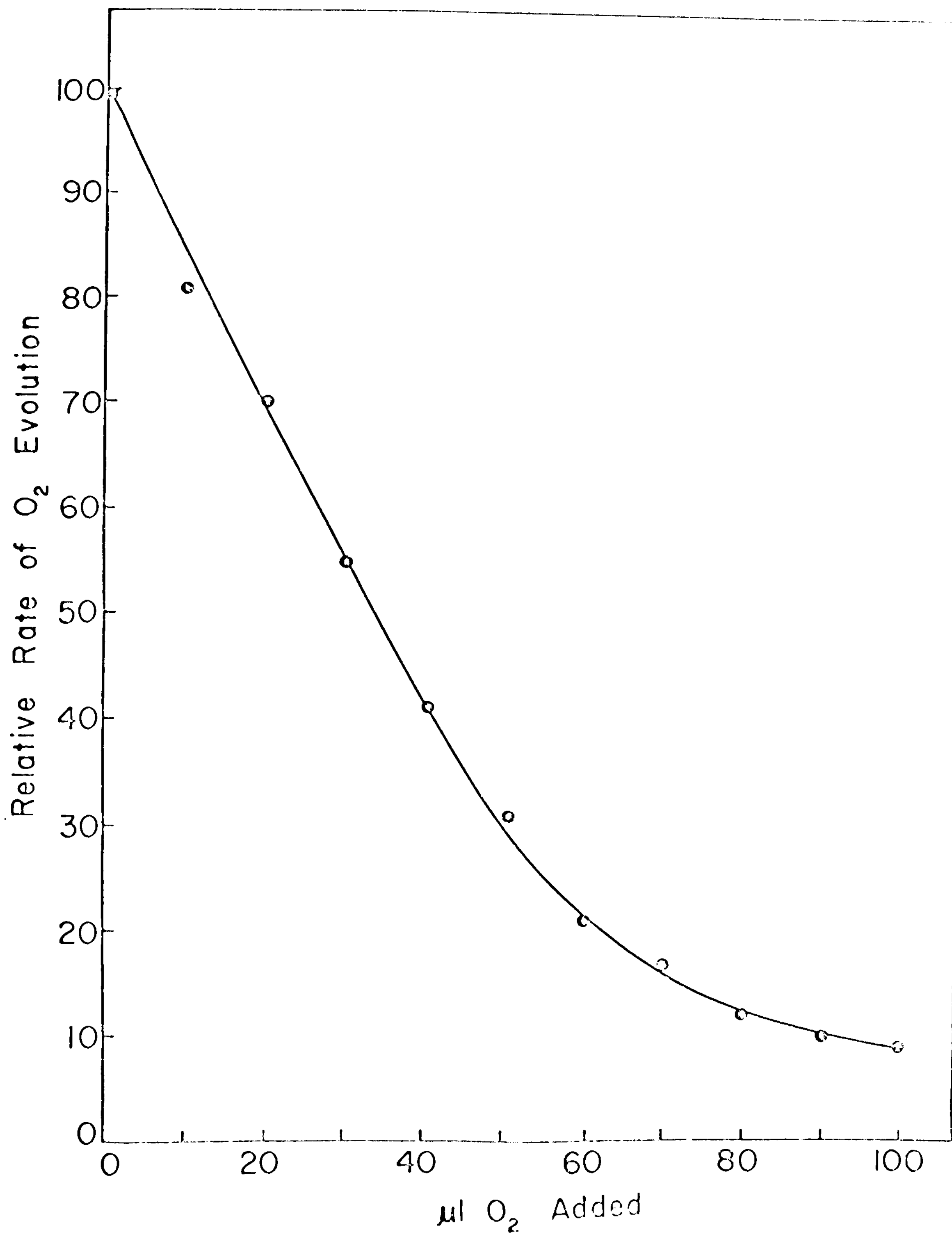

Fig.13

TABLE XII

The n number and ratios of increase in dry weight and protein of *Chlorella ellipsoidea* at various ascorbate concentrations in the open and closed systems.

System	Ascorbate (mM)	Cell Number/ml (in millions)		Cell Size (μ^3)		Ratios of Increase In		
		Initial	Final	Initial	Final	Cell Number (n number)	Dry Wt	Protein
Open	7.2	24.0	97.0	16.6	16.5	4.0	4.1	4.2
	180.0	23.0	94.0	16.6	16.6	4.0	4.0	4.1
	540.0	24.0	96.0	16.6	16.6	3.8	3.9	4.0
Closed	7.2	24.0	27.0	16.6	16.6	1.1	1.2	1.1
	180.0	23.0	24.0	16.6	16.6	1.1	1.4	1.3
	540.0	21.0	21.0	16.6	16.6	1.0	1.0	1.1

cultured in the closed system were below 2 (Table XII).

2. Dithiothreitol

Again, all these experiments were carried out in the usual way except that dithiothreitol at 3.6×10^{-3} and 7.2×10^{-3} M concentrations were added to the cultures.

Synchrony in the open system was unaffected. However, dithiothreitol interferes with the Lowry (29) method of protein determination and it was not possible to measure protein concentration by this method. In the closed system the n number was 1 and the ratio of increase of growth criteria were below 2 (Table XIII).

Dithiothreitol concentrations above 7.2×10^{-3} M had a slight effect on the n number and ratio of dry weight increase in the open system therefore higher concentrations were not used. At 3.6×10^{-3} M dithiothreitol had no effect on cell growth. Consequently lower concentrations were not used.

IV. Hydrostatic Pressure Effects On Cell Division

Even though synchronous cultures could not be maintained in the closed system it was possible to study the effects of hydrostatic pressure on cell division.

Chlorella ellipsoidea cells were harvested from cultures grown in the synchronous apparatus at the end of the 14 hr light period and were then subjected to pressure in the closed vessels in the dark at various O_2 concentrations. O_2 in 0.2 ml increments was injected into the vessels to a

TABLE XIII

The n number and ratios of increase in dry weight and cell size of *Chlorella ellipsoidea* at two concentrations of dithiothreitol (DTT).

System	DTT (mM)	Cell Number/ml (in millions)		Ratios of Increase In	
		Initial	Final	Cell Number (n number)	Dry Wt
Open	3.6	26.0	103.0	4.0	4.1
	7.2	26.0	95.0	3.6	3.7
Closed	3.6	25.0	28.0	1.1	1.6
	7.2	25.0	27.0	1.1	1.2

to a maximum of 1.6 ml of O_2. Pressures of up to 465 atm were applied, vessels darkened and then immersed in the shaker water bath (see Fig. 5) for 10 hr. The usual cell growth criteria were determined at the beginning and end of each 10 hr dark period.

Figure 14 shows the effect of pressure on the n number. At atmospheric pressure with from 0.4 to 1.2 ml O_2 added the n number was 4. At pressures of up to 200 atm with O_2 as high as 1.0 ml O_2 per vessel the n number remained at approximately 4. Pressures of more than 200 atm reduced the n number at all O_2 concentrations. At 330 atm and above there was no cell division (Fig. 15).

The O_2 concentration influenced the n number under pressure in various ways. If no O_2 was supplied to the cells they failed to divide. Low O_2 permitted some divisions when pressure was not inhibitory, but for n to equal 4 a minimum of 0.4 ml of O_2 was required (Fig. 14).

Figure 14 indicates that if there is sufficient O_2 in the closed system (0.4-1.2 ml/vessel) an n number of 4 is achieved under pressures of up to 200 atm. High O_2 concentrations (above 1.2 ml/vessel) reduced the number of cell divisions at any pressure (Fig. 14).

Total protein content and dry weight at the end of any of these experiments were essentially the same as the initial values regardless of whether or not cell division occurred (Table XIV).

Fig. 14 Cell division of synchronous cultures of *Chlorella ellipsoidea* grown in the open system for 14 hr and exposed to various hydrostatic pressures under different O_2 concentrations. The duration of each experiment was 10 hr in the dark.

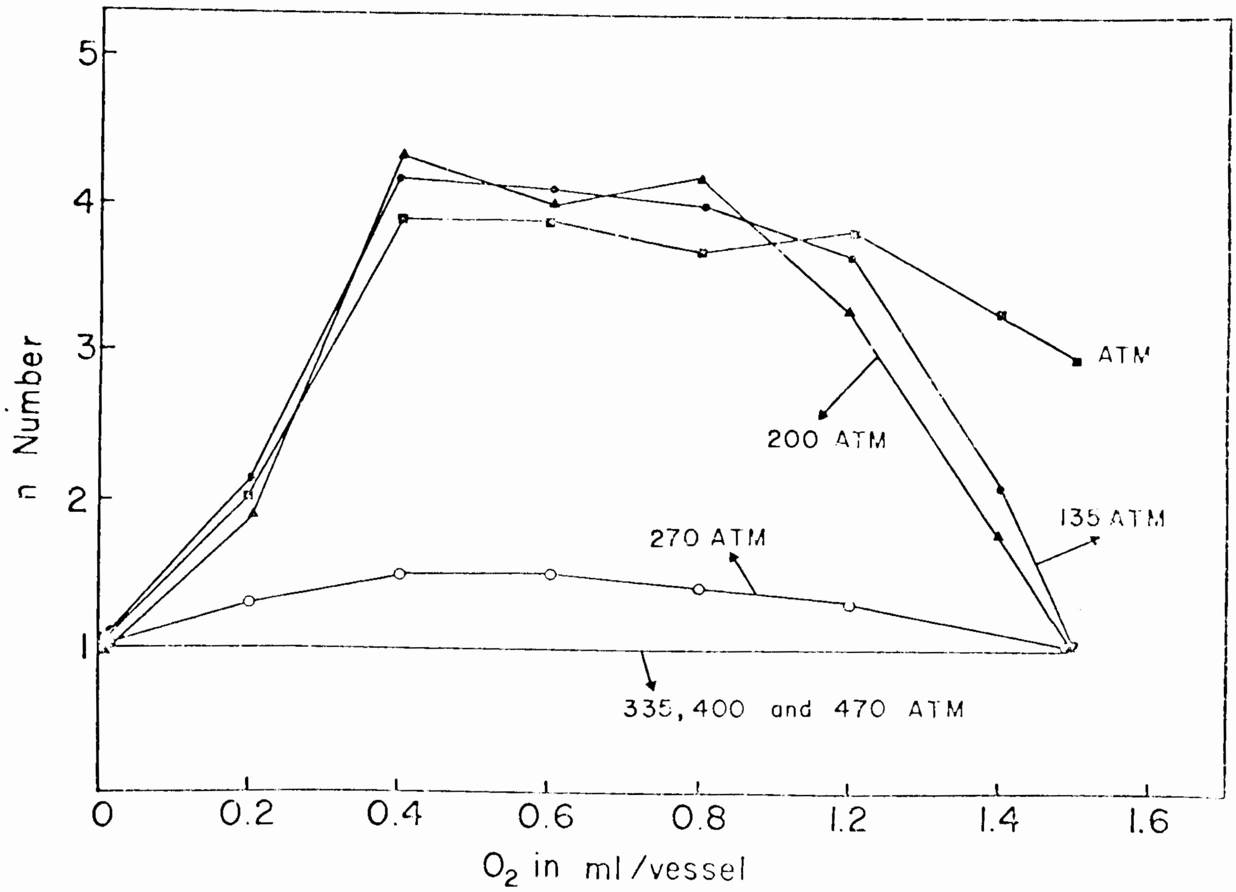

Fig. 14

Fig. 15 Cell division of _Chlorella ellipsoidea_ under hydrostatic pressures and various O_2 con-centrations in the dark for 10 hr. Each symbol represents a specific amount of O_2 added to each vessel. (X = 0.4 ml O_2/vessel, ○ = 0.8 ml O_2/vessel, ○ = 1.0 ml O_2/vessel, □ = 1.2 ml O_2/vessel, and + = 1.6 ml O_2/vessel).

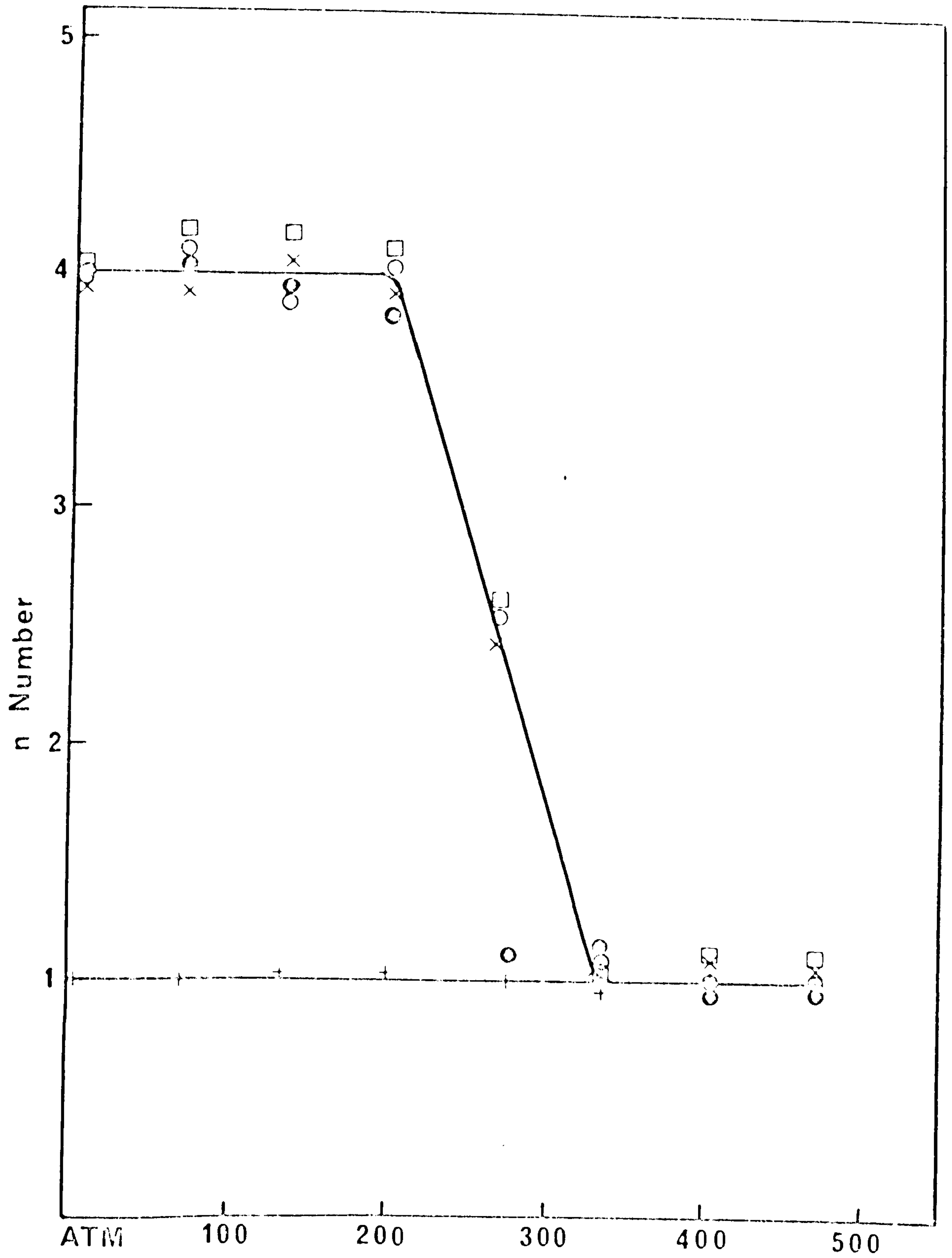

Fig. 15

TABLE XIV

Dry weight and protein concentration at the beginning and end of a typical pressure experiment on cell division of <u>Chlorella ellipsoidea</u>.

Hydrostatic Pressure (in atm)	Dry Weight (in mg)		Protein Concentration (in γ units/ml)	
	Initial	Final	Initial	Final
1	3.9	3.8	260.0	268.0
200	4.2	4.1	285.0	280.0
400	3.9	3.8	252.0	258.0
600	4.1	4.0	260.0	265.0

V. Discussion

Chlorella ellipsoidea has been physiologically well defined (53, 54). Synchronous cultures of this alga were used for this research. This has the advantage in that the life history of each cell is known and cells at any stage in the life cycle may be used for experiments. Indeed, the use of large populations of synchronized algal cells represent an enormously amplified model of a single cell which may be used to investigate various events taking place during the life cycle (54).

In this study synchronous cultures were established for use as controls for similar cultures in the pressure vessels. It was therefore necessary to have information on some of the metabolic activities of synchronous cultures grown in the conventional way.

The effects of hydrostatic pressure on some of the metabolic activities of bacteria and some algae are well known (see Chapter 2). This work was undertaken to investigate some of those metabolic processes using synchronous cultures grown under pressure. Although most of the pressure research reported has been done on bacteria, it is highly probable that similar pressure effects are to be found in algae. While pressure effects on algal photosynthesis have been reported (59) it became of interest to learn what effects growth under pressure might have in producing photosynthetic variants of a photoautotrophic organism. The use of synchronous cultures grown under pressure could also enhance

the understanding of pressure effects on the physiology and biochemistry of algae.

Whereas synchronous cultures were easily maintained in the open system, except when cells were grown under high partial pressures of O_2 (50-95% O_2) (see Table XI), various alterations in the environmental parameters of media, gases, light-dark regimes, light intensity and additional nitrate had little effect on the open system cultures and did not improve cell growth in the closed system significantly.

Glycolate added to cultures in the closed system appeared to stimulate cell growth somewhat. The excretion of glycolate by Chlorella cells during photosynthesis is reported in the literature (56). Miller et al (32) found that glycolate excretion was influenced by the partial pressures of CO_2 and O_2. These authors found that maximum excretion of glycolate occurred at 0.2% CO_2 in air, followed by a rapid decrease at a CO_2 concentration as high as used in this study. Tolbert and Zill (56) observed that glycolate was not excreted if Chlorella cells were gased with a mixture of CO_2 and N_2. Some observations are reported on the utilization of added glycolate by Chlorella cells (32, 40, 43). The uptake of glycolate appears to be controlled by the photosynthetic production of glycolate. Nalewajko et al (40) and Sen and Fogg (43) reported that low concentrations of exogenously supplied glycolate to dilute Chlorella cultures at light limiting conditions shortened the duration of the growth lag phase. It is difficult to explain the results reported here

in terms of the above findings.

Of all the substances added to the medium in the closed system glycolate was the only stimulatory one. Glycolate could serve as a source of reduced carbon in which case the cells could grow heterotrophically even though illuminated and therefore evolve no or limited amounts of O_2. However, due to the complex relation of glycolate to the metabolism of the cells it remains highly uncertain as to how this stimulation came about.

At the end of the 24 hr growth cycle in the pressure vessels there were cells of two different sizes (see Tables IX and X). It is not known whether this resulted from growth of only some of the cells and division of part of these or of some growth of all the cells and division of some of these. Since these cells are presumed to be clonal, growth and division of only part of the population seems unlikely. Consequently, the possibility that all the cells grew and some divided is favoured. Microscopic examination revealed that the large cells contained daughter cells.

Tables IX and X show that 3.6 x 10^3 M glycolate added to the cultures in the closed system produced an n number of 2 and the ratios of increase in growth criteria were near 2. The ratio of divided and undivided cells was not determined but a reasonable possibility is that 1/3 of the cells divided into 4 autospores while 2/3 did not divide. Hence an initial concentration of 2.5 x 10^7 cells/ml of which 1/3 dividing into 4 would produce 3.3 x 10^7 cells/ml to which

adding the remaining undivided cells ($2.5 \times 10^7 - 0.83 \times 10^7$) would give a total of 5.0×10^7 cells/ml or an n number of 2. The ratio of small to large cells would be 3.3:1.7 or about 2:1.

Clearly the difficulty in maintaining synchronous cultures results from the differences between the open and closed systems. Results obtained in the synchronous apparatus and when the pressure vessels were used as open systems are identical.

High concentrations of O_2 photosynthetically evolved appeared to be the major obstacle to the maintenance of synchronous cultures in the pressure vessels. Despite the various changes made in the growing conditions in the closed system the Warburg Effect was not overcome. The results of a number of experiments conducted suggest that there are certain minimal growing conditions (initial cell number of 2.5×10^7/ml, 700 ft-c, 6 ml CO_2/vessel, 3.6×10^3 M glycolate 14: 10 regime and 25°) in the closed system under which maximum growth and/or cell division occurred.

Synchronous cultures could probably be maintained in the closed system provided the photosynthetically evolved O_2 was removed. The addition of ascorbate and dithiothreitol as reducing agents was of little effect. Nevertheless, the studies on the Warburg Effect using the Biological Oxygen Monitor have indicated a possible means of maintaining synchronous cultures in a closed system. Figure 12 indicates a high rate of O_2 evolution starting at zero O_2 concentration.

This rate decreased with time and increasing O_2 concentration, but remained relatively high for an hour until the O_2 concentration had reached 40% of saturation. The cell concentration and light intensity were the same as in the closed vessels (2.5×10^7 cells/ml and 700 ft-c respectively). If initial cell concentration was decreased sufficiently so that O_2 evolved by the cells did not result in O_2 concentrations high enough to inhibit photosynthesis to the extent that enough metabolites are not produced to permit four-fold increases in the growth criteria, the Warburg Effect might be overcome. Dilution of the inocula by a factor of 10-15 should accomplish this. Such a dilution would result in far more light being absorbed by individual cells. Since it is known that excess light can be lethal, the light intensity would have to be adjusted accordingly. To pursue this investigation of pressure effects on synchronous cultures the relationship between the Warburg Effect, light intensity and cell concentration should be examined.

Hydrostatic pressure and O_2 concentration clearly interact to influence cell division of _Chlorella ellipsoidea_. Cells do not divide unless sufficient O_2 is present even at atmospheric pressure in the closed system (see Fig. 14). It is important to make a distinction between the open and closed systems. In a closed system as the cells respire the O_2 concentration decreases. The initial amount of O_2 supplied to the vessels must be sufficient for respiration and to allow cell division to proceed to completion. On the

other hand, the O_2 concentration in an open system remains constant and does not limit division. The decreased cell division in cultures with less than 0.4 ml O_2/vessel added to pressures from 1 to 200 atm is probably due to inadequate O_2 (see Fig. 14). There is an O_2 concentration range (0.4-1.2 ml/vessel) at which cell division occurs up to 335 atm (see Fig. 14 and 15). When the O_2 concentration increased above 1.2 ml/vessel cell division is inhibited at atmospheric pressure and more so at higher pressures. Regardless of the amount of O_2 added to the vessel cell division did not occur above 335 atm (see Fig. 15). An explanation for the inhibition of cell division by O_2 in the dark cannot be suggested. The effect probably contributes to the inhibition of division by cells grown in the closed system in the light.

In the open system synchronous division was completed within 2 hr after the start of the 10 hr dark period (Fig. 16). The time course of cell division under pressure and at various O_2 concentrations was not determined. Such studies should be carried out. This would answer questions as to whether a longer dark period would be required for cell division under different pressures and O_2 concentrations. The pressure/O_2 combinations under which division does not occur regardless of the length of the dark period could also be determined.

This study demonstrates that the Warburg Effect may prevent *Chlorella ellipsoidea* cells from attaining that critical cell mass which is necessary for subsequent division. The gas exchanges within the closed system lead

Fig. 16 Synchronous division of the entire population of *Chlorella ellipsoidea* cells after the onset of the dark period. Synchrony was induced by a programmed 14: 10 light-dark regime. The n number was 4 within 2 hours of the dark period.

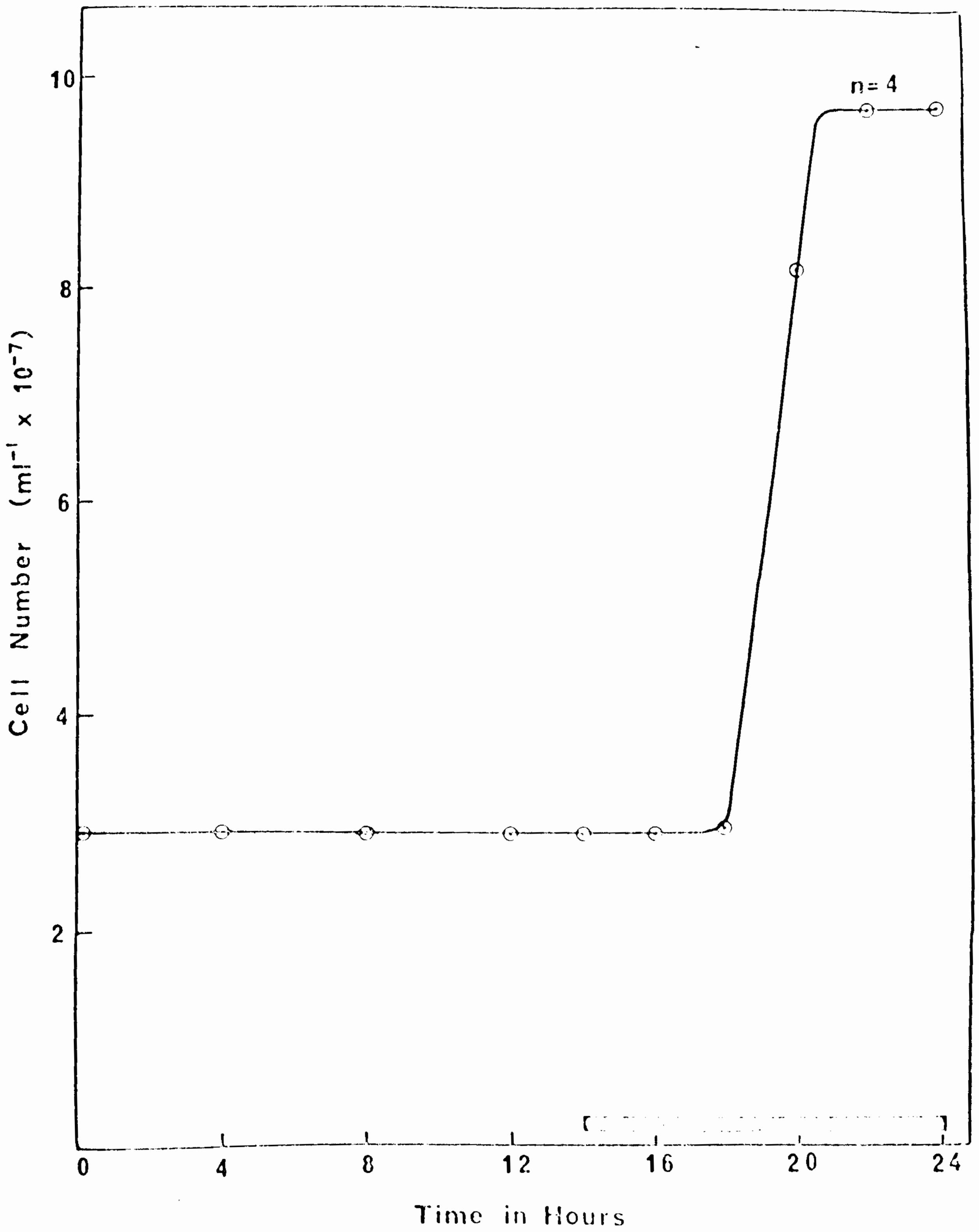

Fig. 10

to the Warburg Effect and possible ways of overcoming this effect have been suggested. If this problem of gas exchange in the closed system can be solved, there is no reason why synchronous cultures of photoautotrophic cells could not be maintained in a closed system and under hydrostatic pressure. It is possible, however that other effects (i.e. Pasteur Effect) may contribute to inadequate cell growth and hence prevent cell division.

CHAPTER 4

HYDROSTATIC PRESSURE EFFECTS NOT RELATED TO SYNCHRONOUS CULTURES

No previous attempts appear to have been made to culture photoautotrophic organisms under hydrostatic pressure using light as the energy source. In the course of developing the techniques of pressure application to synchronous algal cultures some preliminary experiments were carried out in which *Chlorella pyrenoidosa* cells were exposed to different pressures for various times, but no attempt to obtain synchronous growth was made.

I. Pressure Effects On Asynchronous Chlorella Cells

Preliminary experiments were carried out with asynchronous cultures of *Chlorella pyrenoidosa*. The pressure vessels were essentially prepared in the same way as described in Chapter 2. After they were filled with Beijerinck's medium they were inoculated with *Chlorella pyrenoidosa* cells and mounted on the shaker water bath. The free piston cylinder had not yet been constructed and therefore did not constitute a part of the pressure vessel assembly. *Chlorella pyrenoidosa* does not consistently produce an n number of 4 and the cultures cannot be considered to be synchronous. Cell concentration in each vessel was approximately 1 x 10^6 cells/ml. Pressures of up to 700 atm was applied for periods as long as 48 hours. Pressure checks were made 24 hours after pressure application and at the end of an experiment. Cells were illuminated

with 300 ft-c from two 20 watt warm white Westinghouse fluorescent lamps placed on one side of the water bath.

At the end of an experiment the cell concentrations were determined with a haemocytometer. In all experiments, however, at no time did cell number increase above the starting concentration.

Agar medium prepared by dissolving 15 g of Difco agar in 1 litre of Beijerinck's medium was used for culture plates. For a nutrient medium 15 g/l glucose was added to the agar. Both types of media were autoclaved and then approximately 15 ml poured into 100 x 15 mm sterile petri dishes. The streaked plates were incubated at room temperature with light of 700 ft-c from 2 banks of warm white Westinghouse fluorescent lamps.

From unpressurized controls within 7-10 days there was prolific growth of _Chlorella_ colonies, some contaminated with bacteria, on the organic agar (1.5% glucose medium). The inorganic agar supported _Chlorella_ growth in the light. With inorganic agar bacterial growth was less than on the organic medium. _Chlorella_ cells subjected to pressures of up to 300 atm for 48 hr grew on both types of agar about as well as the 1 atm controls. With pressure exposures of up to 700 atm cell growth on organic and inorganic media was slow and fewer colonies developed compared to the controls. It was assumed that approximately the same number of cells were transferred to each agar plate. Since fewer colonies grew on agar streaked with cells exposed to 700 atm it appeared

that the number of viable cells had decreased. In the controls, colonies appeared within 5 to 7 days but after 700 atm for 48 hr colonies, visible to the naked eye, did not appear for 10 to 14 days. Cells exposed to pressures of up to 300 atm for 48 hr were intermediate in the time required for colonies to appear. The organic agar supported faster growth; those cells exposed to 700 atm for 48 hr produced colonies within 10 days (Table XV).

As was expected, the organic medium supported considerable bacterial growth. Attempts were made to suppress bacterial growth by the use of antibiotics. Dihydrostreptomycin at a concentration of 500 parts per million (ppm) suppressed not only bacterial growth but also that of _Chlorella_. Streptomycin and penicillin G at 500 ppm had only a slight effect on both bacterial and _Chlorella_ growth (Table XVI). The results shown in Table XVI indicate that bacteria-free algal colonies might be obtained using 500 ppm dihydrostreptomycin. However, no further attempts were made to get bacteria-free isolates of _Chlorella_ cells using antibiotics.

Cells previously exposed to pressures of 500 to 700 atm grew slowly on either organic or inorganic agar. Some _Chlorella_ colonies exposed to the higher pressures differed in colour from the initial cultures; they appeared either pale to bright yellow or intensely green. These cells were transferred to sterile agar of both types and subcultured through several generations. The intensely

TABLE XV

Growth of *Chlorella pyrenoidosa* cells 10 days after exposure to various pressures for 48 hours. Growth was measured visually and arbitrarily scored as +4= good growth, +1= poor growth, 0= no growth.

	Pressure In Atm						
	1	200	300	400	500	600	700
Growth on inorganic agar	+4	+4	+3	+2	+1	+1-0	0
Growth on organic agar	+4	+4	+4	+3	+2	+1	+1

TABLE XVI

Growth of Chlorella pyrenoidosa and bacteria in the presence of antibiotics of various concentrations on organic agar. +4= good growth, +1= poor growth, 0= no growth.

	Dihydrostreptomycin in ppm			Streptomycin in ppm			Penicillin G in ppm		
	10	100	500	10	100	500	10	100	500
Chlorella	+4	+2	+1	+4	+3	+3	+4	+3	+3
Bacteria	+4	+2	0	+4	+3	+2	+4	+4	+3

green form was eventually isolated in what appeared to be a pure culture. Further work on this isolate, which may have been a pressure-induced colour mutant strain, was not pursued. The yellow-coloured isolate became green after about 2 series of sub-cultures.

II. Discussion

Pressures of up to 700 atm for as long as 48 hr retarded the subsequent growth of *Chlorella* and appeared to reduce the total number of viable cells compared to the 1 atm controls.

Gessner (13) found that 312 hours exposure to 400 atm inhibited subsequent growth of *Chlorella* populations by 50%. The results obtained in this work support those of Gessner to some extent. Although no quantitative studies were carried out in these preliminary experiments pressurization if high enough and for long enough time intervals adversely affected the subsequent growth of *Chlorella* cells.

Pressure might be a mutagen of importance (see Chapter I). Pigment mutants of *Euglena* have been isolated following pressure exposures between 500 and 1,000 atm (14). The yellow and intensely green colonies observed in this study following pressurization of up to 700 atm could well be pigment mutants. It is unlikely that the yellow isolate was indeed a pressure mutant since it became green after only two generations.

The preliminary experiments involving bacterial contamination demonstrated a need for a technique to eliminate

contaminants within the pressure vessels. The free piston cylinders were designed to overcome the problem (see Fig. 3). Use of the cylinders has resulted in uncontaminated algal cultures (see Chapter 2).

SUMMARY

Synchronous cultures of *Chlorella ellipsoidea* was maintained in an open system using conventional culture techniques.

Under identical growing conditions but in a closed system designed to investigate hydrostatic pressure effects on synchronous algal cultures, synchronous cell division was not maintained.

The most significant difference in the culture conditions between the open and the closed systems arises since the concentrations of CO_2 and O_2 remain relatively constant throughout the growth cycle in the open system while the gases vary in concentration continuously during the cycle in the closed system. In the closed system O_2 increases and CO_2 decreases during the light period; in the dark the reverse is true.

Since the intent of this study was to investigate the effects of hydrostatic pressure on the physiology and morphology of algae, it was first essential to develop techniques for successful culture of algae in the closed system. Various modifications of culture conditions were made to obtain equal growth in both systems. Growth conditions of light intensity, programmed light-dark regimes, media, CO_2 concentration, and addition of organic substances were varied in the two systems in attempts to establish and maintain synchronous cultures. The n number and ratios of

increase in dry weight and protein were 4.0 in the open system but never more than 2.0 in the closed system.

Results have been obtained which indicate that high O_2 might be responsible for the descrepancies in the n number and ratios of increase of the other growth criteria between the open and closed systems. Photosynthetically-evolved O_2 in the closed system during the light period might be sufficient to cause the Warburg Effect. If the problem of gas exchange in the closed system can be solved, there is no reason why synchronous cultures of the algae could not be maintained in a closed system. This study has suggested possible ways of overcoming the Warburg Effect.

The effects of pressure on cell division of *Chlorella ellipsoidea* have been studied. Both pressure and O_2 concentration interact to influence the division of cells. A possible mutagenic effect of pressure exposure on algal cells was also considered.

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APPENDIX

GROWTH MEDIA

I Sorokin's Medium (44)

Substance	g/l
KNO_3	1.2500
KH_2PO_4	1.2500
$MgSO_4.7H_2O$	1.0000
$CaCl_2$	0.0835
H_3BO_3	0.1142
$FeSO_4.7H_2O$	0.0498
$ZnSO_4.7H_2O$	0.0882
$MnCl_2.4H_2O$	0.0144
MoO_3	0.0071
$CuSO_4.5H_2O$	0.0157
$Co(NO_3)_2.6H_2O$	0.0049
EDTA*	0.5000
pH	6.8

*$Na_2C_{10}H_{14}O_8N_2.2H_2O$ (Disodium ethylenediaminetetra-acetate)

II Modified Beijerinck's Medium- Bicarbonate Medium

Stock mineral solution	g/l
NH_4NO_3	1.5
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.1
Glass distilled water	1.0 l
Na_2CO_3	0.2 M
$NaHCO_3$	0.2 M

Micronutrient solution was made as described in Table 1

	ml/l of medium
Stock mineral solution	100
Na_2CO_3	25
$NaHCO_3$	25
Micronutrient solution	1

The pH of this medium was adjusted to 6.7 using HCl

III Tamiya and Morimura's medium (55)

Substance	g/l
KNO_3	5.000
$MgSO_4.7H_2O$	2.500
KH_2PO_4	1.250
$FeSO_4.7H_2O$	0.028
Arnon's A 5 solution	1.000 ml/l

Arnon's A 5 solution contains (2)

	g/l
H_3BO_3	2.8600
$MnCl_2.4H_2O$	1.8100
$ZnSO_4.7H_2O$	0.2220
$CuSO_4.5H_2O$	0.0790
MoO_3	0.0071

IV Burr's Medium (8)

Substance		/1	
KNO_3		1.5000	g
Glucose		7.5000	g
$CaCl_2$		0.0800	g
$MgSO_4 \cdot 7H_2O$		0.2400	g
$FeSO_4 \cdot 7H_2O$		0.0070	g
EDTA	as compex	0.0120	g
$ZnSO_4 \cdot 7H_2O$		0.0001	g
H_3BO_3		0.0010	g
$MnSO_4 \cdot 4H_2O$		0.0010	g
$CuSO_4 \cdot 5H_2O$		0.0010	g
$Na_2HPO_4 \cdot 2H_2O$		0.75×10^{-2}	mole
$NaH_2PO_4 \cdot H_2O$		2.25×10^{-2}	mole
pH		6.4-6.5	

In this medium, the phosphate and the glucose were autoclaved separately from the rest of the media. They were added just before use in an experiment. This medium was mainly used in the pressure vessel culture experiment.