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COMPARATIVE EFFECTS OF OXYGEN AT HIGH PARTIAL PRESSURES ON *CHLORELLA SOROKINIANA* AND ON OXYGEN RESISTANT MUTANT

by

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Comparative Effects of Oxygen at High Partial Pressures on *Chlorella sorokiniana* and an Oxygen Resistant Mutant

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THESIS ABSTRACT

Chlorella sorokiniana (+) and an oxygen resistant strain (ORS) isolated from the (+) culture under high oxygen partial pressures were compared on the ultrastructural, physiological and biochemical levels. Results indicate widespread effects of oxygen on the (+) alga. Its growth rate is decreased at all stages of development. The photosynthetic capacity of the cells is immediately lowered (Warburg effect), but continued exposure causes progressive permanent damage to the photosynthetic machinery. Respiratory metabolism is eventually destroyed. Cellular integrity is disrupted and division processes are thwarted resulting in cell degeneration. Pigment content declines, nucleic acid synthesis decreases, and it is probable that cell processes in general are adversely affected by exposure to high partial pressures of oxygen.

General physiological and biochemical parameters including growth rate, temperature optimum, pigment content and DNA compliment as well as general appearance demonstrate the similarity of and provide the basis for a valid comparison between the two organisms. The ORS culture did not show any of the adverse symptoms of oxygen poisoning except for the Warburg effect which reduces apparent photosynthesis by

40%. It does, however, demonstrate subtle structural, physiological, and biochemical differences. The cells of ORS are larger, divide into fewer daughters, exhibit more sluggish metabolic function, have more and better developed mitochondria and pyrenoids and demonstrate a different mode of division. All of these differences appear to be slightly augmented by the presence of oxygen, but cannot be described as oxygen induced properties of the culture.

Experiments with synchronized cultures indicate that early development and autospore formation are severely inhibited by the presence of high oxygen partial pressures in the (+) organism. ORS cultures are not inhibited by the same treatment at any stage of the life cycle.

The presence of (+), and the absence in ORS of osmiophilic storage material indicates a very basic difference in the metabolism of these two organisms. All indications point to inorganic polyphosphate as the major component of these deposits.

Based on these comparative findings, it is apparent that the mutational event(s) which occurred in the development of ORS provided a broad based protection against the adverse effects of high partial pressures of oxygen. It is postulated that such defenses could be provided by mutational events which altered the formation and/or structure of the cellular membranes.

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TABLE OF CONTENTS

INTRODUCTION

The evolution of advanced life forms, i. e. the metazoa, depended on the presence of molecular oxygen and its role in high level energy production through oxidative metabolism. Oxygen, however, is capable of destroying the forms of life which so vitally depend upon it. It is this toxic potential of oxygen and the apparent ability of organisms to resist it which forms the basis for this study.

That the present atmosphere of the earth arose subsequent to its agglomeration is geologically evident. Except for oxygen, the early atmosphere arose from the earth's core during volcanic outgassing. The gases evolved included nitrogen, methane, ammonia, hydrogen, carbon dioxide, and water vapor (Berkner and Marshall, 1969). It was originally thought that the oxygen present in the atmosphere arose from the photodissociation of water by ultraviolet light. However, through studies of Urey (1959) and Berkner and Marshall (1965), it became apparent that these reactions produced a stable equilibrium (Urey equilibrium) resulting in an oxygen partial pressure equal to only 0.001 of the present atmospheric concentration.

Although there is some disagreement as to the exact geologic times involved, (Berkner and Marshall 1964, 1965, 1969; Cloud and Gibor, 1970) it is evident that the appear-

ance of increased atmospheric oxygen depended on the evolution and abundance of photosynthetic organisms. It is also thought that this increase in the oxygenic concentration was responsible for the subsequent evolution of complex life. The following is a brief account of this evolutionary dependence on the atmospheric oxygen level as proposed by several authors (Gilbert 1960, 1963, 1966; Dole, 1965; Berkner and Marshall 1964, 1965, 1969; Cloud and Gibor, 1970).

Because of the lethal levels of ultraviolet radiation with oxygen at 0.1 percent present atmospheric level, the earliest photosynthetic organisms were limited to pools where they were protected by about 10 meters of water. To overcome the stable Urey equilibrium, the abundance of these organisms was sufficiently high to produce oxygen faster than it could be dissociated in the upper atmosphere.

When oxygen reached one percent present atmospheric level, ozone in the atmosphere protected all but the surface waters and the land from lethal radiation. This then allowed the oceans to become inhabited and provided the opportunity for tremendous evolutionary advances. One percent oxygen was also the amount of oxygen necessary for the appearance of oxidative metabolism by fermenting organisms (Pasteur point). Present evidence suggests that this level was reached between 700 and 600 million years ago and corresponds to the appearance of metazoan organisms in the fossil record.

An increase in the photosynthetic oxygen production by marine organisms raised the oxygenic level to 10 percent present atmospheric level.

At this point ozone in the upper atmosphere would be at concentrations adequate to protect the land masses from lethal radiation. Organisms could thus evolve on land. This oxygen level was probably reached about 400 million years ago and eventually gave rise to the development of large land plants. Photosynthesis would then increase rapidly and provide the oxygen levels present in today's atmosphere.

There is an important concept underlying this model for evolution which is pointed out by Olsen (1970), Cloud and Gibor (1970), and Gershman (1964). The development of oxidative metabolism, and even the evolution of organisms which were capable of splitting water photosynthetically to produce oxygen must have been precluded by the evolution of some form of oxygen resistance mechanisms. Cloud and Gibor (1970) state that "it would not have been possible for the essential chemical precursors of life - or life itself - to have originated and persisted in the presence of free oxygen before the evolution of suitable oxygen-mediating enzymes".

It is concluded then that the appearance and accumulation of oxygen in the atmosphere and the evolution of organisms capable of oxidative metabolism depended on the

prior and/or concurrent perfection of mechanisms for resisting auto-oxidation. That these defenses may not be perfect, and that they are inadequate for most organisms above present atmospheric oxygen levels, is the reason for the widespread phenomenon of oxygen toxicity.

As is the case with most areas of biological endeavor, the study of oxygen toxicity is rooted in the 19th century. Paul Bert must be considered "the father of oxygen toxicity" based on the publication, in 1878, of a book describing the "poisonous" effects of pure oxygen on a variety of plants and animals. It is interesting to note that among his conclusions he suggested that the effects of oxygen might be due to the inhibition of what he called "fermentative" reactions.

The next 75 years of study in this field produced an abundance of literature on the physiological effects of oxygen at high partial pressures. These investigations centered around the exposure of men and laboratory animals to oxygen at various concentrations and pressures and observing the progressive effects. The results of this work, presented in a review article by John Bean (1945), indicate that "breathing of pure oxygen or hyperoxygenated air will cause not only general physiological changes, but also pathological alterations".

Since that time, increased interest in underwater research, aerospace medicine, oxygen therapy, and metabolic

control have stimulated research on the biological effects of oxygen. A number of review articles and two symposia have appeared recently which deal with the chemistry, physiology, and biochemistry of oxygen toxicity (Haugaard, 1968; Fishman, 1965; Dickens and Neil, 1964; Fenn, 1965; Brown and Cox, 1966; Gottlieb, 1965; Lambertsen, 1964; Davies and Davies, 1964). It can be generally concluded from these extensive investigations that the effects of oxygen will vary to a great extent depending upon the partial pressure, the length of exposure, the organism studied (even the species), the tissue, the cell type, the organelle and the biochemical substance or pathway investigated. Also, the biological effects of oxygen are highly relevant to the problems of cell division, aging, growth, mutagenesis, radiobiology, and pathology (Gershman, 1964).

There is a basic question underlying all of the research on the biological effects of oxygen which remains unanswered. What are the fundamental mechanisms of oxygen toxicity and resistance?

The physical and chemical properties of the oxygen molecule make it uniquely qualified as a biological source of energy. Gilbert (1960) points out that its high potential, availability, abundance, and sluggish rate of destruction make oxygen the most likely form for storing energy in living systems. He is quick to emphasize however, that this energy source "will tend to be dissipated and thus destroy life

itself." The reduction (destruction) of molecular oxygen is accomplished through a series of one electron additions which result in the formation of oxidizing free radicals (Michaelis 1946). The free radical states form energy barriers to further oxidation thus providing the "sluggish" nature of energy release from oxygen. Once formed, however, these species are highly reactive releasing potential energy upon chemical combination (Gershman 1964).

The "Gershman theory" suggests that it is the presence of oxidizing free radicals in living systems which is the basis for the toxic effects of oxygen. The free radicals are formed either in the reduction of oxygen by hydrogen or through the reaction of oxygen with metabolically produced organic radicals. Once formed, these highly reactive compounds can give rise to non-specific propagating chain reactions producing peroxides and additional free radicals. These in turn could attack vital cellular constituents such as enzymes, DNA, lipids, etc., which would alter the physiological responses of the organism. High concentrations of oxygen would increase the rate of the auto-oxidative processes (Law of Mass action) producing more damaging molecular species.

Haugaard (1968), as well as other investigators (Davies and Davies, 1965); Barron and Singer, 1943), postulate that the most oxygen sensitive components of the cell, and the ones most likely to be directly implicated in oxygen toxicity are the sulfhydryl or SH containing enzymes and co-

factors. The tremendous diversity of these compounds and their relative inactivation by or resistance to oxidation by molecular oxygen in different species and under different experimental conditions indicates that their role in oxygen toxicity is extremely complex.

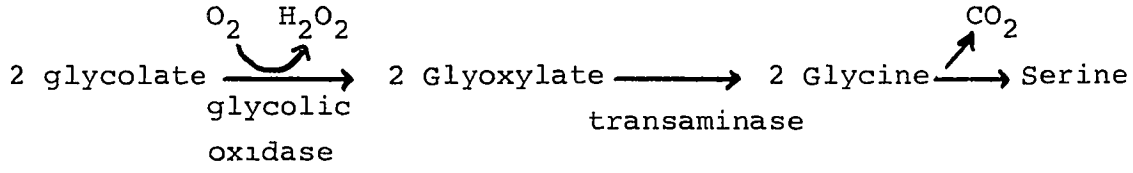
As pointed out earlier, protection against the adverse effects of oxygen was a prequisite to its production by photosynthesis and use in oxidative energy metabolism. The form that these antioxidant mechanisms take is varied. It can be through the production or accumulation of chemicals such as phenols, carotenoids, vitamin E, or cobalt (II) which inhibit oxidative reactions. It can be mediated by enzymes such as catalases which function in the removal of peroxides, intermediates in many oxidative reactions. Or protection can be achieved by mechanisms which keep the dissolved oxygen concentration low in the cell through physical barriers to gas exchange (Gilbert 1963). The mechanism of action in these defenses in vivo has, in many cases, only been postulated. It is agreed, however, that the relative effectiveness of these antioxidant mechanisms establishes the ability of an organism or cell to withstand various oxygen concentrations ranging from zero in the case of obligate anerobes to 100 atmospheres of oxygen tension in the gas gland of a deep sea fish swim bladder (Ball, et al 1955).

The growth and development of plants of many species have been studied in relation to high oxygen partial pressures. Adverse effects include inhibition of seed germination, retardation of root development, loss of vitality, reduced respiratory rates, impaired Krebs cycle activity, inhibition of water uptake, and death (Siegel et. al., 1963; Begin-Heich, 1970; Siegel and Gerschman, 1959; Hew and Krotkov, 1968; Ullrich, 1970; Haugaard, 1968). These effects are mediated through the oxidative metabolism of the plant cells, and are thus analogous to the toxicity seen in animals. Siegel reported that antioxidants such as cobalt (II) could retard or eliminate the symptoms of oxygen poisoning in certain cases.

The inhibition of photosynthesis by oxygen, called the "Warburg effect" after Otto Warburg, who first observed it in 1920, has been seen and studied in a wide variety of plant species. Leaves of higher plants and algae, when exposed to high oxygen partial pressures, demonstrate an immediate drop in photosynthesis between 30 and 100 percent. The degree of inhibition, measured by gas exchange, depends on the species studied, carbon dioxide concentration, light intensity, and temperature. Short term experiments, from a few seconds up to a few hours, indicate that the Warburg effect occurs rapidly and is reversible upon return to low oxygen.

Although no definate conclusions concerning the actual site of action of oxygen on the photosynthetic machinery have been reached, most evidence indicates that it is within the enzymatic "dark" reactions of the Calvin cycle and its related pathways. Oxygen can also act as a Hill reagent (Mehler, 1951) in photosystem II and can react directly with ferrodoxin (Arnon, 1967) in the first step of electron transport from photo-system I. These reactions may take place to some greater extent upon long exposure to high oxygen serving to block photophosphorylation and NADPH production necessary for the function of the dark reactions.

Much interest has been shown in recent years in the process of photorespiration (Jackson and Volk, 1970) and its effect on the efficiency of the photosynthetic reactions. It has been shown that carbon dioxide production and oxygen uptake, in the light, occur through a different process than that shown through typical mitochondrial respiration. In fact, low light intensities appear to completely inhibit "dark respiration". Most investigators agree that photorespiration occurs through the glycolate pathway:



This pathway is stimulated by high oxygen, and has been implicated by many investigators as the reason for reduced photosynthesis under high oxygen. The conditions which produce the greatest degree of photosynthetic inhibition, i.e., high light, low carbon dioxide, high oxygen, and high temperature, are the optimum conditions for photorespiration.

That this is only a partial answer is evidenced by the facts that: 1) green algae do not contain glycolate oxidase and have a pronounced Warburg effect; 2) corn and some other monocots have a very limited glycolate pathway and do not demonstrate photorespiration, but also exhibit the Warburg effect.

It is obvious that the effects of oxygen on plants cannot be limited to increased photorespiration. In fact, as Franck suggested, photorespiration may be a method of protection against auto-oxidation which evolved in the plant kingdom (1951). Perhaps, as suggested by Tamiya and Huzisige (1949), oxygen ultimately causes the inactivation of enzymes associated with the dark reactions of photosynthetic carbon dioxide reduction.

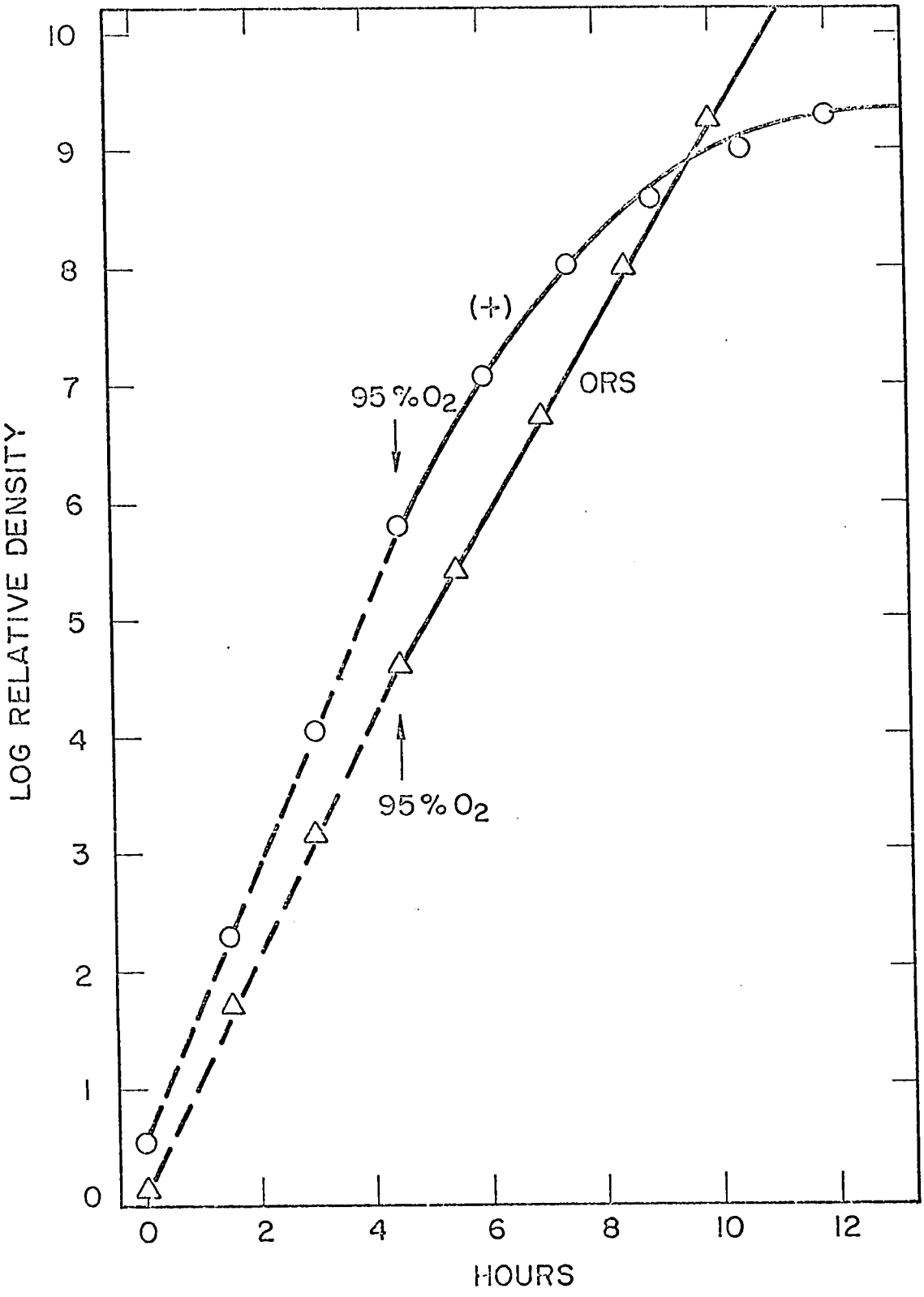
The problem of oxygen toxicity is ubiquitous and complex. It involves all living systems and all aspects of metabolism. Mechanisms of resistance to oxygen were a prerequisite to its utilization and thus have evolutionary implications. Elucidation of this toxicity-resistance pheno-

menon will be of basic and practical interest in all areas of theoretical and applied biology.

The experimental system used in this study is unique in that it provides a tool for the comparative study of toxicity and resistance in an oxygen environment. It involves two strains of Chlorella sorokiniana (Shihira and Krauss, 1963) a thermo-tolerant green alga. The parent strain or wild type (+), has been widely used in studies of algal physiology (Sorokin, 1959, 1960; Sorokin and Krauss, 1959, 1962, 1965). It is characterized by its rapid growth rate at 38° — 39°C (Shihira and Krauss, 1963). The oxygen resistant strain (ORS) was isolated by C. H. Ward from liquid cultures of (+) exposed to high partial pressures of oxygen. The effects of oxygen on the growth rate of the two organisms is shown graphically in Figure 1. Random cultures grown at 39° C under saturating light in air-5% CO_2 were transferred during the logarithmic phase of growth. The growth of the cultures was followed turbidimetrically for four hours and then the gas was changed to 95% O_2-5% CO_2 and the growth study continued. The (+) culture shows immediate and severe oxygen inhibition. ORS continues to grow logarithmically at only a slightly lower rate. This effect has been previously demonstrated by Morhardt (1968).

Stock cultures of both organisms have been maintained in our laboratory for several years through repeated transfer and reisolation without any change in the toxicity-resistance

Figure 1. - Comparison of growth rates on high and low oxygen. (+) cultures represented by O; ORS cultures represented by Δ. Air + 5% CO_2 ----; 95% O_2 + 5% CO_2 O—O.


LOG RELATIVE DENSITY
HOURS
(+)
ORS
95% O2
95% O2
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12

phenomenon. ORS cultures, transferred from solid to liquid medium, require no induction period for autotrophic growth in 95% oxygen. Growth on 20% oxygen for several days does not affect its resistance. Although others have reported the ability to isolate ORS from (+) cultures after exposure to high oxygen (Wagner and Welch, 1969), this has not been accomplished in our laboratory. Wild type will adapt to 95% oxygen, after long exposure, and grow at a much reduced rate. Return of the culture to air level oxygen, however, eventually restores the original growth rate and reexposure to oxygen results in the same toxic response seen in Figure 1. Morhardt (1968) made repeated attempts to reisolate ORS from (+) cultures under various conditions without success.

From this and other evidence it is felt that ORS resulted from a mutation in the (+) strain and that the rates of mutation both from (+) to ORS and the corresponding back mutation are low.

It is this difference in the response of the two organisms to high partial pressures of oxygen which provides a good system for comparative study of oxygen effects at the cellular level.

The complexity of the oxygen toxicity problem and the ubiquity of the toxic effects of oxygen on almost all known organisms provided few solid clues to the mechanisms of oxygen resistance. Hence a starting point for this study was difficult to establish.

The existance of a toxic element in the environment of an organism may elicit a series of responses. The first such responses would be on the molecular level and would include various chemical interactions. The results of these biochemical effects would soon affect the physiological responses of the total organism. Eventually, if no matural means of combating the toxic effect was available to the organism, structural damage and eventually death, or at least suspension of growth and development would result. However, if the organism possesses a natural resistance to the toxic substance, the chemical interactions would be negated biochemically. These reactions may or may not have a profound effect on the physiological response of the organism. Its structure should remain essentially unchanged and its growth and development should continue at a substantially normal rate.

Such a "natural" resistance may be adaptive, i.e. the organism responds to the presence of the toxic substance only. This "toxin induced" resistance would only manifest itself under normally toxic conditions, and the organism would return to normal biochemical and physiological conditions when the toxic substance was removed. On the other hand, the resistance may be the effect of a permanent mutation in the organism. In such a case, one would expect the mechanism responsible for resistance to be present even in the absence of toxin, although perhaps at a reduced level. One would also expect in such a case to see little physio-

logical or structural change in the organism when it is subjected to the toxic substance in question.

Based on this series of responses, and oxygen as a toxic substance, structural differences in the two organisms should show up gross effects of oxygen toxicity and could provide clues to the location of the resistance phenomonon. Various physiological studies comparing the two organisms as they react to the oxygen environment would provide a better understanding of the toxic response, and point the way to selected biochemical comparisons.

It is this basic approach that I have undertaken to provide some insight into the toxicity-resistance phenomonon demonstrated by these two organisms.

A summary of the basic facts concerning the system of study follows:

1. ORS grows well under 95% O_2-5% CO_2 while (+) is immediately and severely inhibited by the same treatment.

2. ORS is a permanent mutation. It will not revert to (+) under air or after many transfers with reisolation.

3. (+) cannot be adapted to growth under high oxygen except after prolonged exposure. Adaptation thus achieved is not complete and is not permanent.

4. Both organisms appear to be Chlorella sorokiniana when grown autotrophically under air level oxygen.

These facts provide the basis for a comparative study of these two organisms as they react to oxygen. Such a comparison should provide information concerning:

1. Toxic effects of oxygen to *C. sorokiniana*;
2. Oxygen resistance in *C. sorokiniana*;
3. Mechanisms of oxygen toxicity and resistance; and
4. Means of protection against high oxygen which are natural in origin.

It is hoped that such information will shed new and significant light on the general problem of oxygen toxicity.

METHODS

The experimental system used in these comparative studies involved two strains of C. sorokiniana, a thermotolerant unicellular green alga. The two strains were originally separated, based on their resistance to high partial pressures of oxygen, by C. H. Ward. The normal strain, which is sensitive to high oxygen levels, is referred to as wild type and abbreviated (+). The mutant or oxygen resistant strain has been called ORS (Morhardt, 1968).

Unless otherwise stated, all cultures were grown autotrophically on Myers' Knops medium containing per liter the following salts: $MgSO_4.7H_2O$, 1.25g ; KNO_3, 1.25g ; KH_2PO_4, 1.25g ; $CaCl_2$, 0.084g ; H_3BO_3, 0.114g ; $ZnSO_4.7H_2O$, 88.2mg ; $MnCl_2.4H_2O$, 14.4mg ; H_2MoO_4(85%), 9.24mg; $CuSO_4.5H_2O$, 15.7mg; $Co(NO_3)_2.6H_2O$, 4.9mg; $FeSO_4.7H_2O$, 0.05g ; EDTA, 0.5g; KOH, 0.31g. The pH of the medium was routinely adjusted to 6.8 by addition of 1N potassium hydroxide. Stock cultures were maintained on 1.2% tryptic soy broth slants solidified with 1.5% agar. All media for algal growth were autoclaved in growth vessles prior to inoculation.

Cultures were grown at 39°C under constant illumination of 1800-2000 ft-c provided by cool-white flourescent tubes. Light was measured using a YSI Radiometer and/or Weston foot candle meter. These conditions permitted maximum logarithmic growth of the algal cultures. Gas of known composition was provided either from commercial mixtures or

through continuous pre-mixing by the use of flow meters. The standard mixtures were 5% carbon dioxide in air and 5% carbon dioxide in oxygen. These gases were bubbled through the culture medium after filtering through cotton.

Two procedures were used extensively for growing experimental material. For large volumes, cells were grown in continuous batch cultures in 3 liter culture flasks which were either shaken in a Psycotherm Constant Environment Chamber (New Brunswick Scientific) adapted for subillumination or stirred magnetically in a Shearer plant growth chamber. Either of these methods provided approximately 1 liter of cell suspension containing up to 2 mg/ml dry weight of algae daily. When smaller samples were needed, cultures were grown in test tubes containing 20 ml of media.

Several methods were employed to quantify cellular material. Growth of cultures was measured turbidimetrically using an Evelyn Colormeter with a 600 mμ filter. Using the optical density thus established in the formula:

$$\log_n \frac{C_2}{C_1} = K\ (T_2 - T_1)$$

where C_1 is the O.D. at time T_1 , C_2 the O.D. at a later time T_2 , and using logs to the base 2 ($3.3 \times \log_{10}$) the generation or doubling time was determined. Specific growth rates were expressed as doublings/day.

Dry weights were taken by pipetting a known volume of culture through a membrane filter which was previously washed,

dried at 100°C and tared. The filter and cell material were then dried to constant weight at 100°C. Results were expressed as mg dry wt/ml of cell suspension. Cell numbers were determined visually using hemocytometer counts and are expressed as cells/ml of suspension. Cell weight was calculated from dry weight and cell number data.

Synchronization was obtained by subjecting cultures to an alternate light-dark regimen (Sorokin, 1957). The (+) strain achieved synchrony after three cycles of nine hours of light, (1800 ft-c), fifteen hours of darkness. ORS-Air cultures required eleven hours of light, and ORS-O_2 cultures required twelve hours of light to maintain a synchronous population. Under these conditions, the cells matured during the light period and divided in the dark period providing a culture which was more than 95% daughter cells at the beginning of each light period.

Fixation of material for electron microscopy was done by methods similar to McLean (1968). The cells were harvested from culture, washed in fresh medium, and suspended in 6% gluteraldehyde in 0.01 M cacodylate buffer, pH 6.8. Cells were fixed at room temperature for 2 hr, washed 4X over a 1 hr period with 0.02M cacodylate buffer and post-fixed in 2% OsO_4 for 2 hr. After washing 2X with tap water, cells were dehydrated in a graded ethanol series, transferred to propylene oxide and then to a propylene oxide:Epon (1:1) mixture. Infiltration occured in this mixture overnight. Fresh Epon was exchanged the following day; the cells were

placed in geletin capsules and polymerized at 60°C. Thin sections were cut with a diamond knife on a Porter-Blum ultra microtome. The sections were stained with uranyl acetate, post stained with lead citrate and photographed in an RCA EMU 3 electron microscope.

DNA and RNA were determined simultaneously using the method of Pederson (1969). Samples were homogenized with a Bronwill Homogenizer using 0.01 mm glass beads (4 g) and 2 ml 100% acetone in a Mico-Chamber. The homogenate was then diluted to 11 ml with 100% acetone and mixed. Aliquots were then centrifiged, washed with acetone (to remove pigments and lipids), washed with cold 10% TCA and hydrolized for 15 minutes at 90°C in 5.0% TCA. A 2 ml aliquot of sample was mixed with 4 ml of diphenylamine reagent, (1 g diphenylamine in 100 ml glacial acetic acid plus 2.75 ml concentrated sulfuric acid (Dische, 1955) and reacted for 4 hr at 100°C. The samples were cooled and read on a Beckman DB-G spectrophotometer against a blank which contained 2 ml distilled water in place of sample. Concentrations of nucleic acids were calculated using the two-wave length method of analysis (Hickey, 1955) from readings at 650 mm and 550 mm.

Photosynthesis and respiration measurements were made using an oxygen electrode (YSI Model 53 Oxygen Monitor) or manometrically using standard methodology. Measurements were made on freshly harvested cells. The pH was adjusted

to 4.8 - 5.0 with dilute HCl. A 4 ml sample was placed in a constant temperature sample container and equilibrated for three minutes. Gas of known composition was bubbled through the sample during equilibriation.

Readings were continuously recorded as per cent saturation on a Beckman 10" log-linear recorder. Rates of oxygen evolution or uptake were calculated using the Bunsen coefficient for dissolved oxygen at the temperature of the experiment, and expressed on a per cell or dry weight basis.

Pigment concentrations were determined spectrophotometrically from acetone extracts. Cells were homogenized in 100% acetone, and an aliquot of the resulting suspension was centrifuged to remove cell debris. The resulting supernatant was diluted to known volume in 80% acetone and water. Chlorophyll concentrations were calculated from the pigment spectra using the following formulae:

$$\text{ug/ml Chl a} = 12.7 \times D_{663} - 2.69 \times D_{645}$$

$$\text{ug/ml Chl b} = 22.9 \times D_{645} - 4.68 \times D_{663}$$

$$\text{ug/ml Total Chl} = \frac{D_{652}}{.0345}$$

Total carotenoids were estimated from the same spectra using the following formula:

$$\text{ug/ml Carotenoids} = \frac{(D_{460} - 0.195) \times 1000}{200}$$

Chromatographic separation of pigments was done by the method of Jeffrey (1961). Acetone extracts were partitioned with anhydrous ether and 10% sodium chloride. The resulting ether-pigment layer was dried and spotted on Whatman # 3 paper or silica gel (Eastman) thin layer plates. Separation was obtained using two-dimensions. The first dimension was run in 4% n-proponal in petroleum ether (Bp 60-90°C), the second dimension was run in 30% chloroform in petroleum ether. Separated pigments were identified by the characteristic absorption maxima of eluted spots (Goodwin, 1965).

RESULTS

Several techniques have been used to quantify the cell material in these studies. From a comparison of these, it became apparent that there was a significant difference in size between the two strains which appeared to be affected by the oxygen concentration. An understanding of this difference became very important when presenting data based on the amount of cell material involved. For example, DNA on a per cell basis was essentially the same for both strains, but expressed on a dry weight or protein basis it was very different.

A size distribution curve was determined for both strains under 20% oxygen and 95% oxygen. Log growth cells from cultures grown in the appropriate atmosphere were photographed at 430X, enlarged, and printed with a resulting magnification of 2700X. All diameters were measured with a vernier caliper, converted to microns, ranked according to increasing size, grouped in 0.25μ intervals and plotted as percent of total population vs. cell diameter. Figures 2 and 3 show the four resulting histograms. Figure 4 was obtained by "smoothing" the histograms using interval averaging and plotting the resulting curves for comparison of the cultures. These data were confirmed using a Coulter Model B electronic cell counter calibrated with teflon beads.

Figure 2 - Cell size distribution of (+) cultures grown on 20% and 95% oxygen.

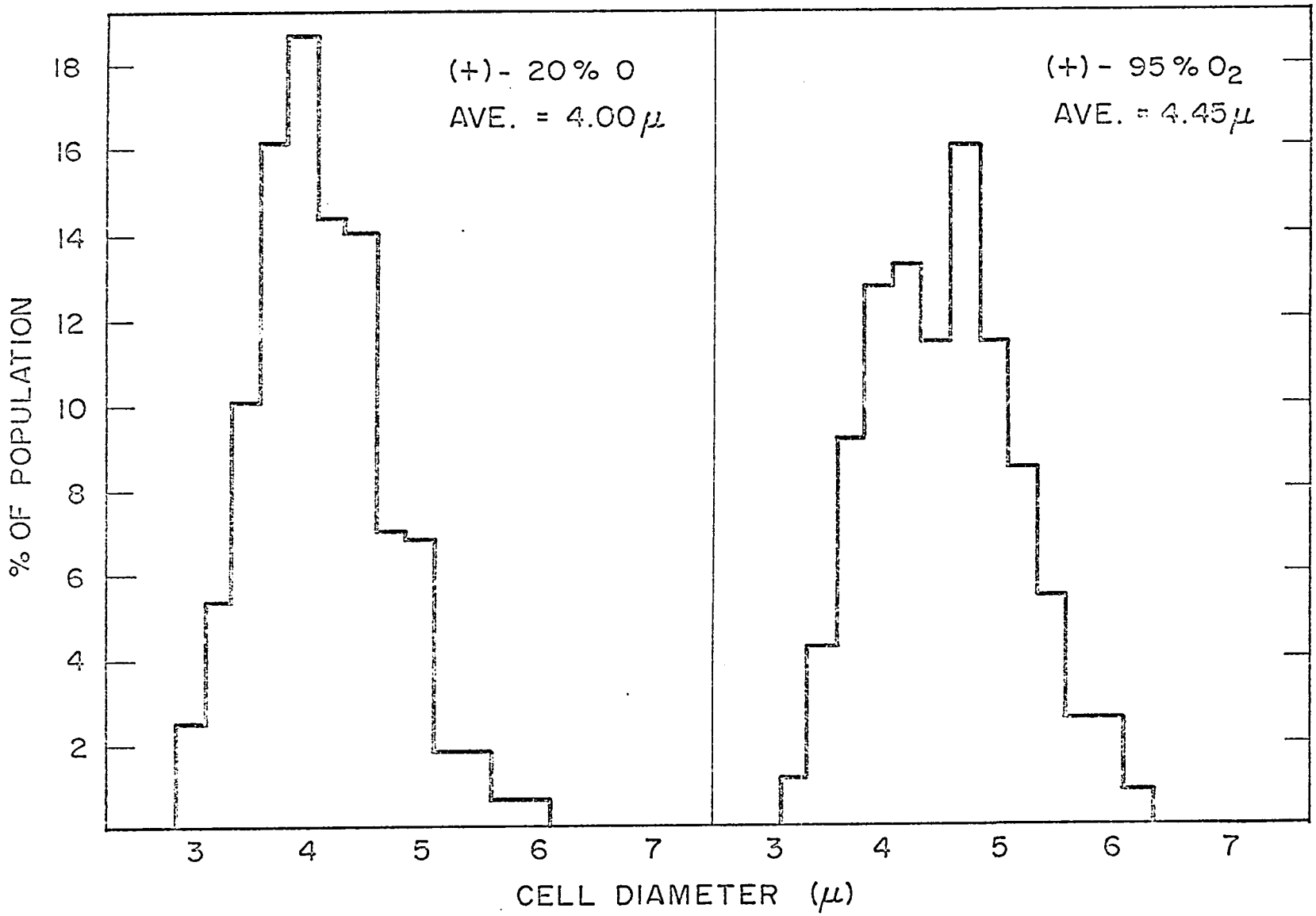

(+) - 20% O
AVE. = 4.00 μ
(+) - 95% O2
AVE. = 4.45 μ
% OF POPULATION
18
16
14
12
10
8
6
4
2
3
4
5
6
7
3
4
5
6
7
CELL DIAMETER (μ)

Figure 3 - Cell size distribution of ORS cultures grown on 20% and 95% oxygen.

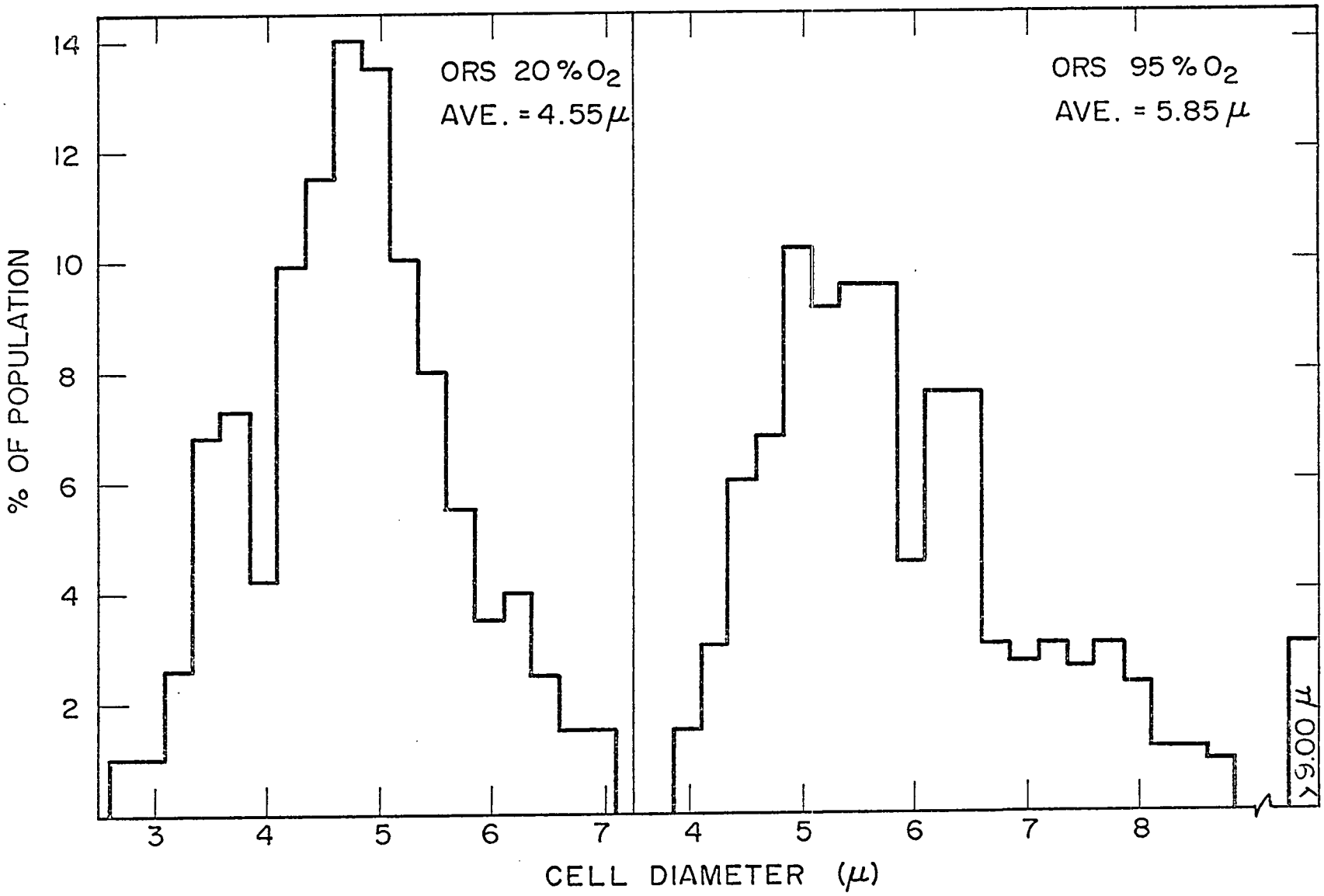
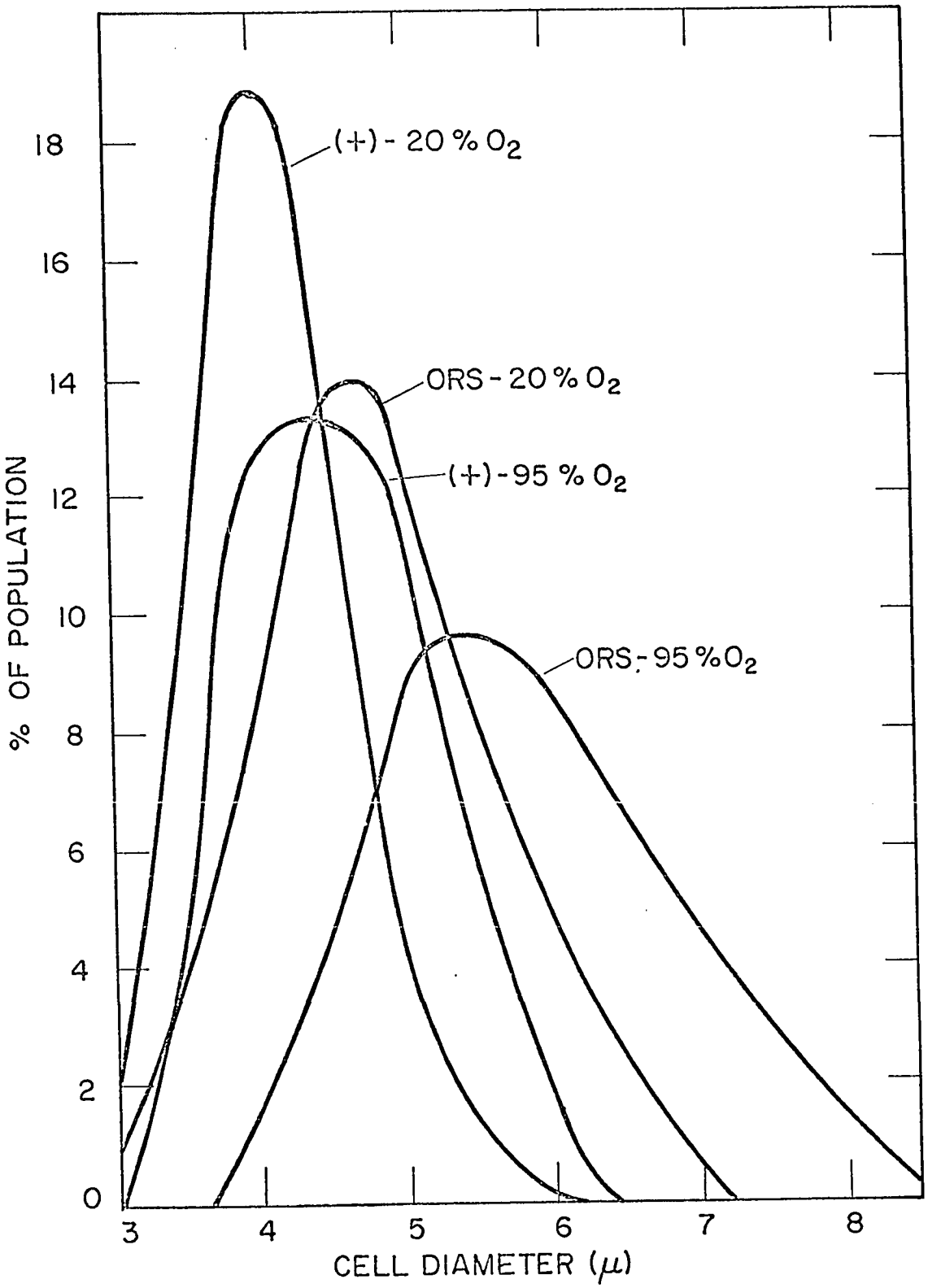

ORS 20 % O_2
AVE. = 4.55 μ
ORS 95 % O_2
AVE. = 5.85 μ
% OF POPULATION
CELL DIAMETER (μ)
14
12
10
8
6
4
2
3
4
5
6
7
4
5
6
7
8

Figure 4 - Comparative size distribution of (+) and ORS cultures grown under 20% and 95% oxygen.


(+) - 20 % O_2
ORS - 20 % O_2
(+) - 95 % O_2
ORS - 95 % O_2
% OF POPULATION
CELL DIAMETER (μ)
0
2
4
6
8
10
12
14
16
18
3
4
5
6
7
8

The (+) culture grown in air level oxygen has a normal distribution of cell diameters. The slightly skewed high end represents dividing cells. Oxygen exposure increased the average size of the (+) cells slightly and spread the distribution curve in both the lower and higher ends.

ORS cultures both on 20% and 95% oxygen contained larger cells with a pronounced skew toward the larger cells. This corresponds well with other quantitative data which indicate ORS cells are larger. ORS grown in 95% oxygen show the greatest proportion of large cells. This is obvious even in routine light microscopic examination of the cultures.

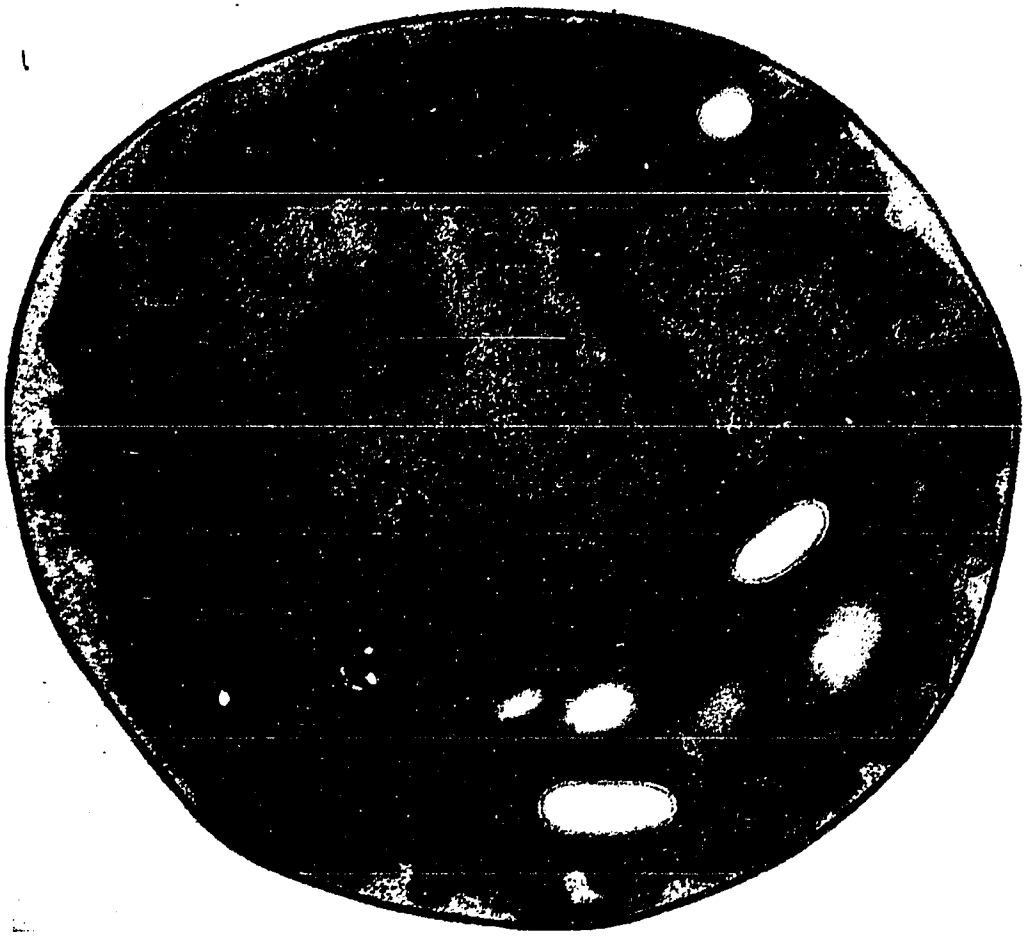
Electron microscopic examination of the two cultures was undertaken to determine what structural differences existed between the two strains of algae. It was also of interest to compare the effect that 95% oxygen had on the morphology of (+) and ORS. Cultures of each organism were grown at optimal conditions under atmospheres of 20% and 95% oxygen, harvested, and prepared for electron microscopy as previously described.

The (+) cells grown under air level oxygen show structures typical of actively growing Chlorella. These normal cells, pictured in Figures 5, 6, and 7, have a prominant, centrally located nucleus (N) which usually contains the nucleolus (nuc); a large characteristically cup shaped chlorplast (Chl) with starch bodies (S) and a pyrenoid (P); numerous small mitochrondria (M); golgi apparatus (G);

Figure 5 - Typical (+) cell grown on 20% oxygen. Nucleus (N); nucleous (nuc); chloroplast (Chl); mitochrondrion (M); starch (S); osmiophilic vacuolar material (OSM). 40,000 X


S

Figure 6 - Typical (+) cells grown on 20% oxygen. Nucleus (N); golgi apparatus (G); endoplasmic reticulum (ER); mitochondrion (M). 35,600X, 28,600 X



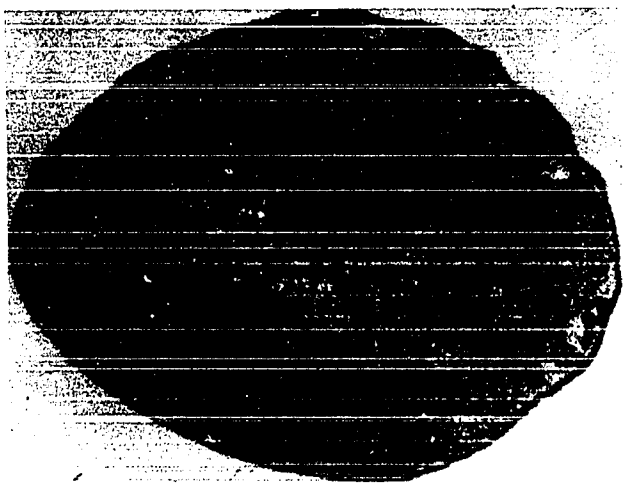
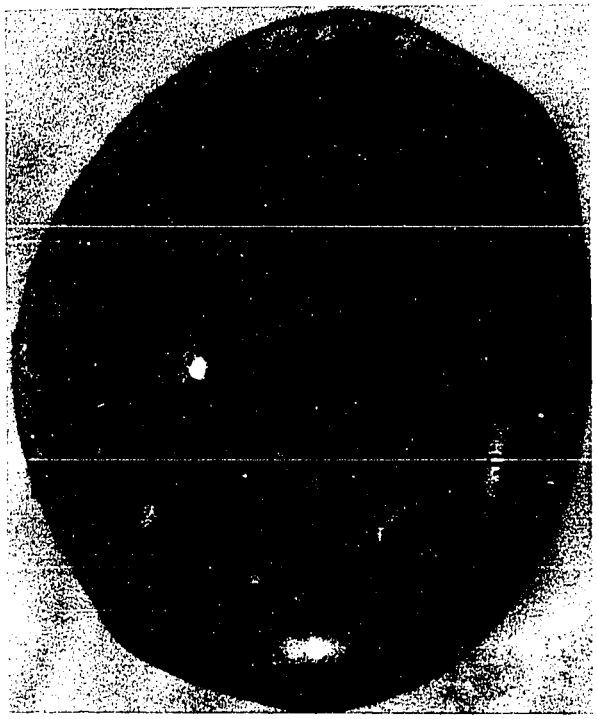
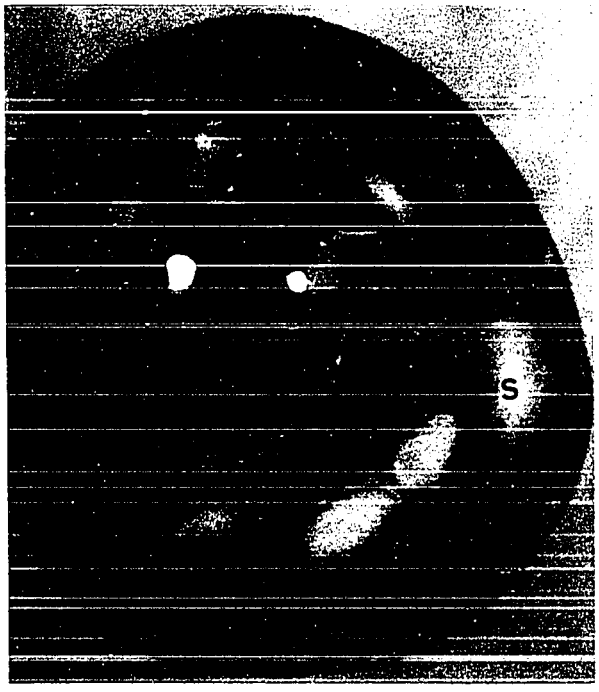
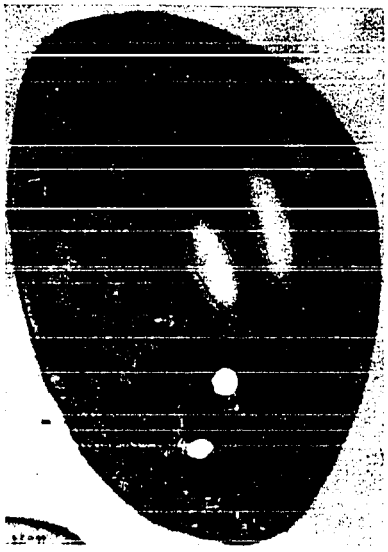


Figure 7 - Typical (+) cells grown on 20% oxygen. Rough endoplasmic reticulum (ER); pyrenoid (P); starch (S); osmiophilic material (OSM); chloroplast (Chl); nucleus (N) 29,000X, 29,000X, 28,600X




S



endoplasmic reticulum (ER); and abundant ribosomes (r). In addition, these cells typically contain abundant osmiophilic material within vacuolar spaces in the cytoplasm (OSM).

High partial pressures of oxygen have a dramatic effect on the ultrastructure of (+) cells. A general loss of internal integrity is seen in the cultures grown in 95% oxygen (Figures 8, 9, 10, 11, 12). The chlorplasts (Chl) are much smaller and contain little starch and fewer lamellae (Figures 8, 9, and 12); the pyrenoid (P), when present, is more pronounced as seen in Figures 10 and 11; mitochrondria are larger and fewer in number (Figures 8 and 9); the osmiophilic material (OSM), seen as discrete granules in air grown cells, is more abundant, amorphous and globular (Figures 9, 10, 11, and 12); and there is a noticeable increase in the amount of vacuolar space (VS) seen in particular around the cell border. Golgi activity is very low or lacking and the amount of endoplasmic reticulum is significantly reduced.

Typical cells from ORS cultures grown on air level oxygen are very similar in almost all respects to air grown (+). Figures 13, 14, 15, and 16 are representative of this strain and show the same structures seen in the normal (+) Chlorella. The most obvious difference between the cultures is the complete lack of osmiophilic material (OSM) in the ORS cells. Mitochondrial activity appears to be more pronounced in ORS (Figures 13, 14, and 15), and the pyrenoid

Figure 8 - Wild type cell grown on 95% oxygen. Two daughter cells just released from the mother cell wall (MCW). Chloroplast (Chl); nucleus (N); mitochondria (M). 45,000X

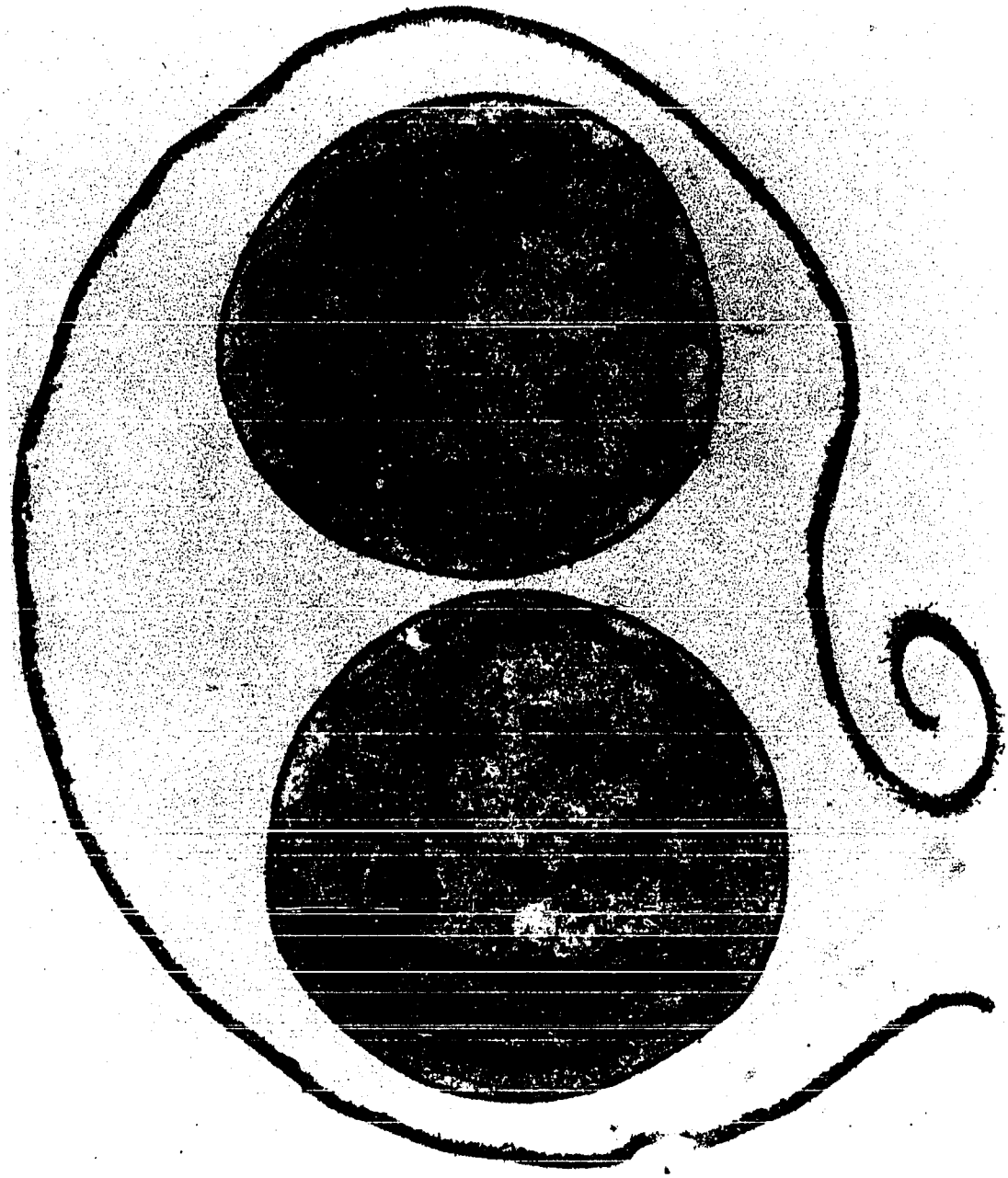
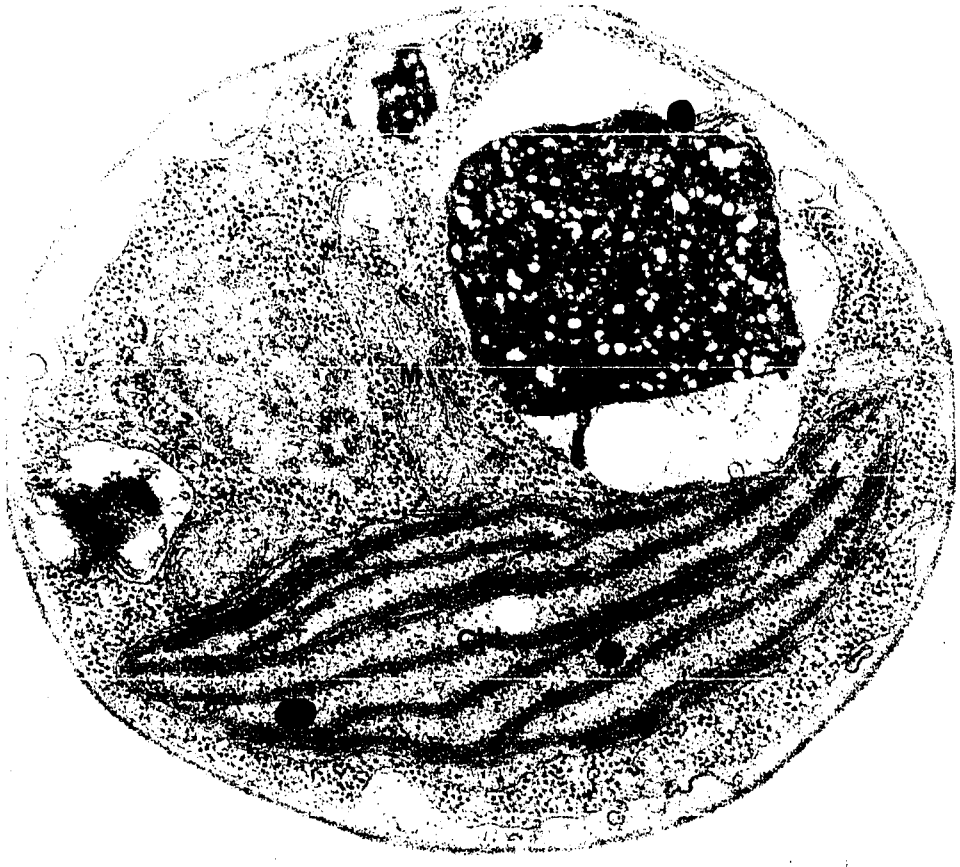


Figure 9 - Typical cell grown on 95% oxygen. Large mitochonria (M); osmiophilic material (OSM); extensive vacuolar space (vs); Chloroplasts (Chl); nucleus (N). 40,00X, 30,000X, 25,200X




VS

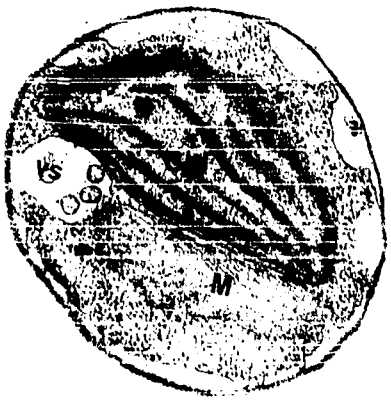
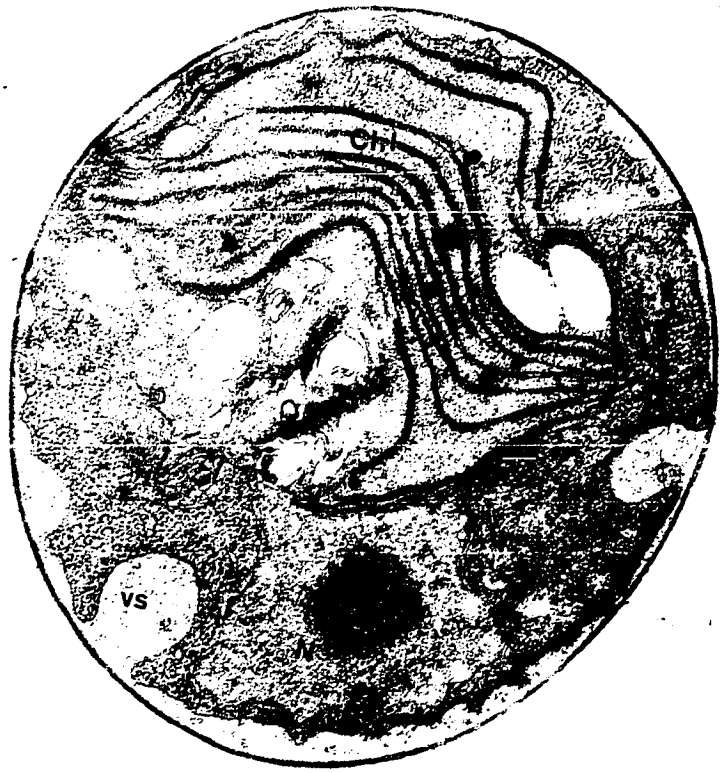

VS
M

Figure 10 - (+) cells grown on 95% oxygen showing breakdown of internal structural integrity. Pyrenoid (P); vacuolar space (vs); chloroplast (Chl); large deposits of osmiophilic material (OSM); starch (S); nucleus (N). 25,200X, 28,600X, 23,800X


Chr
vs
N


S

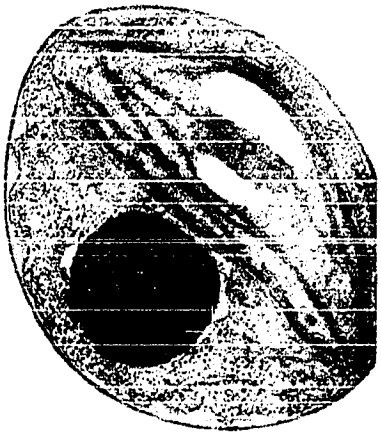


Figure 11 - Wild type 95% oxygen. Increased vacuolar space (vs); large deposits of osmiophilic material (OSM) laid down in three different forms; pyrenoid (P); starch (S); chloroplast (Chl); nucleus (N). 28,000X

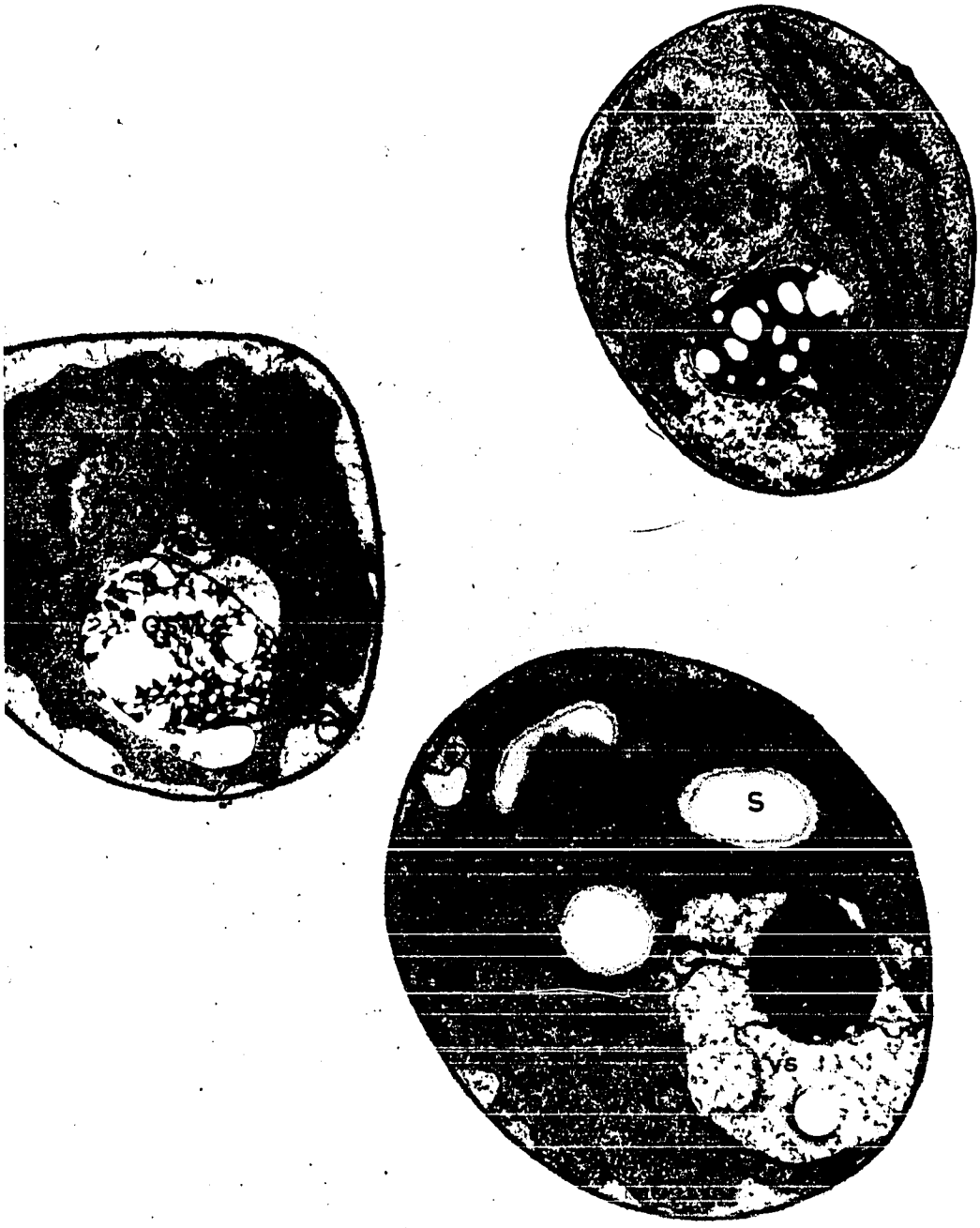

S
Vs

Figure 12 - Typical (+) cells grown on 95% oxygen. Cells show loss of integrity, much vacuolar space (vs); osmiophilic deposits (OSM); small chloroplasts (Chl); mitochondrion (M); nucleus (N). 29,100X, 29,100X, 28,800X


VS
VS
VS

Figure 13 - Cell of ORS grown on air level oxygen. Chloroplast (Chl); nucleus (N); nucleolus (nuc); endoplasmic reticulum (ER); starch (S); mitochondira (M). 39,500X

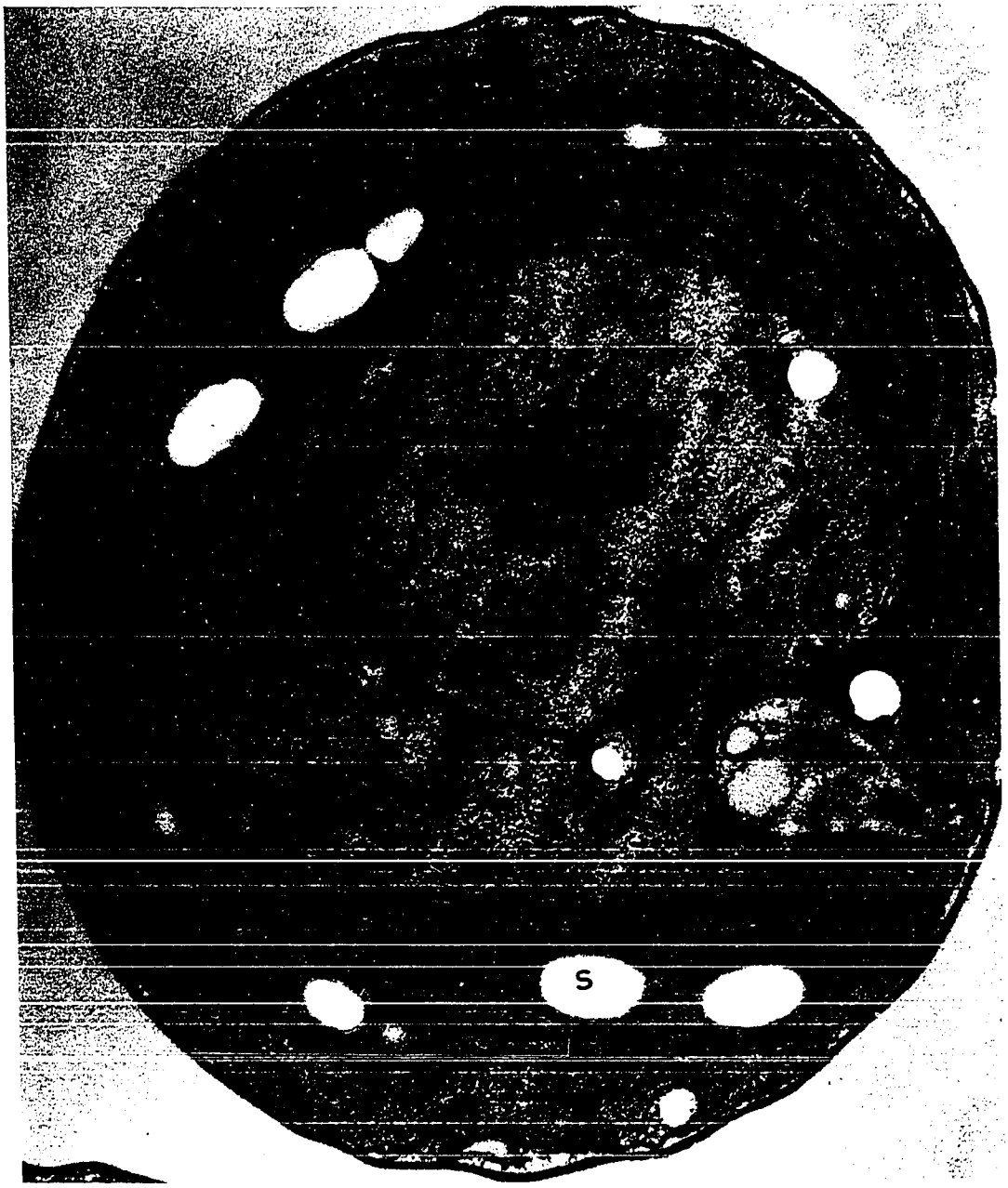

S

Figure 14 - Typical ORS cell grown on air level oxygen. Pyrenoid (P); chloroplast (Chl); nucleus (N); mitochondrion (M). 39,500X

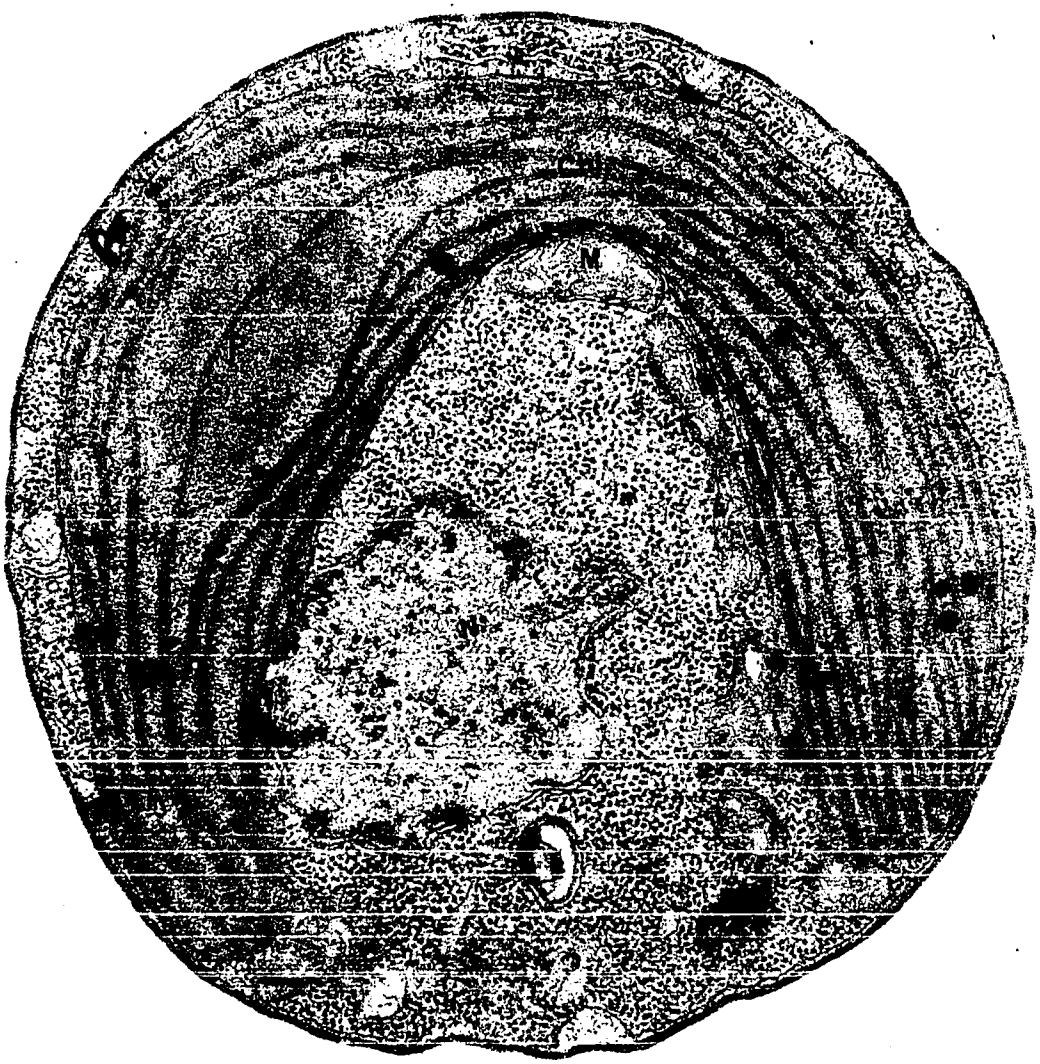
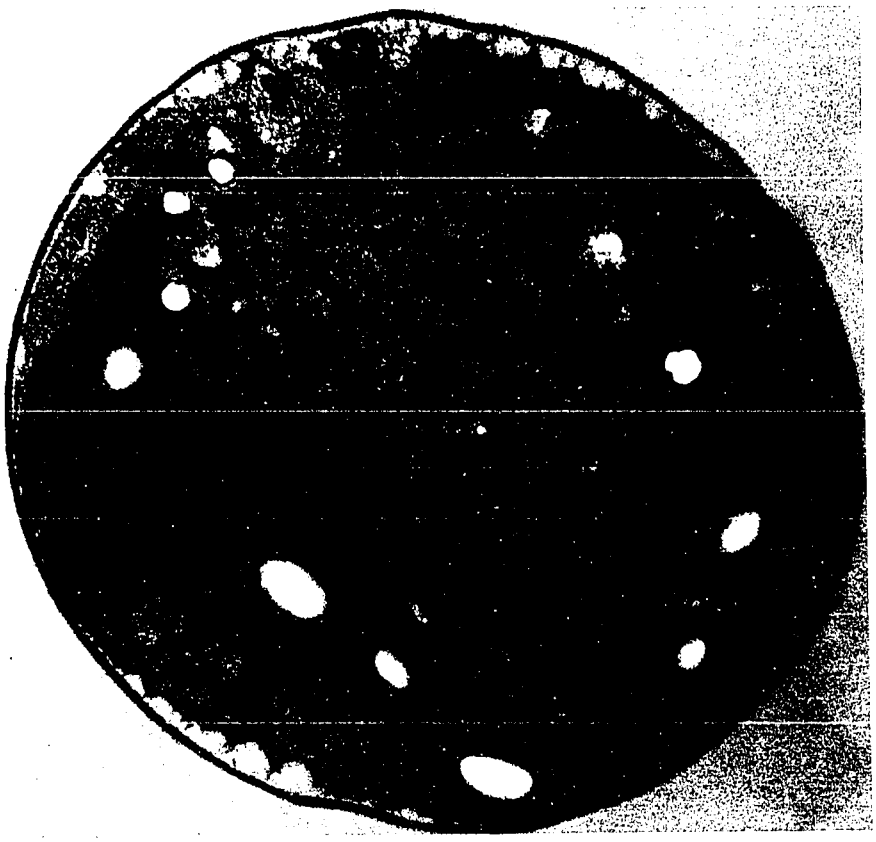
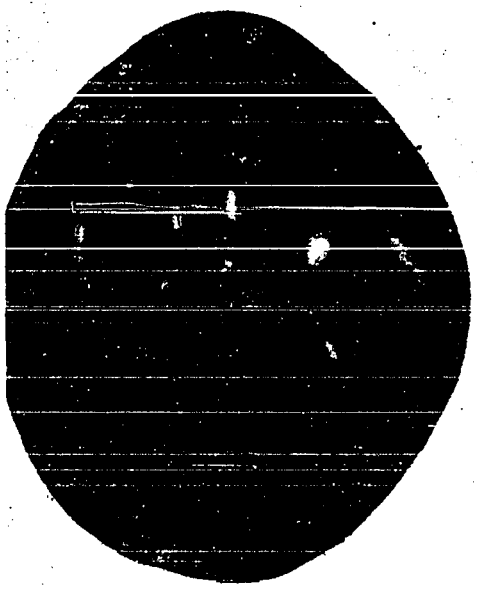


Figure 15 - Cells of air grown ORS showing typical structures including chloroplasts (Chl); golgi (G); endoplasmic reticulum (ER); nucleus (N); mitochondrial activity (M); starch deposits (S). 37,500X, 28,200X




S

Figure 16 - ORS cells grown on 20% oxygen showing prominent pyrenoids (P); chloroplasts (Chl); mitochondria (M); and vacuole (vs). 19,300X, 19,400X, 14,000X



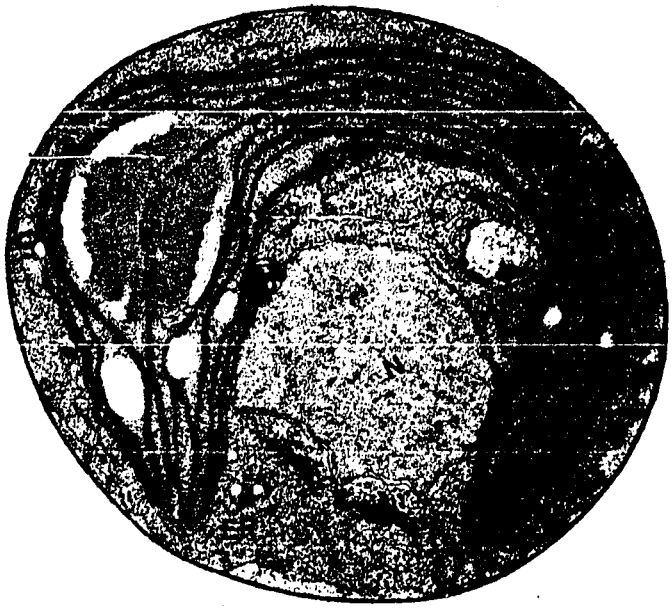
is more obvious within the chloroplast (Figure 16). The remaining organelles are present and appear normal as compared to (+) cells.

The effects of 95% oxygen on ORS cultures are very slight if existant. The protective mechanism(s), which enables it to grow well under high oxygen, also protects the structural integrity of the cells. The presence of enlarged mitochondria (Figures 18 and 19) and a very prominent pyrenoid (Figure 20) indicate some oxygen effect is reflected in the morphology as it is in the slightly reduced growth rate of ORS on 95% O_2. Again, there is a complete lack of osmiophilic material seen in (+) cultures. Comparison of these cells, shown in Figures 17, 18, 19, and 20, with those of (+) grown on 95% oxygen again points out the dramatic difference between these two cultures.

It is apparent from these studies that ORS is able to maintain typical cell morphology under high level oxygen while (+) cultures show oxygen toxicity resulting in a loss of cellular integrity. These results along with those of the cell size and growth studies pointed toward a series of comparative studies to see how oxygen affected the algae on the physiological level. It was also hoped that basic differences between the two strains would become evident through their physiological responses.

The optimum temperature for growth of algal cultures varies over a wide range. It is indicative of the alga studied and of its metabolic machinery in general. A series

Figure 17 - Typical ORS cells grown with 95% oxygen. Structure is intact and organelles are well developed and show no toxic effects of high oxygen. Nucleus (N); pyrenoid (P); endoplasmic reticulum (ER); mitochondria (M); chloroplasts (Chl); starch (S); golgi apparatus (G). 20,000X, 28,300X


N

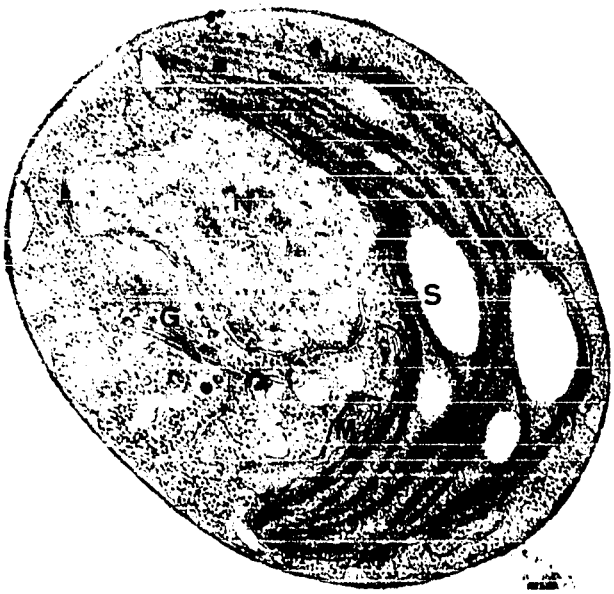
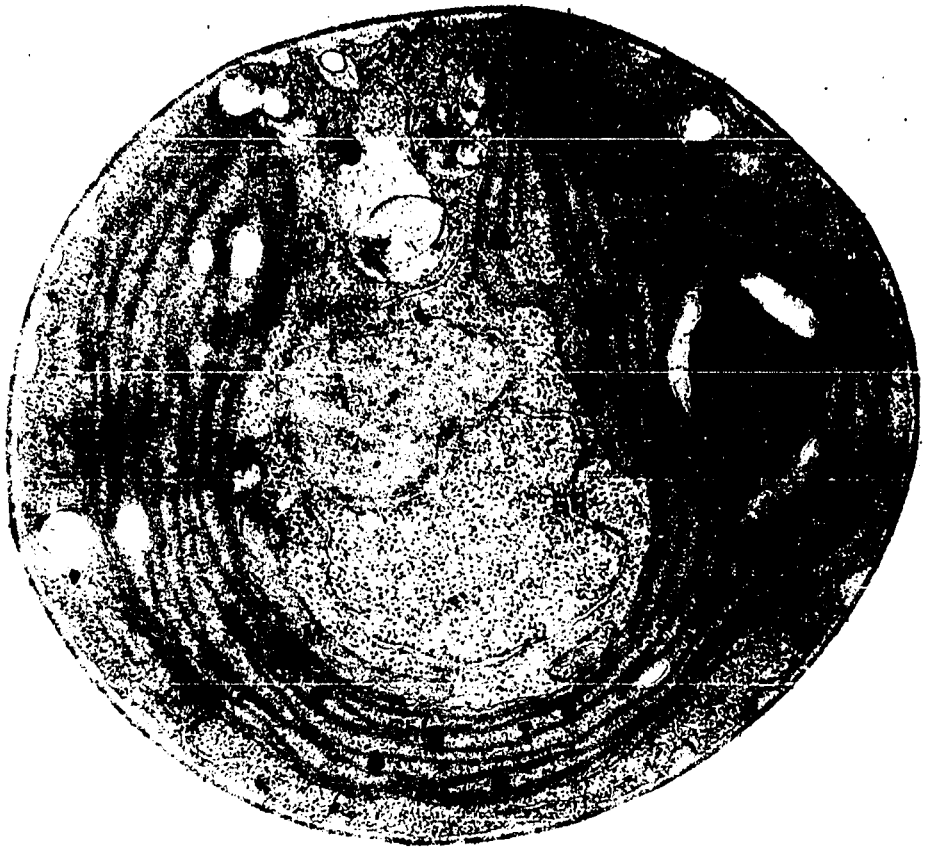

N
S
G

Figure 18 - Typical cells of ORS grown on 95% oxygen showing large, strange shaped mitochondria (M); chloroplasts (Chl); abundant starch deposits (S); pyrenoid (P). 30,000X, 19,500X

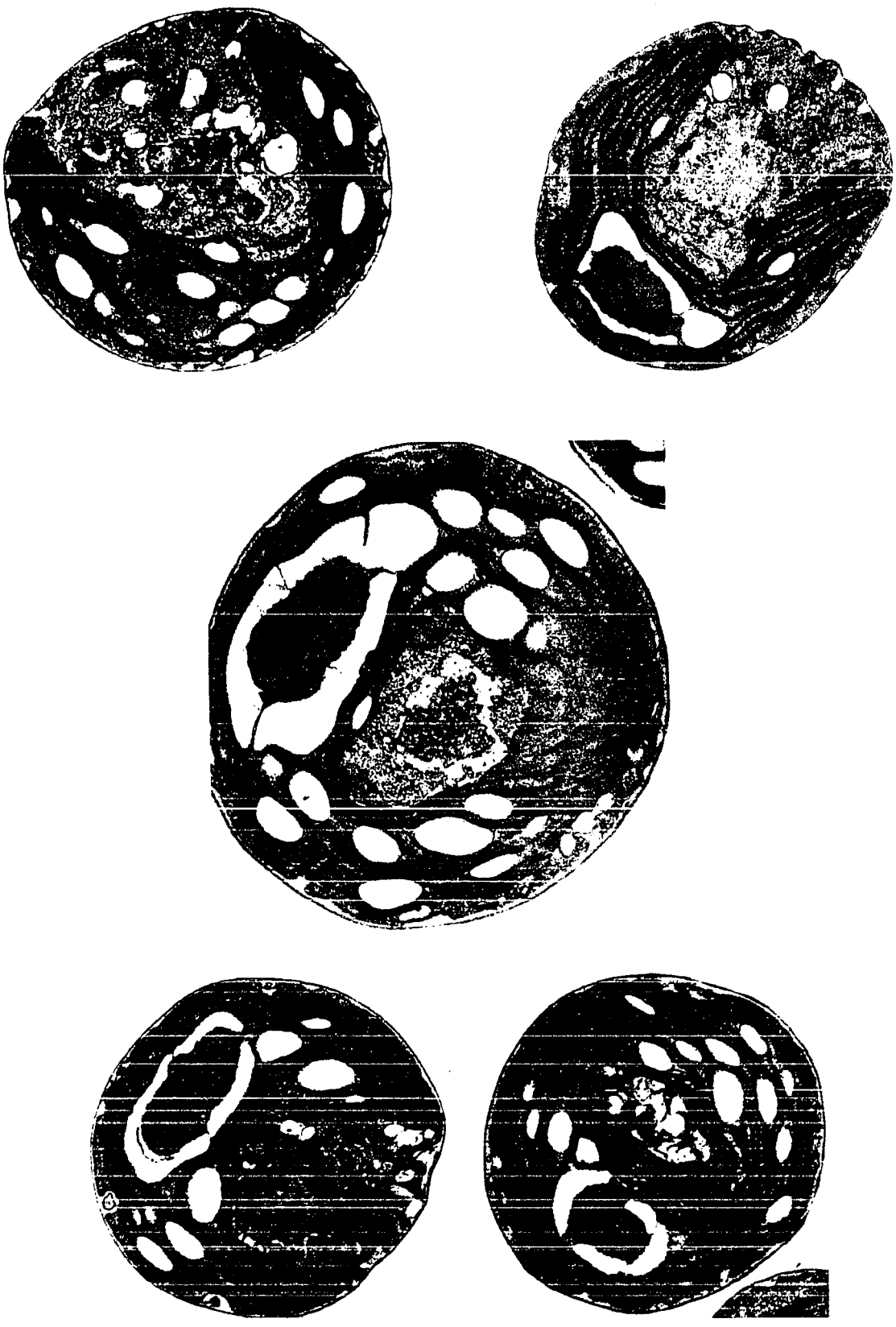



G
nuc
N
S

Figure 19 - ORS cell grown on 95% oxygen demonstrating increased mitochondrial activity (M), and well developed chloroplasts (Chl) with abundant starch deposits (S), and pyrenoid (P). 38,900X


S

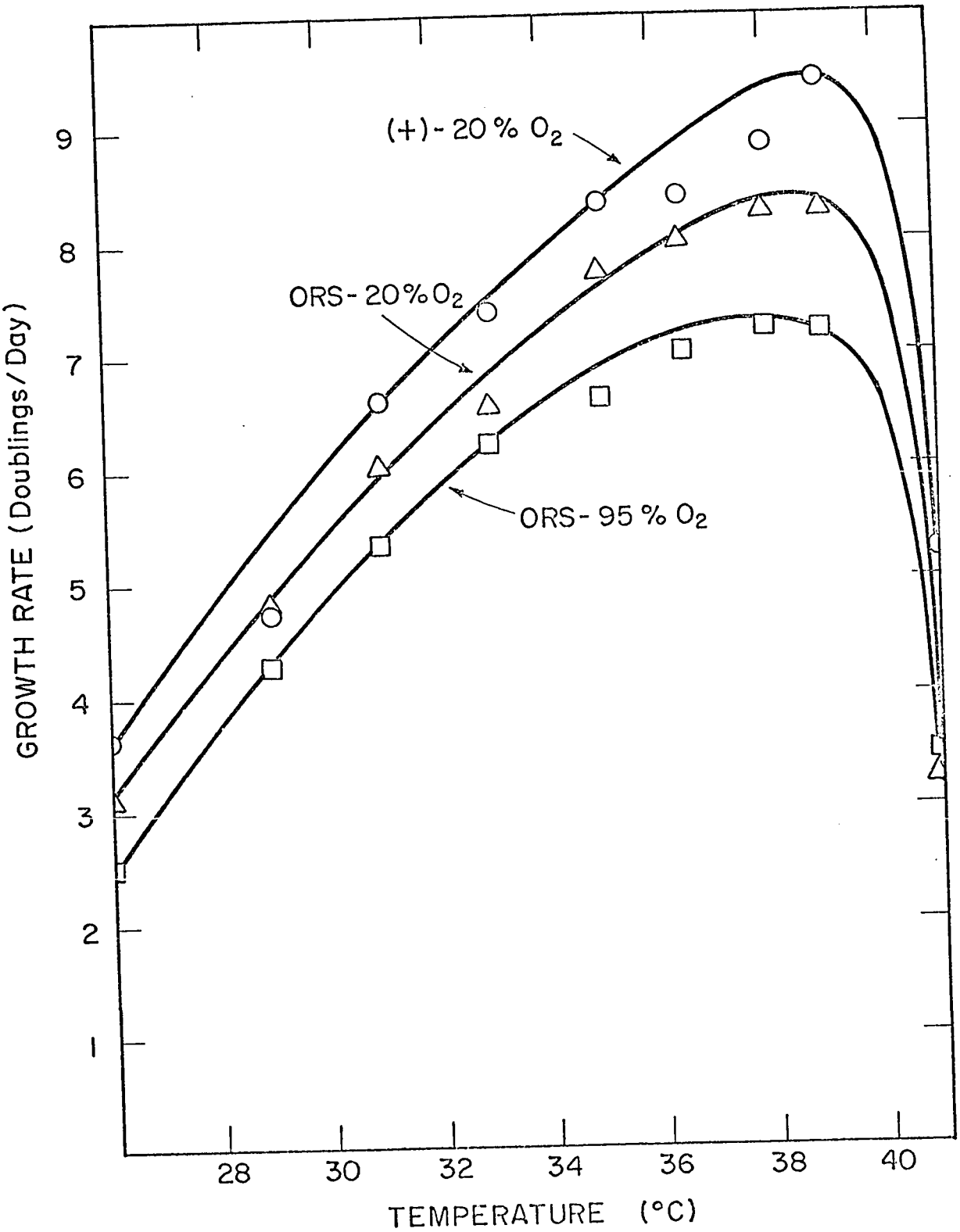
Figure 20 - Typical cells of ORS grown under high oxygen showing prominent pyrenoids (P) in well developed chloroplasts (Chl). 10,400X



of experiments was done to determine the temperature optimum for ORS on 20% and 95% oxygen and to observe the effects of temperature on the growth rates of the two strains. The light intensity was held constant at 2000 ft-c and the temperature was varied from 26° to 41°C. Growth rates were determined by turbidometric measurements and expressed as doublings/day. Cultures were maintained at maximum growth for at least two days at each temperature prior to measurement of growth rate. Figure 21 shows that 39°C is the optimal temperature for growth of both (+) and ORS cultures. Growth of (+) on 95% oxygen was not constant at any temperature and log growth rates could not be determined. The temperature curve for (+)-20% oxygen is similar to one previously reported by Sorokin (1959) for that strain and thus serves as a control. The shapes of the three curves are very similar and indicate no difference in response to temperature between the two strains. The lower growth rates of ORS cultures has been pointed out previously.

C. sorokiniana has the capacity for heterotrophic growth on glucose (Shihira and Krauss, 1963). A series of experiments was done to determine if the oxygen resistance of ORS could be demonstrated with equal facility in the dark with an exogenous carbon source. Cells were grown on glucose in the dark at 39°C in 20% oxygen for three days. These cells were then transferred to fresh Knops medium fortified with 0.5% glucose and gassed with 20% and 100%

Figure 21 - Comparative growth rates of (+) and ORS cultures as a function of temperature. Optimum growth temperatures for both strains is 39°C. ○—○ (+) 20% oxygen; △—△ ORS- 20% oxygen; □—□ ORS- 95% oxygen.


(+) - 20 % O2
ORS - 20% O2
ORS - 95 % O2
GROWTH RATE (Doublings / Day)
TEMPERATURE (°C)
1
2
3
4
5
6
7
8
9
28
30
32
34
36
38
40

oxygen atmospheres. Samples were collected periodically for the determination of cell number and dry weight. The results are shown in Figure 22.

Both cultures grew well heterotrophically in air level oxygen. However, growth data indicate that ORS, under these conditions, does not demonstrate the degree of oxygen resistance in the dark that it does in the light. That ORS will grow on exogenous glucose in 100% oxygen was reported in previous studies (Morhardt, 1968); Wagner and Welch, 1969). This suggests a longer adaptation period for oxygen growth to be demonstrated by ORS heterotrophically. Obviously more extensive study in this area is needed.

The lack of an immediate resistance response of ORS grown heterotrophically suggested a possible effect of glucose supplied exogenously on the oxygen resistance mechanism. Photoheterotrophic growth on glucose in the light was measured. Cells were grown in the medium enriched with 0.5 glucose for several days prior to growth rate measurement. Results, presented in Figure 23, show that no change in growth rate was evident when glucose was added to the culture medium. ORS cultures did not require any adaptation for growth in high oxygen atmospheres.

Another set of experiments, designed to determine the effect of light on heterotrophic growth, was accomplished by

Figure 22 - Heterotrophic growth of (+) and ORS cultures on 0.5% glucose in 20% and 100% oxygen atmospheres: a) mg dry weight/ml cell suspension; b) cell number/ml cell suspension.

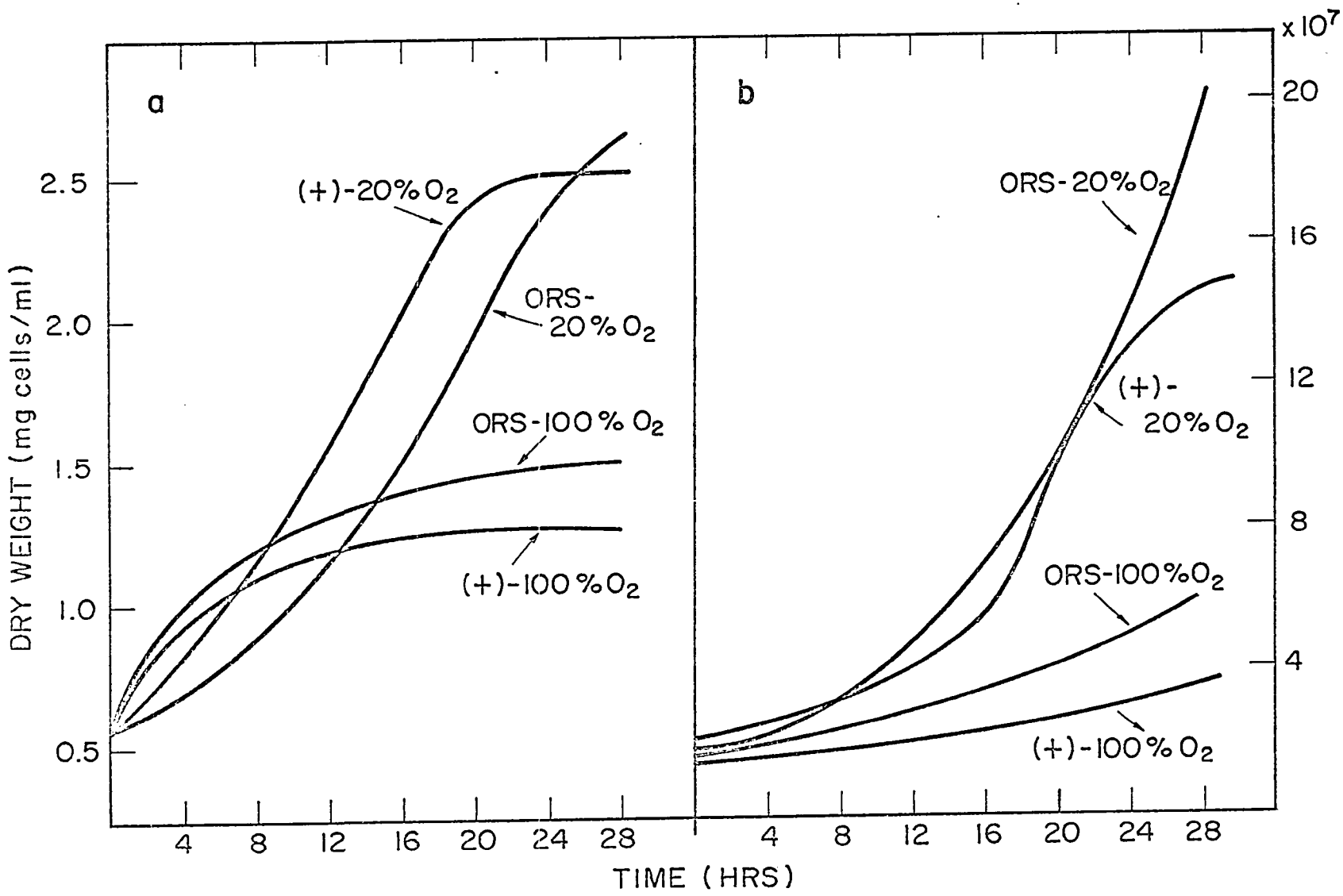
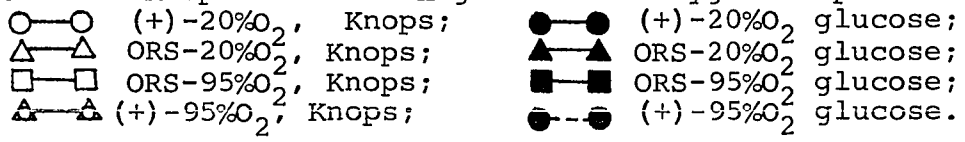
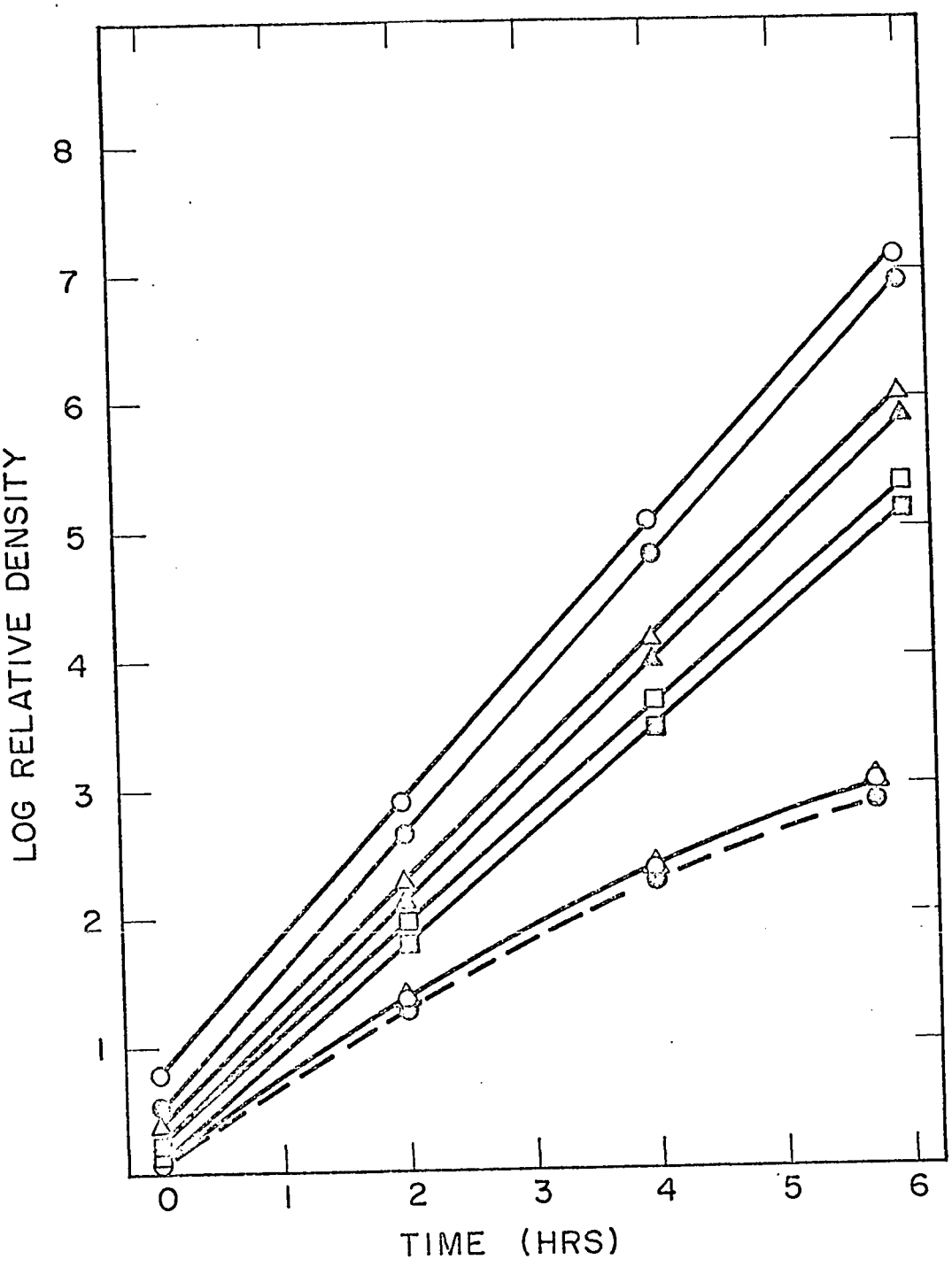

a
b
(+)-20% O2
ORS-20% O2
ORS-100% O2
(+)-100% O2
DRY WEIGHT (mg cells/ml)
2.5
2.0
1.5
1.0
0.5
x 10^7
20
16
12
8
4
4 8 12 16 20 24 28
TIME (HRS)

Figure 23 - Photo+heterotrophic growth of (+) and ORS cultures on 0.5% glucose compared with normal autotrophic growth on Knops medium. High and low oxygen compared.




LOG RELATIVE DENSITY
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
TIME (HRS)

the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), at a concentration of 10^{-5}M, to the culture medium. At this concentration, DCMU is a potent inhibitor of photosynthesis. A control experiment, Figure 24a, showed the inhibitor did not affect heterotrophic growth in the dark.

Media containing DCMU and DCMU+0.5% glucose were inoculated with both cultures and placed at 39°C under 20% and 95% oxygen. The light intensity was held at 2000 ft-c. As expected, the cultures which contained DCMU only did not show any growth (Figure 24b). The glucose +DCMU cultures growing on air behaved in a manner similar to that of dark grown cells. ORS cultures supplied with glucose + DCMU required several days to show appreciable growth on 95% oxygen. Figure 25 shows the growth of (+) and ORS on glucose + DCMU after one week of incubation under high and low oxygen atmospheres.

Although growth studies give a good indication of the general physiology of the cultures, measurements of photosynthesis and respiration should provide more specific information concerning the comparative effects of oxygen on (+) and ORS. Random log growth cells were harvested from tube cultures and the photosynthetic and respiratory rates

Figure 24 - a) Dark heterotrophic growth of (+) and ORS on Knops 0.5% glucose 10^{-5}M DCMU. ●—● α(+)-glucose; ○—○ (+)-DCMU and glucose; ■—■ ORS-glucose; □—□ ORS- DCMU and glucose.
b) Light autotrophic growth of (+) and ORS on Knops and DCMU. ○—○ (+); □—□ ORS.

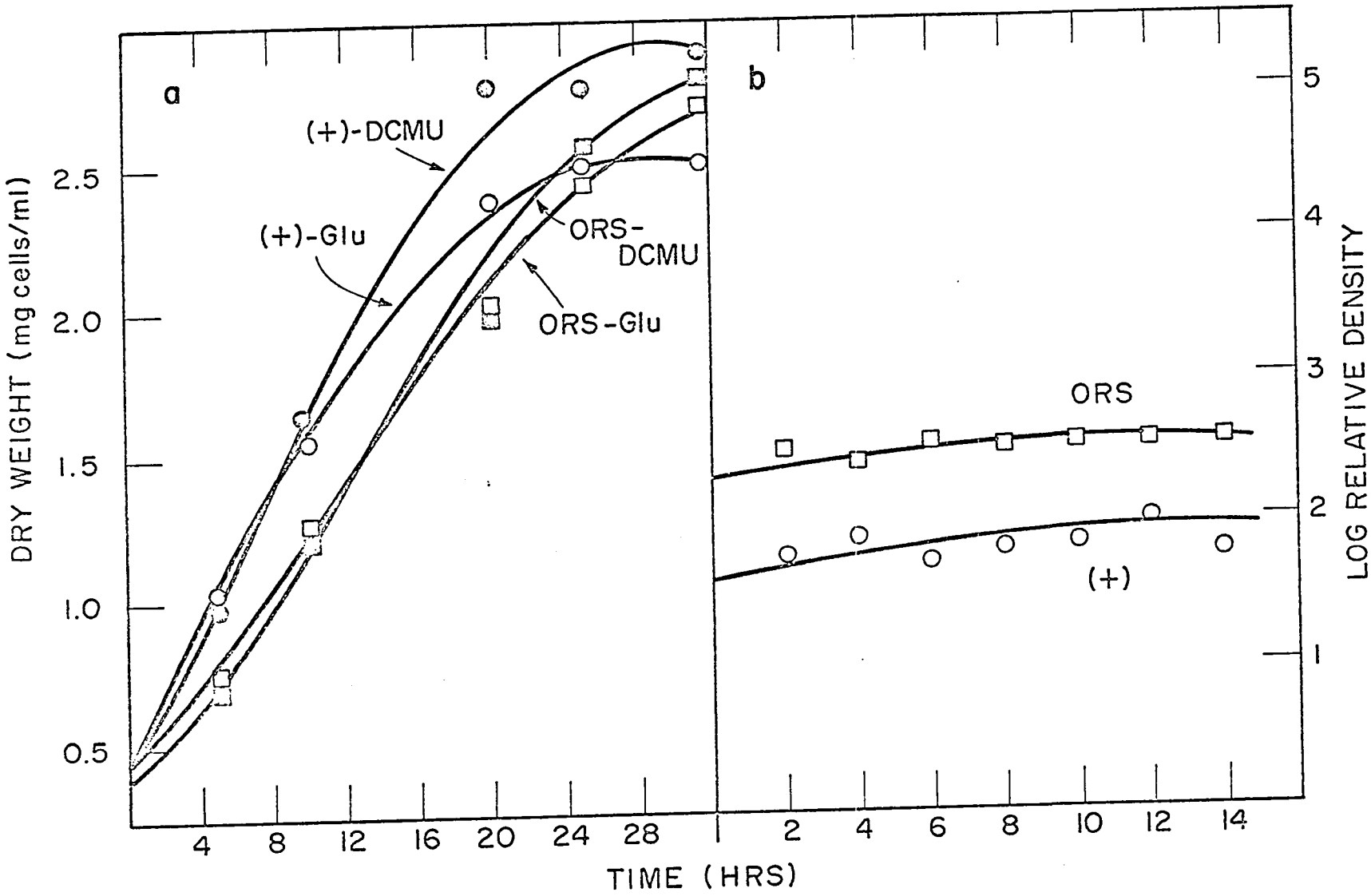
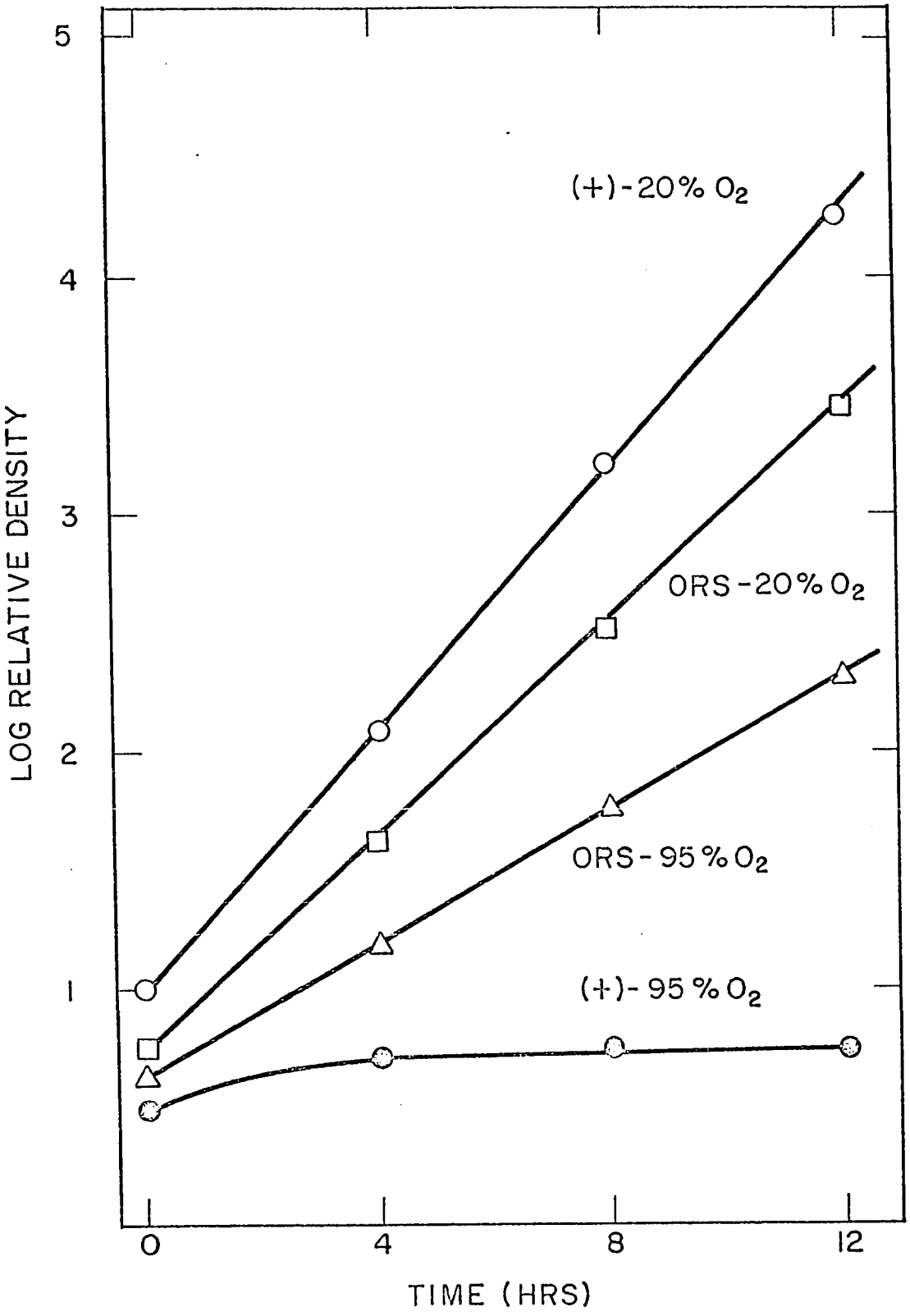

a
b
(+)-DCMU
(+)-Glu
ORS-DCMU
ORS-Glu
ORS
(+)
DRY WEIGHT (mg cells/ml)
LOG RELATIVE DENSITY
TIME (HRS)
0.5
1.0
1.5
2.0
2.5
1
2
3
4
5
4
8
12
16
20
24
28
2
4
6
8
10
12
14

Figure 25 - Heterotrophic growth of (+) and ORS in the light on 0.5% glucose plus 10^{-5}M DCMU. ○—○ (+)-20%O_2; □—□ ORS-20%O_2; △—△ ORS-95%O_2; ●—● (+)-95%O_2.


5
4
3
2
1
LOG RELATIVE DENSITY
(+)-20% O2
ORS-20% O2
ORS-95% O2
(+)-95% O2
0
4
8
12
TIME (HRS)

measured with the YSI oxygen electrode as described in the methods section. Rates were determined under high and low oxygen atmospheres for both strains grown autotrophically at 20% and 95% oxygen.

The photosynthetic rates are presented in Table 1. Wild type cultures show a slightly higher rate than ORS on a weight basis, but the larger ORS cells produce more oxygen when expressed on a cell basis. Both cultures exhibit the Warburg effect when photosynthesis is measured under high oxygen. ORS grown under 95% oxygen retains 40% of its photosynthetic capacity while (+) cultures, exposed for several days to 95% oxygen, are not able to photosynthesize in the high oxygen atmosphere. The ORS cultures grown on 95% oxygen are also capable of complete and immediate recovery of full photosynthetic capacity when switched to air level oxygen. Wild type cultures show permanent damage after extended 95% oxygen exposure and are not capable of recovery over several hours.

The respiratory rates, measured immediately following a photosynthetic period, are presented in Table 2. Characteristically, the respiratory rate was rapid and linear at first and then began to decrease slowly, finally approaching a low steady state endogenous rate.

Table 1. - Photosynthetic rates of (+) and ORS cultures grown on 20% and 95% O_2 - 5% CO_2 and measured in high and low oxygen atmospheres.

Table 2. - Respiratory rates of (+) and ORS cultures grown on 20% and 95% O_2 - 5% CO_2 and measured after a photosynthetic period in high and low oxygen atmospheres.

Culture Condition in % O_2	Measured on 20% O_2		Measured on 80% O_2		% inhibition
	$\mu l\ O_2$/mg cell/hr	$\mu l\ O_2$/cell/hr	$\mu l\ O_2$/mg cell/hr	$\mu l O_2$/cell/hr	
(+)-20% O_2	580	4.0×10^{-6}	260 → 0	$2.3 \times 10^{-6} \rightarrow 0$	45%
(+)-95% O_2	Not Constant Low and Rising	——	NONE	NONE	100%
ORS-20% O_2	480	5.25×10^{-6}	200	2.1×10^{-6}	40%
ORS-95% O_2	500	7.1×10^{-6}	220	2.38×10^{-6}	40%

CULTURE CONDITIONS IN % OXYGEN	MEAS. ON 20% O_2		MEAS. ON 80% O_2		
(+)-20% O_2	90	0.63×10^{-6}	500	3.8×10^{-6}	
(+)-95% O_2	Less than 5	LOW	—	—	
ORS-20% O_2	70	0.5×10^{-6}	340	3.6×10^{-6}	
ORS-95% O_2	20	0.2×10^{-6}	152	2.0×10^{-6}	

These results are similar to those reported by Ward, Pulich and Hall (1969) except that use of the YSI oxygen monitor allowed for a more precise measurement of the post-photosynthetic respiratory burst. As seen in the data, this burst indicated much higher respiratory rates when the cultures were exposed to high oxygen. The ORS cultures grown on high oxygen, however, demonstrated a lower rate generally. This is in agreement with earlier results (Ward et al, 1969). Wild type cultures grown on high oxygen show a very low respiratory rate indicative of oxygen poisoning.

These studies with random cultures pointed to basic differences in size, structure, and physiology between (+) and ORS. It was hoped that the use of synchronized cultures could, through following the effects of oxygen on the development of the cultures, provide additional information concerning the toxicity-resistance phenomenon. This was particularly interesting in light of the large amount of study by Sorokin (1951, 1957, 1960, 1963) and Sorokin and Krauss (1959, 1961, 1962) on the techniques and effects of synchrony on algal cultures using the (+) strain as a study organism.

Culture conditions, as described in the methods section, were established and then various light-dark regimens were tested to arrive at the one most favorable for the

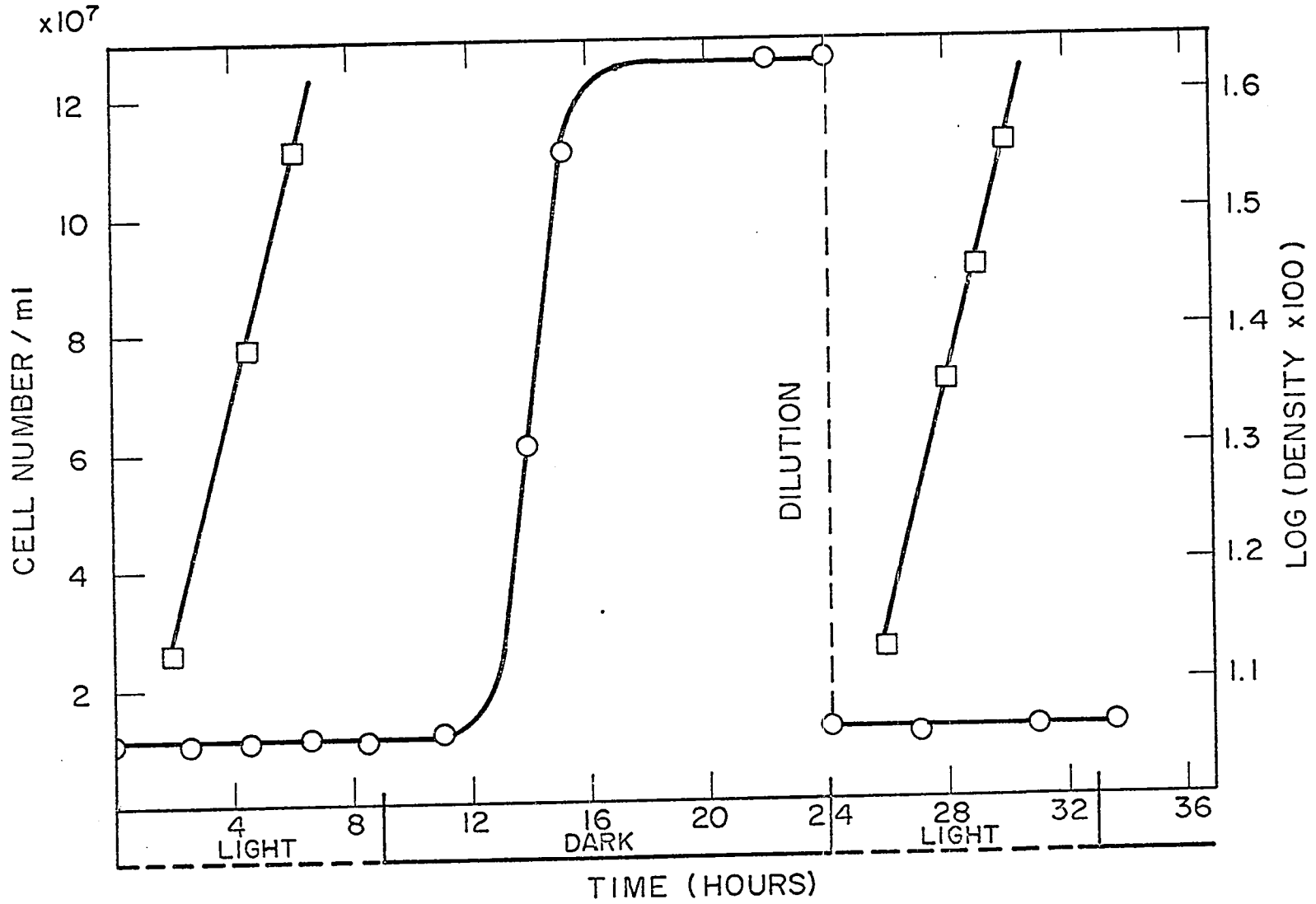
synchronous growth of each culture.

The 9 hour light, 15 hour dark regimen used by Sorokin and Myers (1956) provided good synchrony of the (+) strain on air-5% CO_2. After 9 hours of continuous illumination, the cells began to divide. This division continued in the dark, but the absence of a carbon source halted growth of the autospores until the beginning of the next light period. After three such cycles, 95% of the cells in the culture were autospores at the beginning of the light period. Figure 26 shows the typical synchronous growth for (+)-air at 39°C. The toxic effects of oxygen on this strain precluded any attempt to synchronize cultures under a 95% oxygen atmosphere. Studies of oxygen effects on this strain were done after synchrony was first established in air.

Difficulty was encountered in the attempts to establish comparable synchrony of ORS cultures exposed to air-5% CO_2. The best patterns were obtained after 4 to 5 cycles of 10.5 to 11 hours of light preceeding 13.5 to 13 hours of darkness. A typical synchronous growth pattern for ORS-air is shown in Figure 27. Synchrony of these cultures would "break down" after about four days and it became necessary to re-establish the pattern.

ORS cultures were more easily synchronized under 95% oxygen + 5% CO_2 atmosphere. The presence of high oxygen appeared to stabilize the development of this strain. Eleven to 12 hours of light followed by 13 to 12 hours of darkness were required for synchronization as seen in Figure 28.

Figure 26 - Synchrony of (+) culture on air-5% CO_2 showing cell number (0-0-0) and cell density (□—□—□).


x10⁷
12
10
8
6
4
2
CELL NUMBER / ml
1.6
1.5
1.4
1.3
1.2
1.1
LOG (DENSITY x100)
DILUTION
4
8
12
16
20
24
28
32
36
LIGHT
DARK
LIGHT
TIME (HOURS)

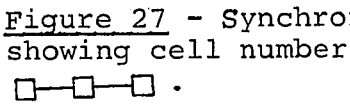


Figure 27 - Synchrony of ORS culture on air-5% CO_2 showing cell number O——O——O ; and cell density □—□—□ .

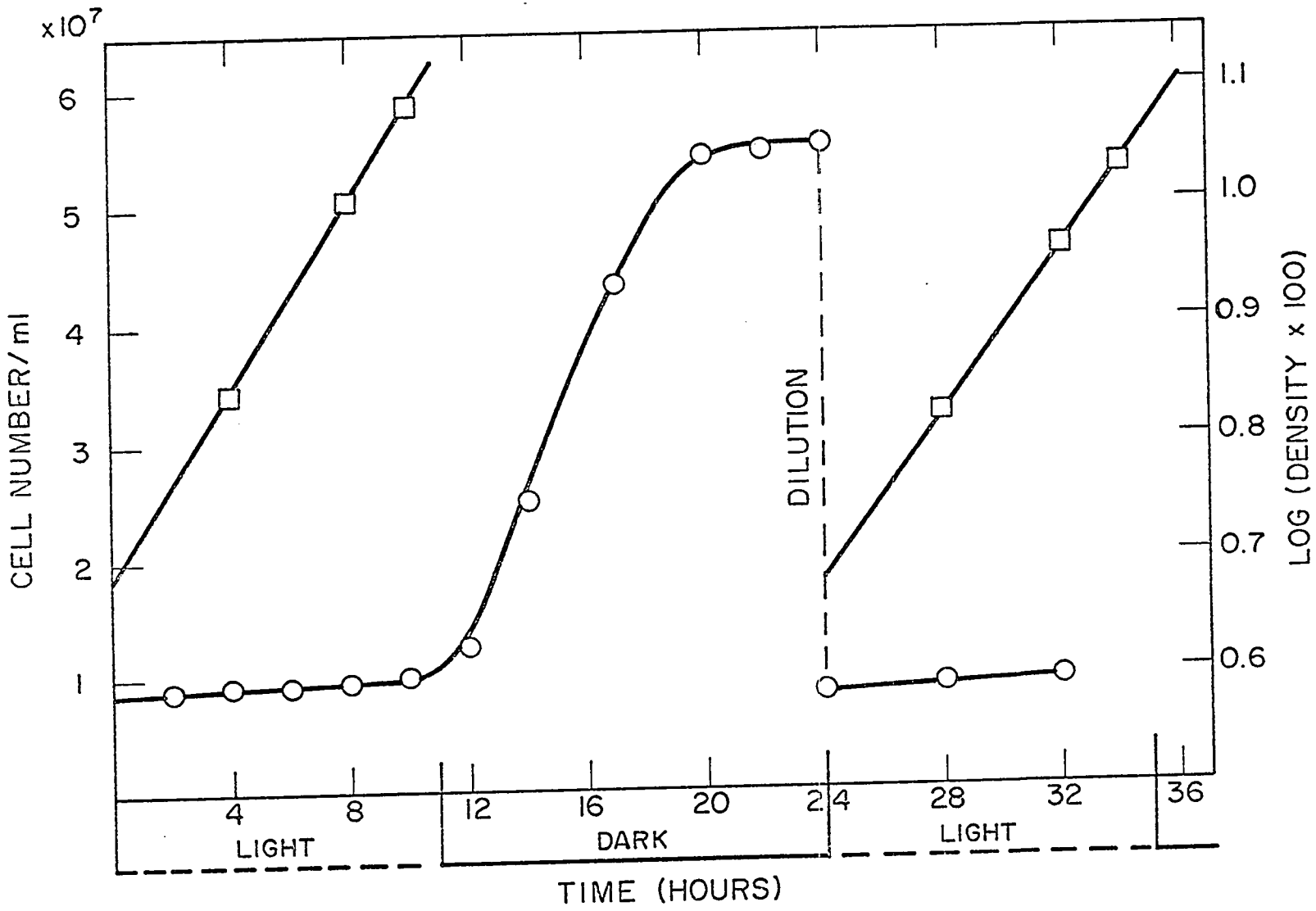
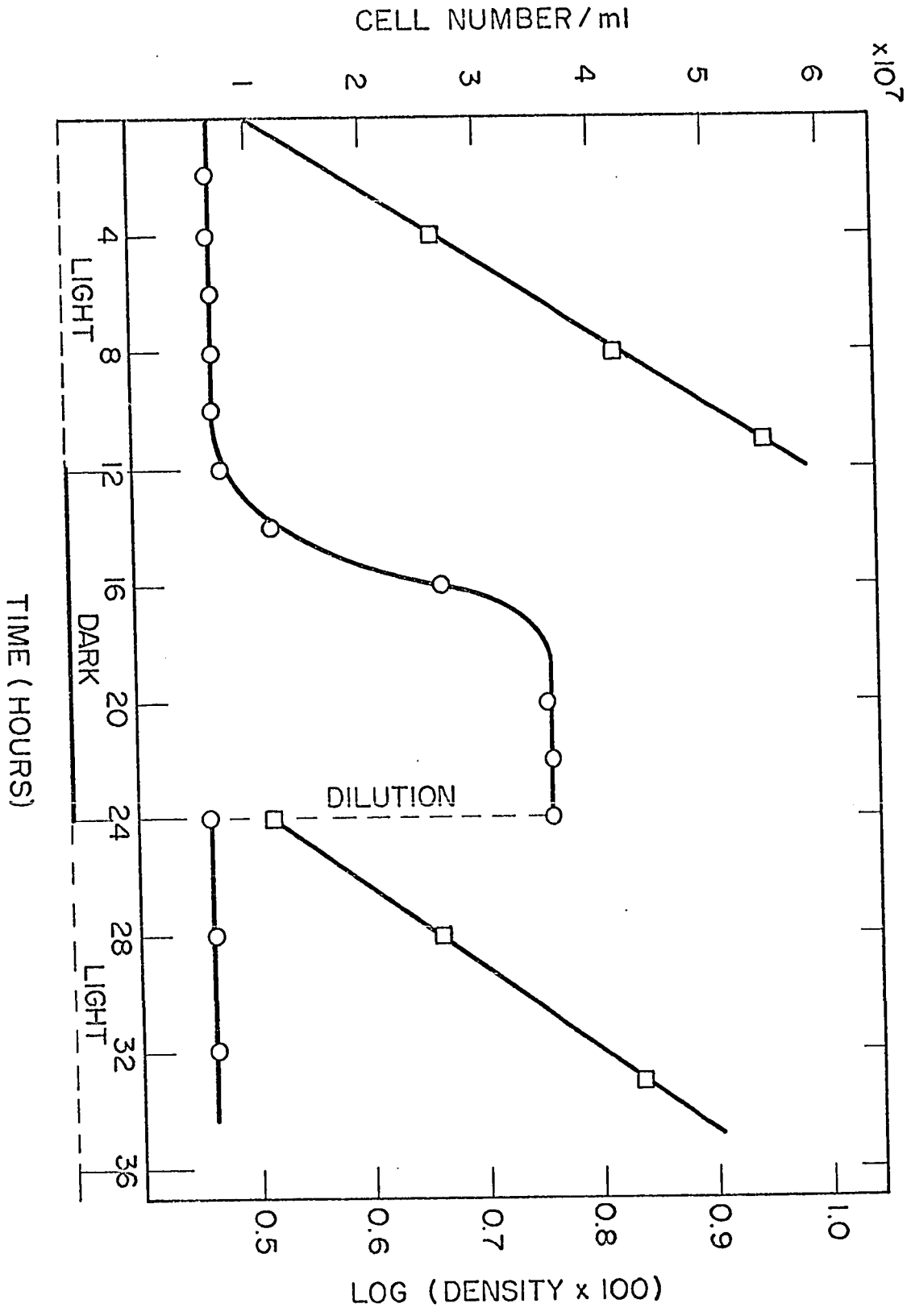

x10⁷
6
5
4
3
2
1
CELL NUMBER/ml
1.1
1.0
0.9
0.8
0.7
0.6
LOG (DENSITY x 100)
DILUTION
4
8
12
16
20
24
28
32
36
LIGHT
DARK
LIGHT
TIME (HOURS)

Figure 28 - Synchrony of ORS culture on 95% oxygen-5% CO_2 showing cell number (O——O——O) and cell density (□—□—□).

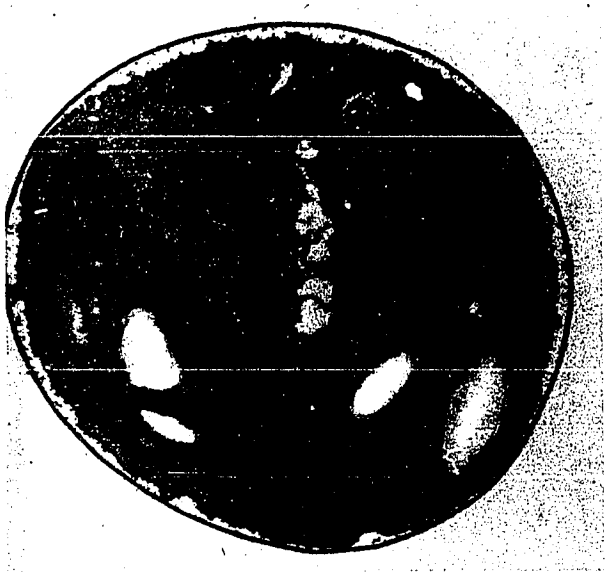

CELL NUMBER / ml
x10^7
1
2
3
4
5
6
TIME (HOURS)
LIGHT
DARK
LIGHT
4
8
12
16
20
24
28
32
36
DILUTION
LOG (DENSITY x 100)
0.5
0.6
0.7
0.8
0.9
1.0

The average number of cells produced during one cycle (division number) provided a means of determining the average number of autospores produced during division of each cell in the culture. The division number for (+)-air was 12 indicating that the cells produced either 8 or 16 autospores per division. The ORS cultures produced less than half as many autospores judging from their division number of between 5 and 6. Cultures grown under air atmosphere showed a slightly higher number (around 6) than those grown in 95% oxygen. Wild type cultures, synchronized under air + 5% CO_2 and then placed under high oxygen, had division numbers which were also low (between 2 and 4) indicating that oxygen had a pronounced effect on division of these cultures.

The synchronized cultures presented an opportunity to compare the division of (+) and ORS cultures by use of electron microscopy. Cells were harvested from synchronous cultures at the time when autospore release was just beginning, and prepared for electron microscopy as described in the methods section. Cultures of (+), synchronized on air and placed under 95% oxygen, were harvested at the same time as the (+)-air cultures.

The normal division process of (+)-air is shown in Figures 29, 30 and 31. The nucleus (N) and chloroplast (CHl) divide, and the first division plane (DP) is formed by a series of vesicles. There is a decrease in the amount of osmiophilic material (OSM). This process con-

Figure 29 - a) Division of (+)-air cells showing the first nuclear (N) and chloroplastic (Chl) divisions and the formation of the first division plane (DP). b) Further stage of (+) division with three nuclei (N) and chloroplasts (Chl). 19,000X



a

b

Figure 30 - Division of (+) cells grown under normal oxygen showing further development of individual autospores. 29,000X, 24,000X

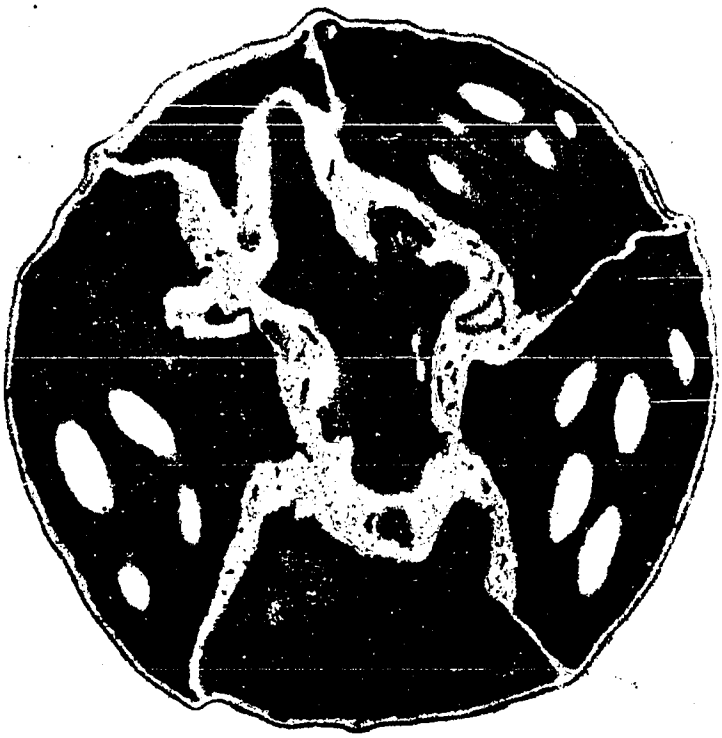
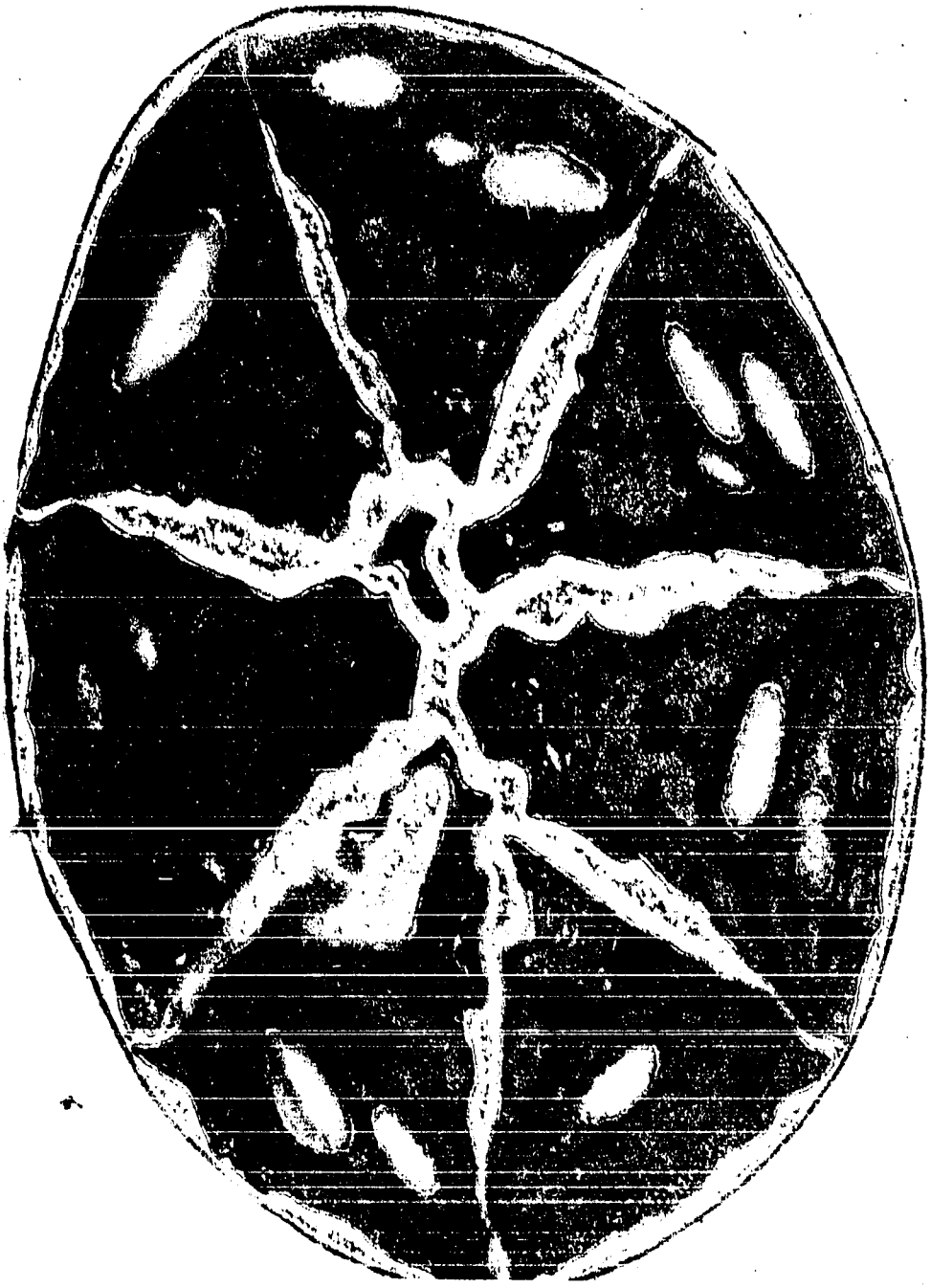


Figure 31 - Mature mother cell of (+) grown on 20% oxygen. This micrograph was taken just prior to autospore release. 31,500X



tinues through a series of divisions until 8 or 16 daughter cells have formed within the mother cell wall. The mother cell then ruptures releasing the preformed daughters.

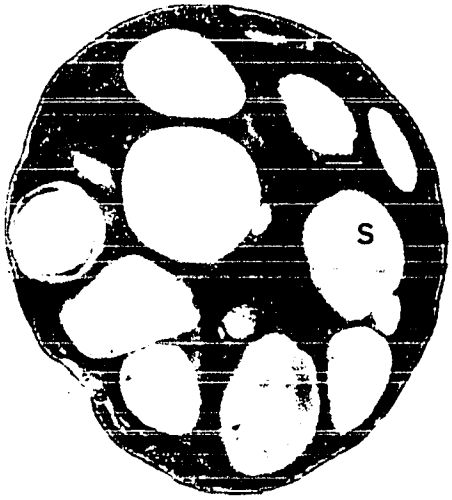
There were very few "dividing" cells in the oxygen exposed (+) cultures. After 9 hours of exposure the majority of the cells were small and gorged with starch (S) and osmiophilic material (OSM) as seen in Figure 32. Those cells which appeared to be dividing, both in this and the previous fixation, show complete disorganization, Figures 33, 34 and 35. The division planes (DP) show no relationship to nuclear and chloroplastic division. Osmiophilic material (OSM) and vacuolization (V) are abundant. When division is successful (Figure 36) it produces few autospores of questionable viability.

ORS-air cultures show "normal" division patterns, but appear to have fewer autospores as would be expected from the division number. Figures 37 and 38 are typical of ORS division. Figures 38 and 39 show a different mode of division which was seen only in ORS cultures. This binary division phenomenon is also observed under the light microscope and appears to occur in about 5% of the culture.

ORS cultures synchronized on 95% oxygen appear normal and similar to the air grown cultures. Figures 40 and 41 show the progressive development of autospores in these cultures. Again, the number of autospores produced agrees with the division number calculated from the synchronized cultures. The progression of the binary mode of division

Figure 32 - Wild type cells after nine hours of exposure to 95% oxygen demonstrating accumulated starch (S) and osmiophylic material (OSM). 28,300X, 19,500X, 19,600X


S


S


S

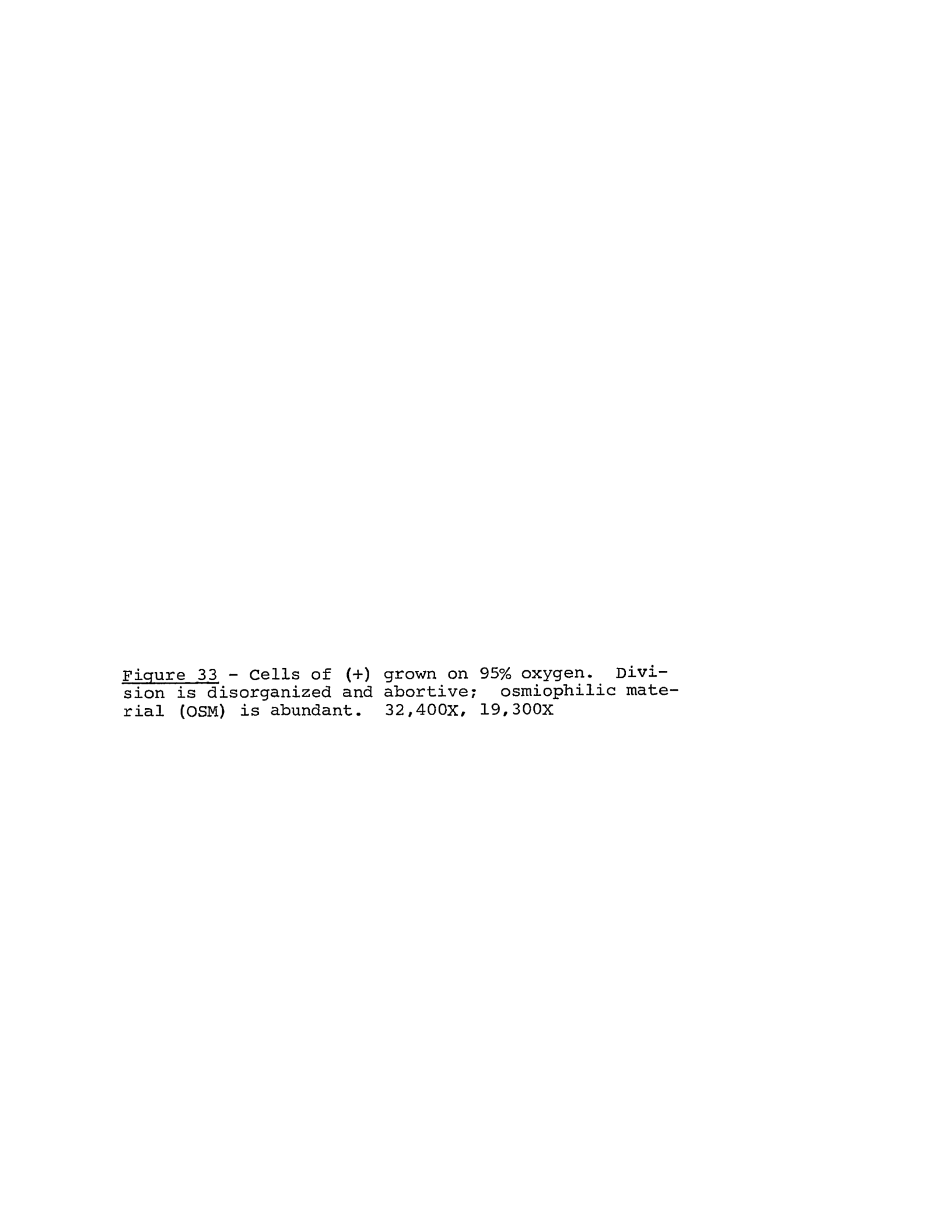


Figure 33 - Cells of (+) grown on 95% oxygen. Division is disorganized and abortive; osmiophilic material (OSM) is abundant. 32,400X, 19,300X

Figure 34 - Further evidence of poor division in (+) cultures grown on high oxygen. Division planes do not correspond with chloroplastic and nuclear division. 24,000X, 28,600X

Figure 35 - Wild type cell grown on high oxygen showing cytoplasmic division (C) prior to nuclear and chloroplastic division. Note large osmiophilic deposits (OSM) and mitochondrion (M). 39,000X

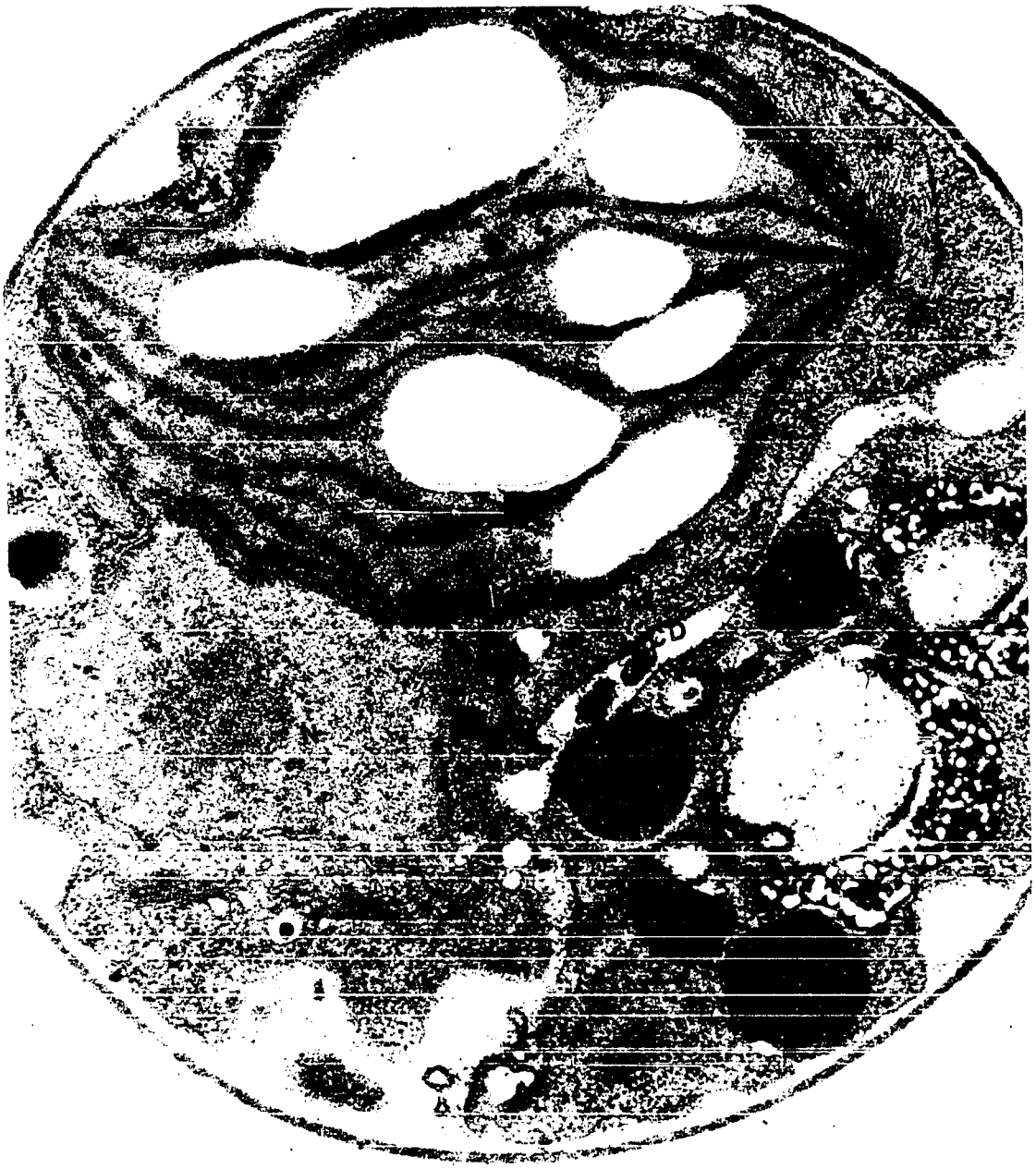


Figure 36 - Micrograph showing "successful" division into two autospores of (+) cell grown on 95% oxygen. The mother cell wall (MCW) has just ruptured. 52,000X

Figure 37 - Typical normal division of ORS cells grown on air level oxygen. Lower number of autospores formed is predicted from division number. 27,400X, 19,400X

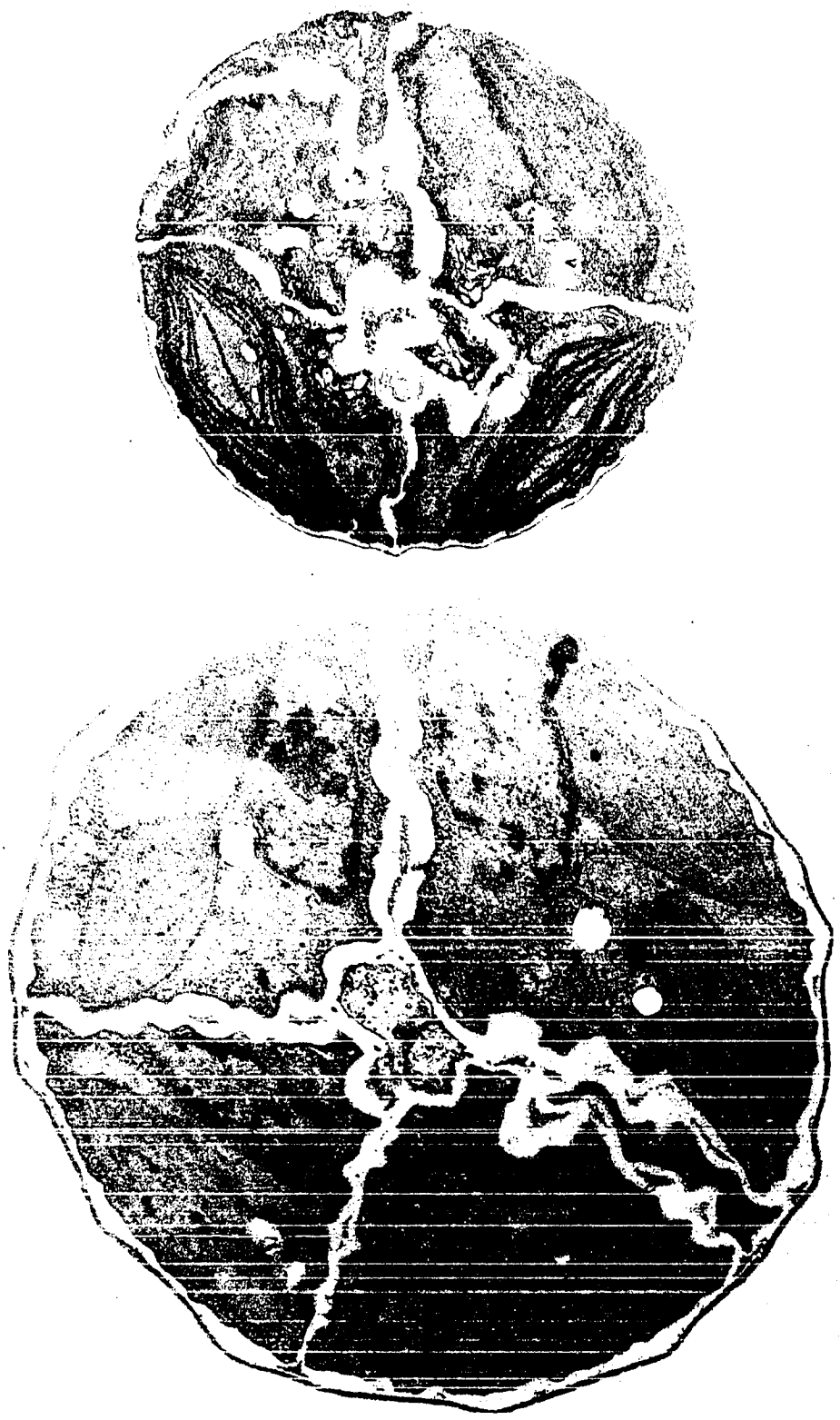
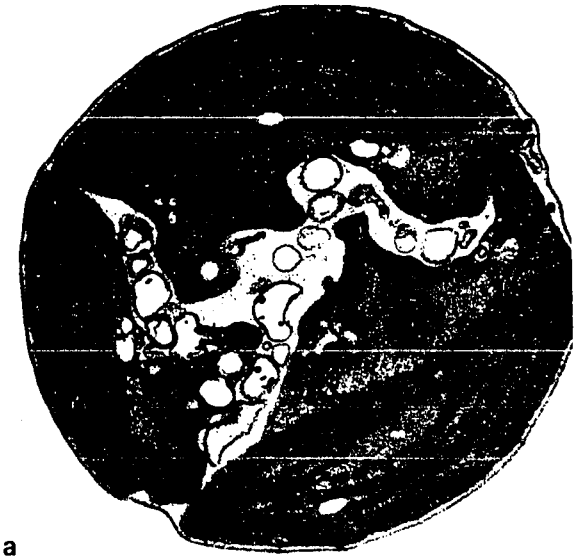


Figure 38 - a) Normal division of ORS cell grown in 20% oxygen. b) Binary division in ORS showing typical "dumbell" shape. 20,000X, 24,300X



a

b

Figure 39 - ORS cell undergoing binary division. Plane of division (DP) is seen and the beginning of the new cell wall has started (arrow). 31,400X

Figure 40 - Early stage of normal autospore formation in ORS grown with 95% oxygen atmosphere. 28,300X

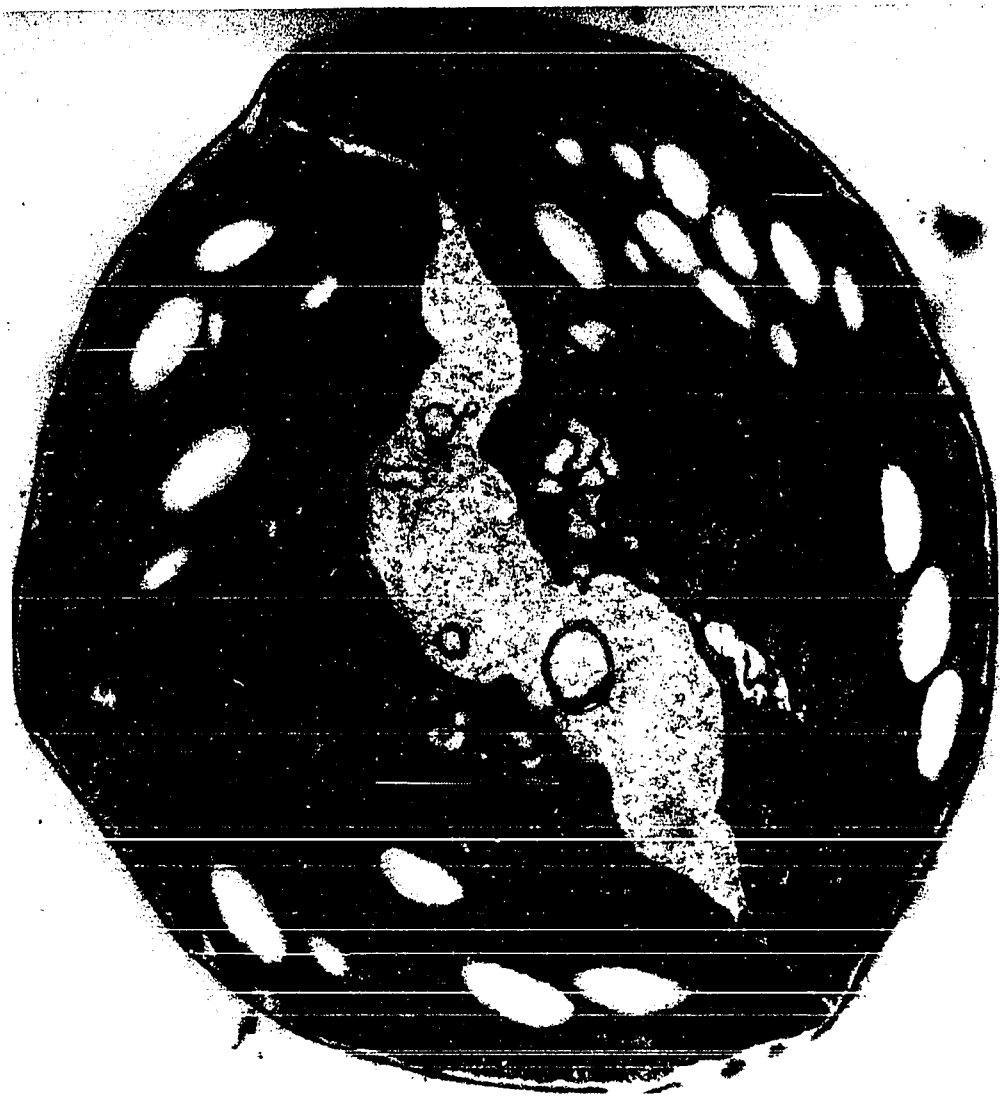
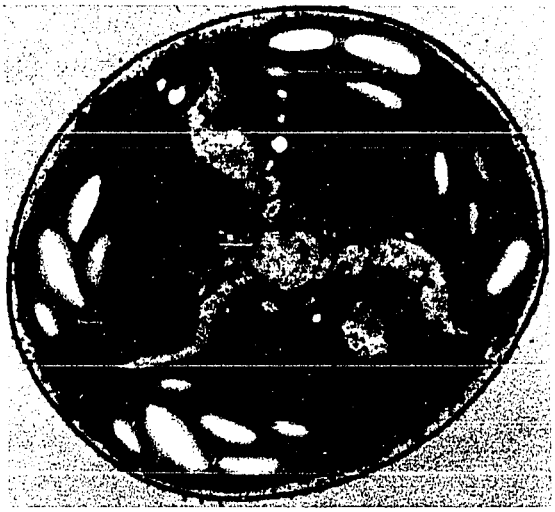
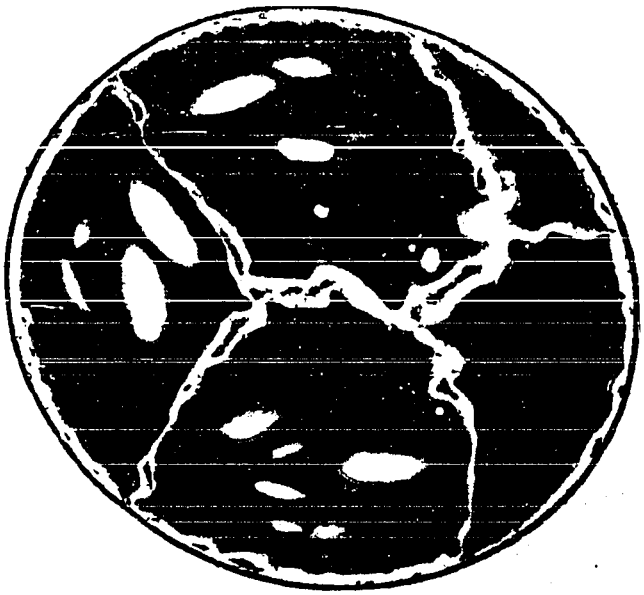


Figure 41 - Progressive stages of cell division in two cells of ORS grown in 95% oxygen. 10,400X, 20,000X





in ORS is shown in Figures 42 and 43. Nuclear and chloroplastic division are followed by the formation of vesicles along the division plane (Figure 43a). Then a cleft (Figure 43b) begins at one edge of the cell and moves progressively across the narrow region finally dividing the two cells (Figure 43c).

These data again demonstrate, on the ultrastructural level, differences between (+) and ORS strains and the toxic effects of oxygen on the (+) cultures.

The synchronized cultures were employed to study the physiology of the two strains under 20% and 95% oxygen during their development. Samples were harvested from the culture flasks at various times during the life cycle and used to measure growth rate, photosynthesis and respiration.

The growth rates of (+)-air, ORS-air and ORS O_2 are graphically presented along with the synchronous cell number patterns (Figures 26, 27 and 28) calculated from culture density as described in the methods section. They are all slightly lower than those calculated from log growth cultures, but are in agreement with similar data presented by Schmidt (1966, 1969). Wild type had a rate of 8 doublings/day, ORS-air, 6.5 doublings/day and ORS O_2, 4.5 doublings/day. Growth of the cultures stopped when the dark period started, and the density and dry weight remained essentially constant until the lights came on again. Growth rates of (+)-O_2 from synchronized cultures will be discussed later.

Figure 42 - Electron micrograph of ORS cell dividing by binary mode. Nuclear and chloroplastic division are complete. 29,500X

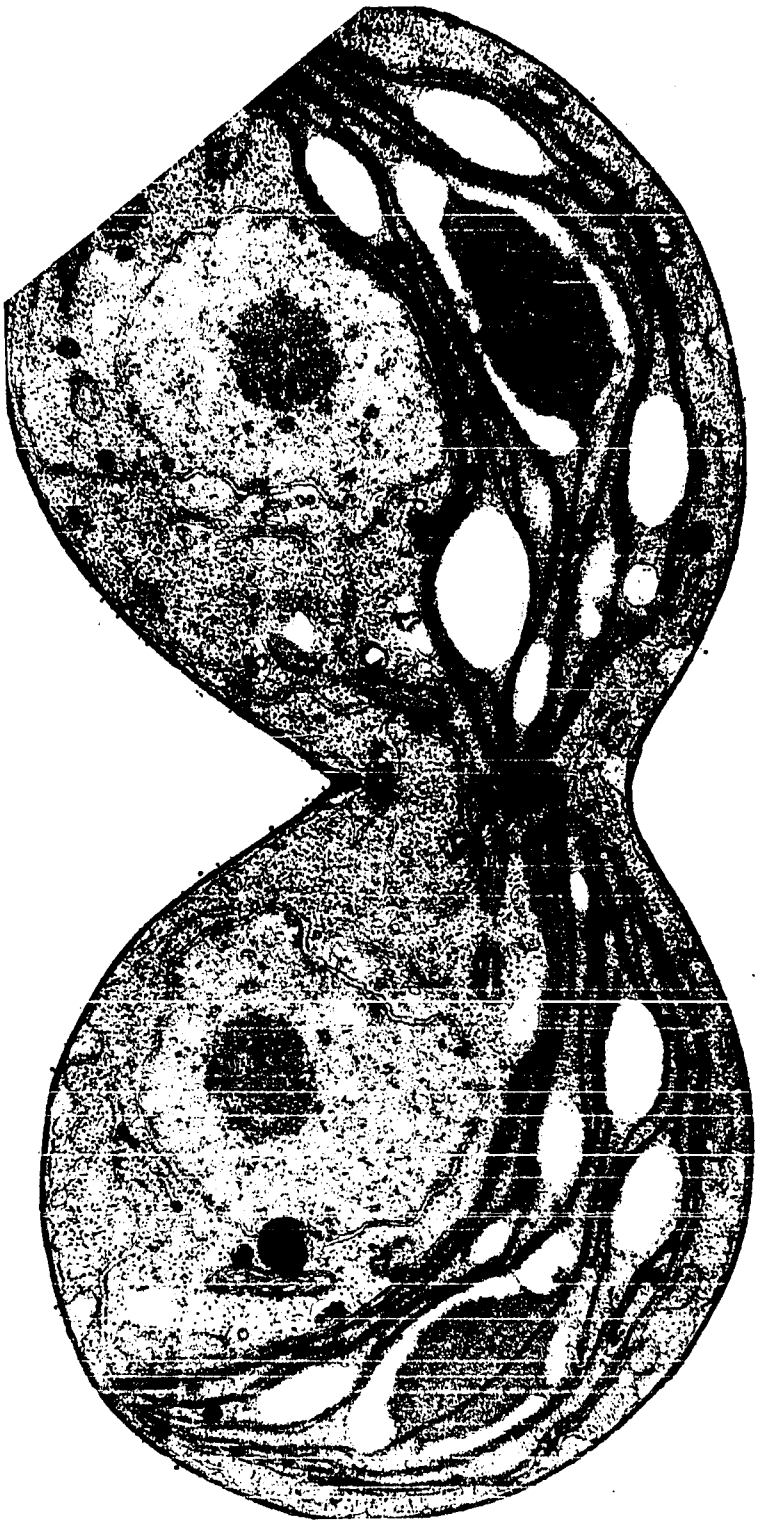
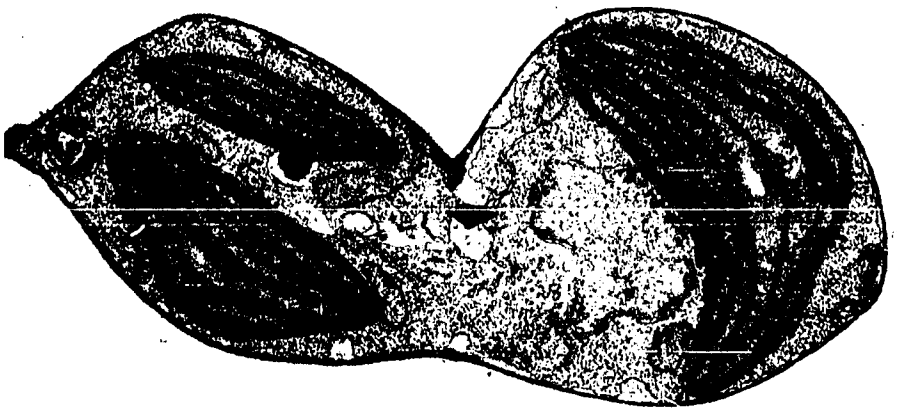
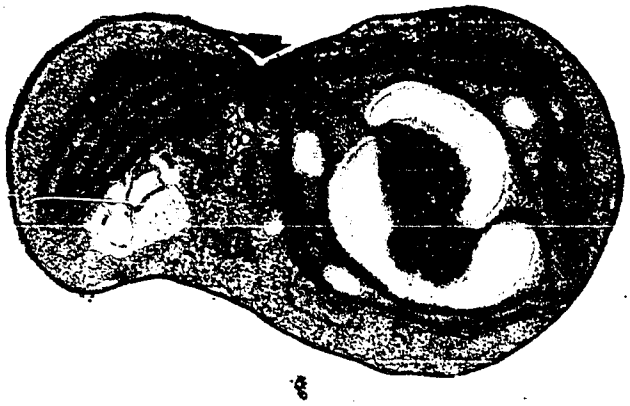


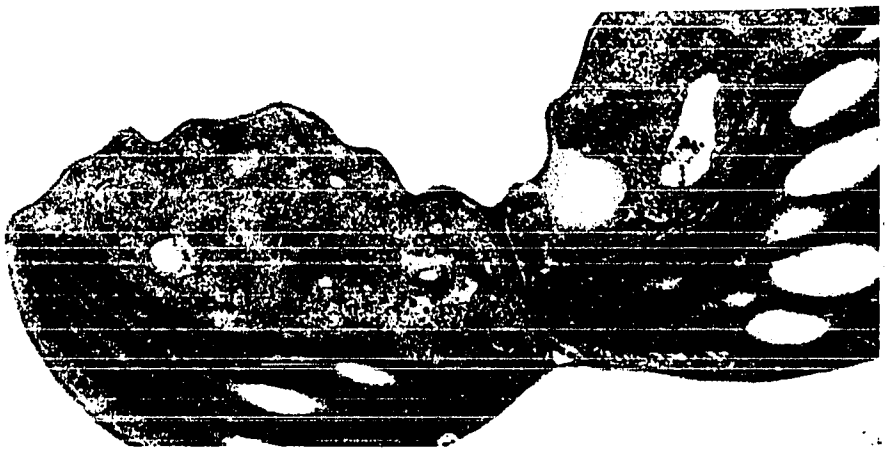
Figure 43 - Stages of binary division. a) vesicle formation b) beginning of cell wall formation c) cytoplasmic separation complete. 20,000X, 20,000X, 26,400X



a



b



c

Cell weights, calculated from dry weight and cell number data, are compared for (+)-air, ORS-air, and ORS-O_2 in Figure 44. The weight increases until the time of division for each culture and then declines as division progresses, reaching a minimum when all the cells in the culture have completed division.

The photosynthetic and respiratory rates of the cultures during development remained fairly constant when expressed on a dry weight basis. Wild type cultures had higher rates than ORS-air which were higher than ORS-O_2 by a small amount. These results compare well with those seen for random cultures. There was an initial increase in both metabolic functions upon illumination, and both decreased in all synchronous cultures following the light period as carbon became limiting.

Photosynthesis and respiration increased throughout the development of the cultures when the rates were expressed on a cellular basis. ORS, because of its larger size, had higher rates than (+) cultures, but the (+) cultures increased faster as expected from the growth rates and synchrony patterns shown previously. Comparative plots of these physiological parameters are presented in Figures 45 and 46. Both photosynthetic and respiratory capacity fell off rapidly upon darkening.

The effect of oxygen on (+) and ORS cultures during development was determined by harvesting the cells from synchrony on air and exposing them to 95% oxygen in test

Figure 44 - Comparative cell weights of (+)-20% O_2, ORS-20% O_2, and ORS-95% O_2 during a 24 hour synchrony cycle. ●—● (+)-20% O_2; ▲—▲ ORS-20% O_2; ■—■ ORS-95% O_2.

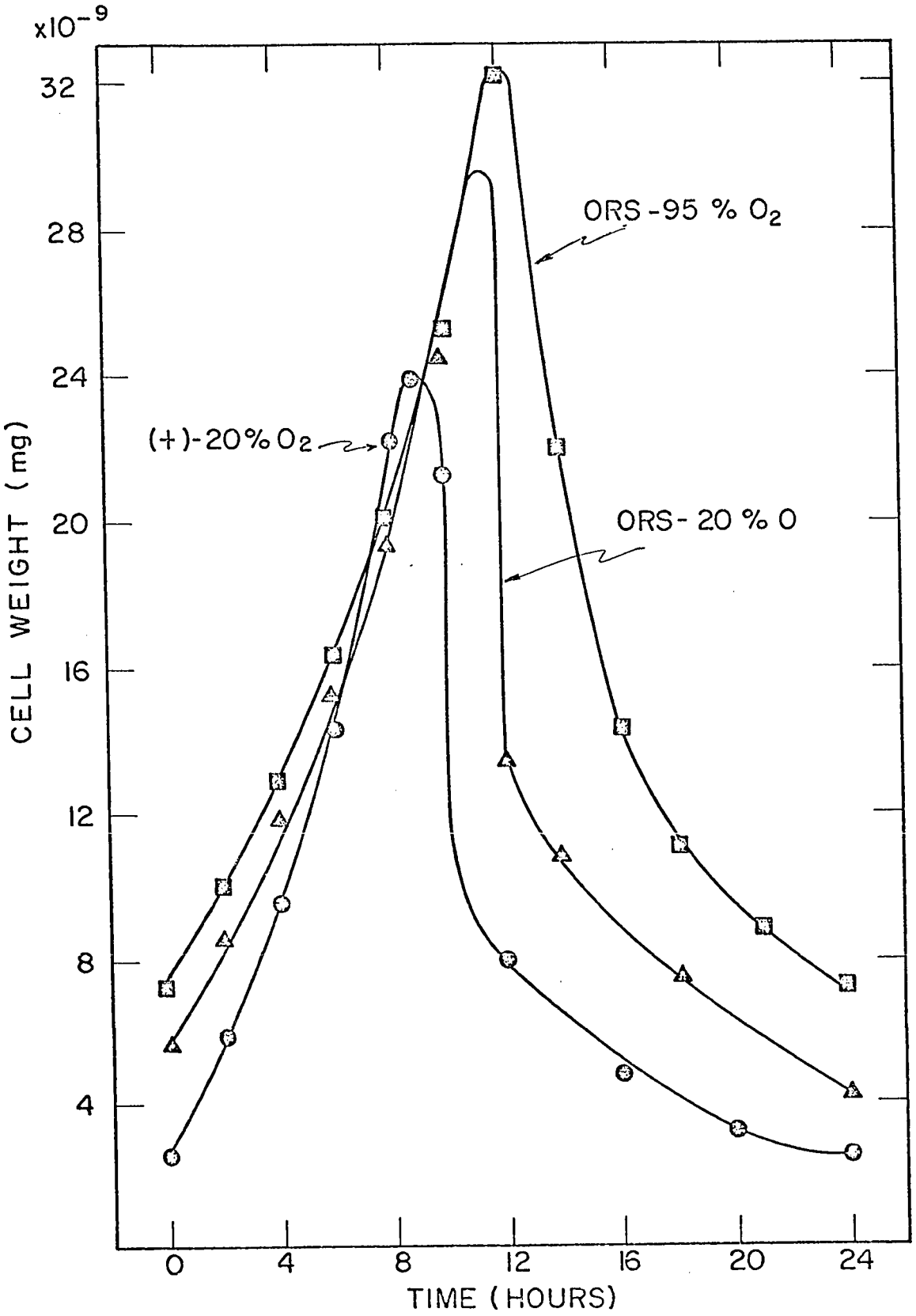

x10⁻⁹
32
28
24
20
16
12
8
4
CELL WEIGHT (mg)
ORS-95 % O2
(+)-20% O2
ORS-20 % O
0
4
8
12
16
20
24
TIME (HOURS)

Figure 45 - Comparative photosynthetic rates per cell of (+)-20% O_2, ORS-20% O_2, ORS-95% O_2 during a 24 hour synchrony cycle. ●—● (+)-20% O_2; ■—■ ORS-20% O_2; ▲—▲ ORS-95% O_2. Solid line during light period, dashed line during dark period.

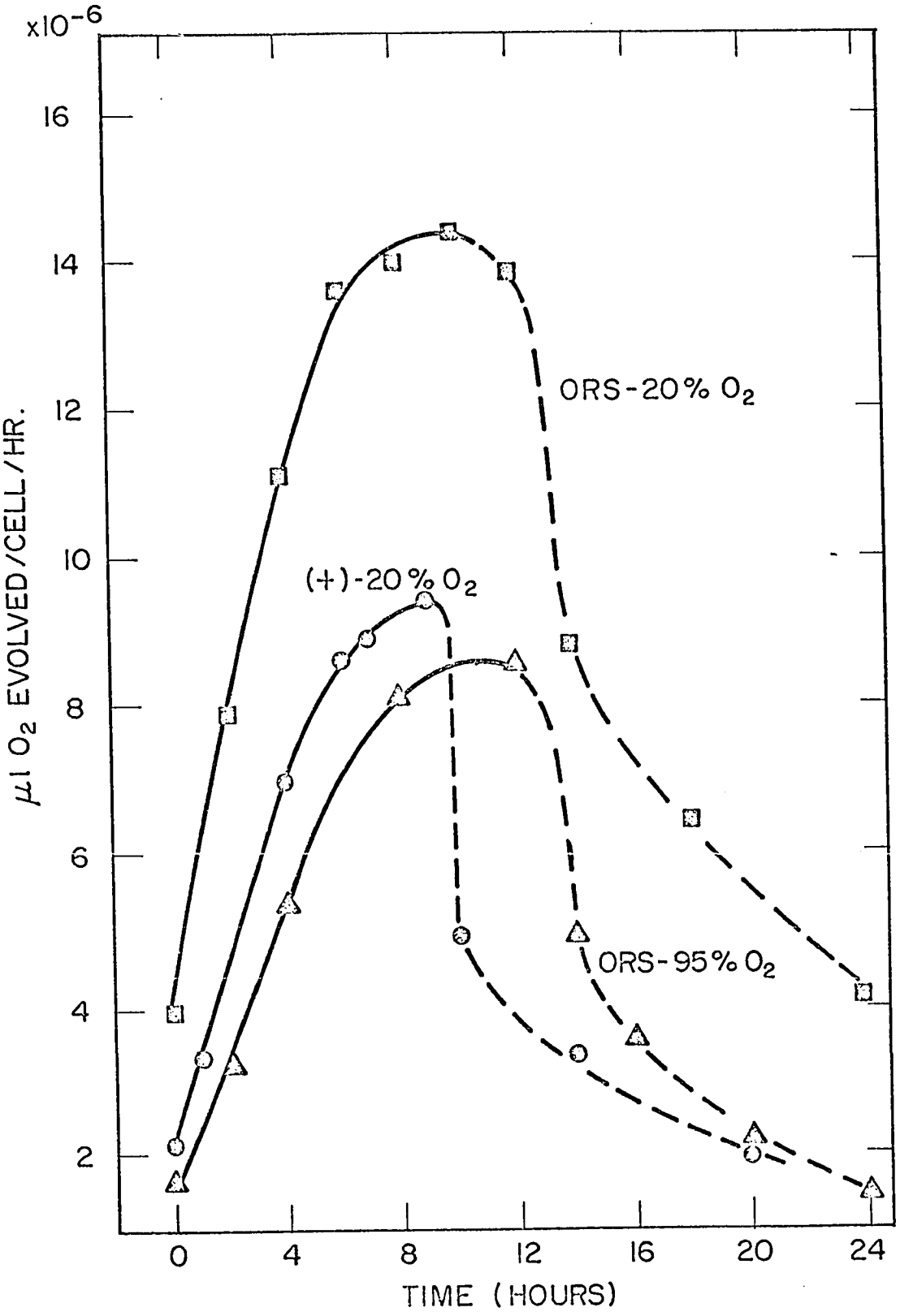
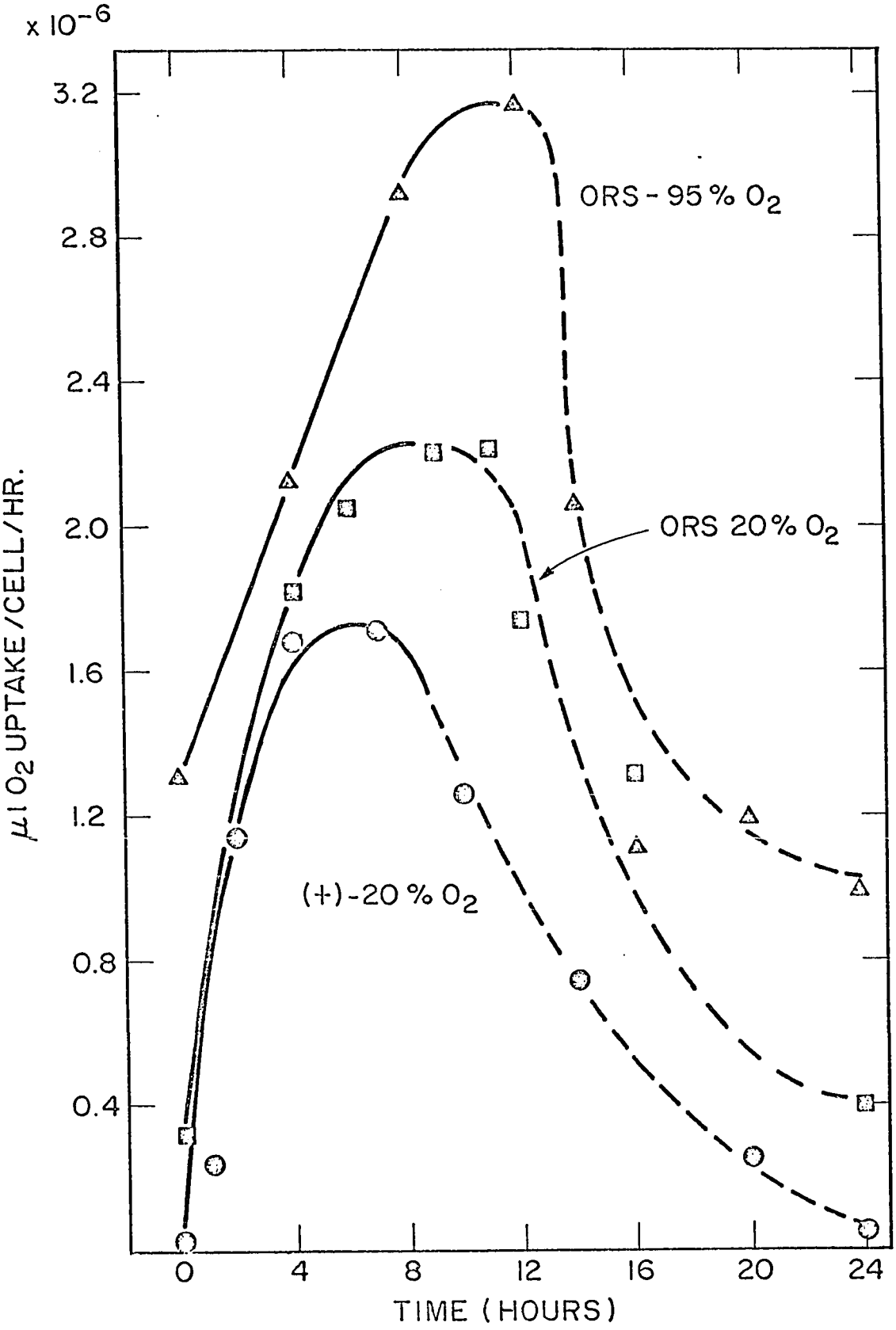

x10⁻⁶
16
14
12
10
8
6
4
2
μl O₂ EVOLVED/CELL/HR.
ORS-20% O₂
(+)-20% O₂
ORS-95% O₂
0
4
8
12
16
20
24
TIME (HOURS)

Figure 46 - Comparative respiratory rates per cell during one 24 hour synchrony cycle. ●—● (+)-20% O_2; ■—■ ORS-20% O_2; ▲—▲ ORS-95% O_2 Solid line is during light period, dashed line is during the dark period.


x 10⁻⁶
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
μl O2 UPTAKE/CELL/HR.
ORS - 95% O2
ORS 20% O2
(+)-20% O2
0
4
8
12
16
20
24
TIME (HOURS)

tube cultures. Growth, photosynthetic, and respiratory rates were followed on cultures harvested at various stages of development. Growth of ORS was not appreciably affected at any stage of growth (Figure 47) while (+) cultures were severely inhibited at all stages of development (Figure 48).

The "rate" of oxygen toxicity on (+) cells exposed to 95% oxygen at various stages of development was linear for several hours as seen in Figure 49. This provided a means of determining the relative severity of the oxygen toxicity phenomenon on (+) cells of different stages of maturity. Figure 49 shows that the young (zero hour) and the older or most mature (8 hour) cells show the most pronounced toxic response while the intermediate stages of development are less dramatically affected. These results correlate well with the effects of oxygen on division seen in the electron microscopic studies of (+) on 95% oxygen.

A similar study was carried out on the effects of oxygen on photosynthesis through the development of the cultures. ORS showed the same type of Warburg effect on all stages of development and the inhibition started immediately and remained constant. Wild type cultures, however, were immediately inhibited and the photosynthetic rate continued to decrease as growth in high oxygen continued. A typical experiment, where zero hour cells of (+) were followed through the nine hour growth cycle, is presented in Table 3. While the photosynthetic rate remained high in the cultures developing under air level oxygen, it dropped continuously

Figure 47 - Effect of oxygen on the growth of ORS cultures at various stages of development. Circles are zero hr cells; squares are 3 hr cells; triangles are 6 hr cells. Open figures and dashed line show growth on 95% oxygen. Solid figures and solid line show growth in 20% oxygen.

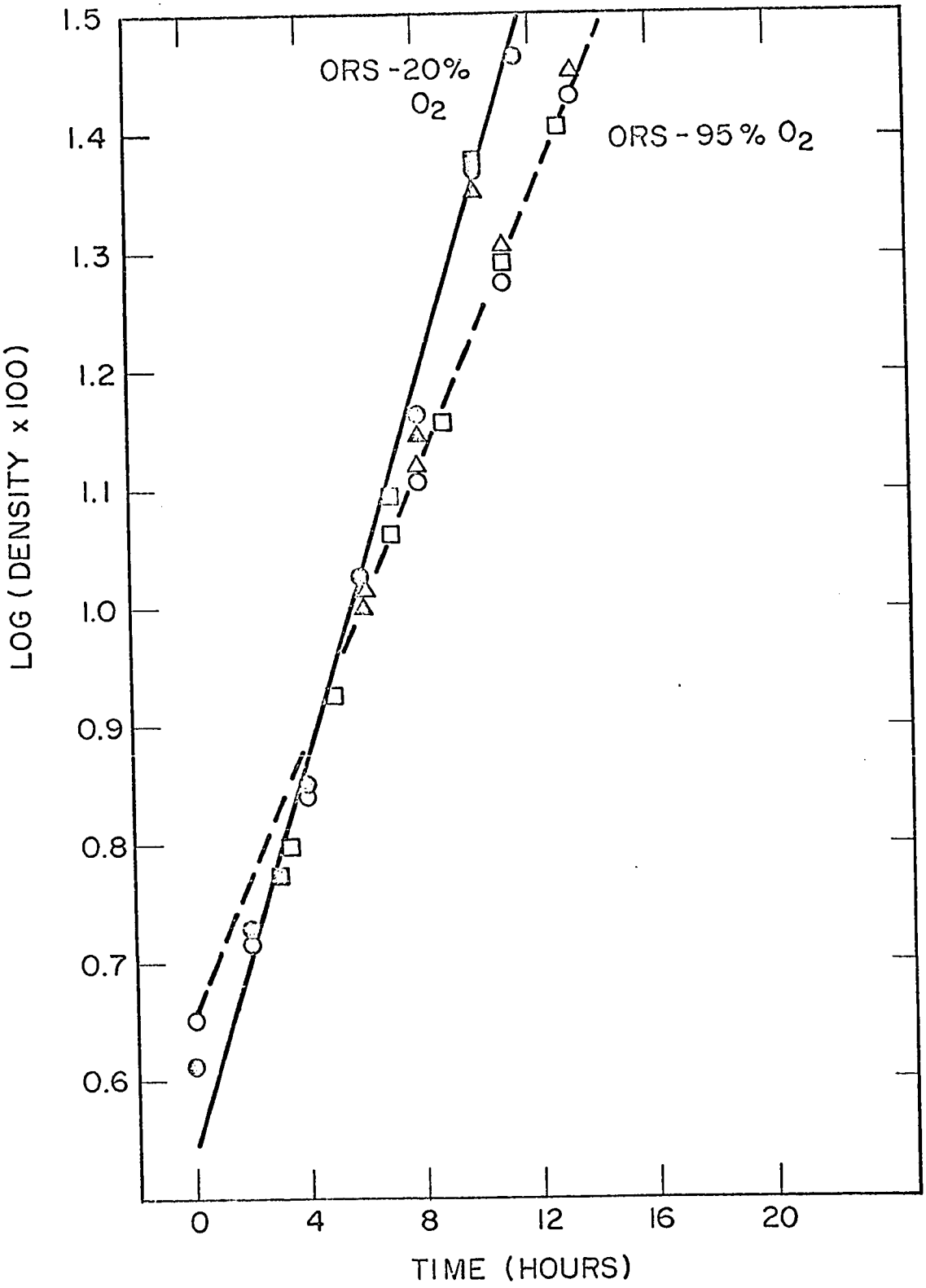

ORS - 20% O2
ORS - 95% O2
LOG (DENSITY x 100)
TIME (HOURS)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0
4
8
12
16
20

Figure 48 - Effect of oxygen on the growth of (+) cultures at various stages of development. Circles are zero hr cells; squares are 3 hr cells; triangles are 6 hr cells. Open figures and dashed lines show growth on 95% O_2. Solid figures and solid line show growth on 20% O_2.

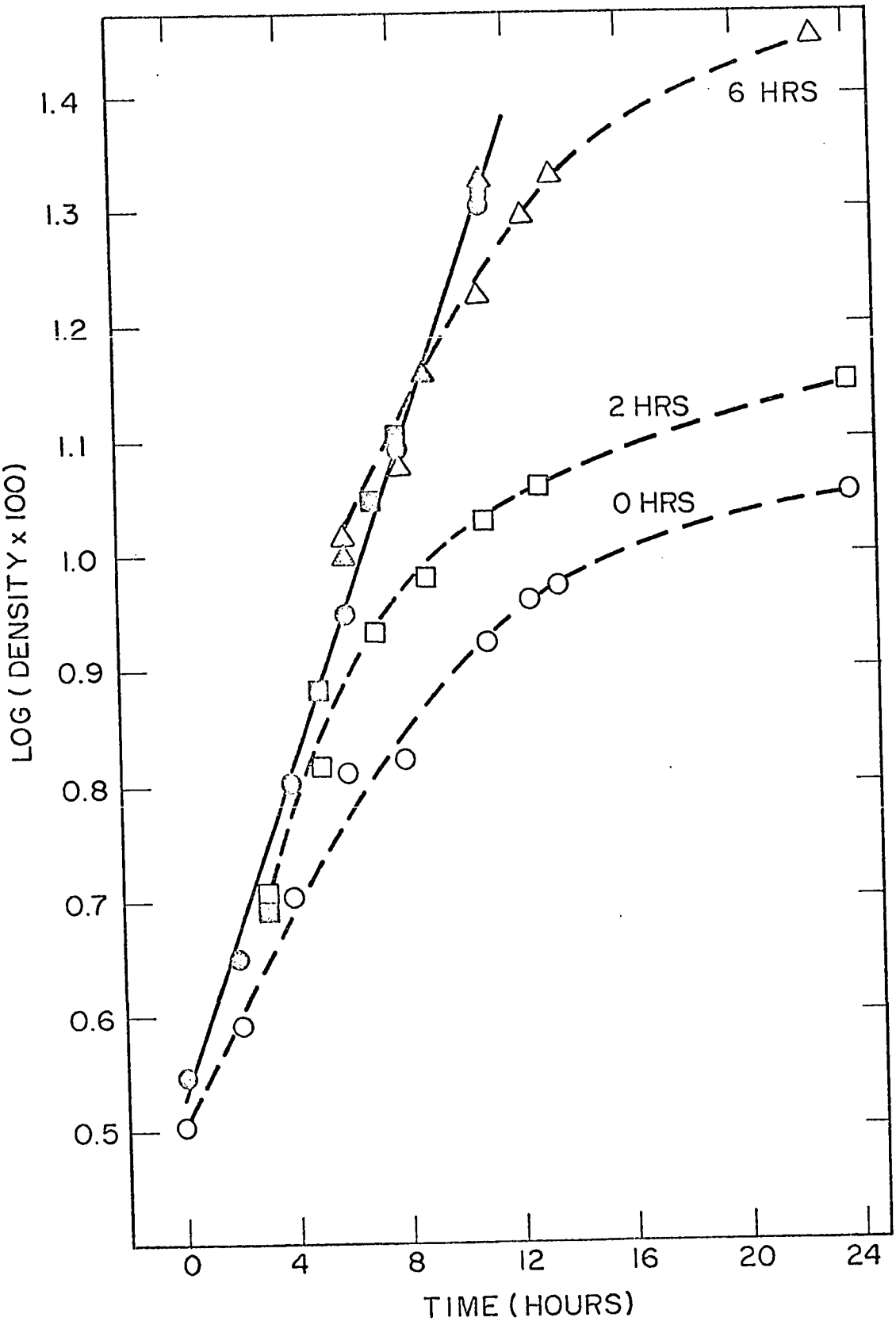

6 HRS
2 HRS
0 HRS
LOG (DENSITY x 100)
TIME (HOURS)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
4
8
12
16
20
24

Figure 49 - The "rate" or degree of oxygen inhibition on the growth of (+) cultures at different stages of development. The slope of the lines is inversely related to the severity of the oxygen effect.

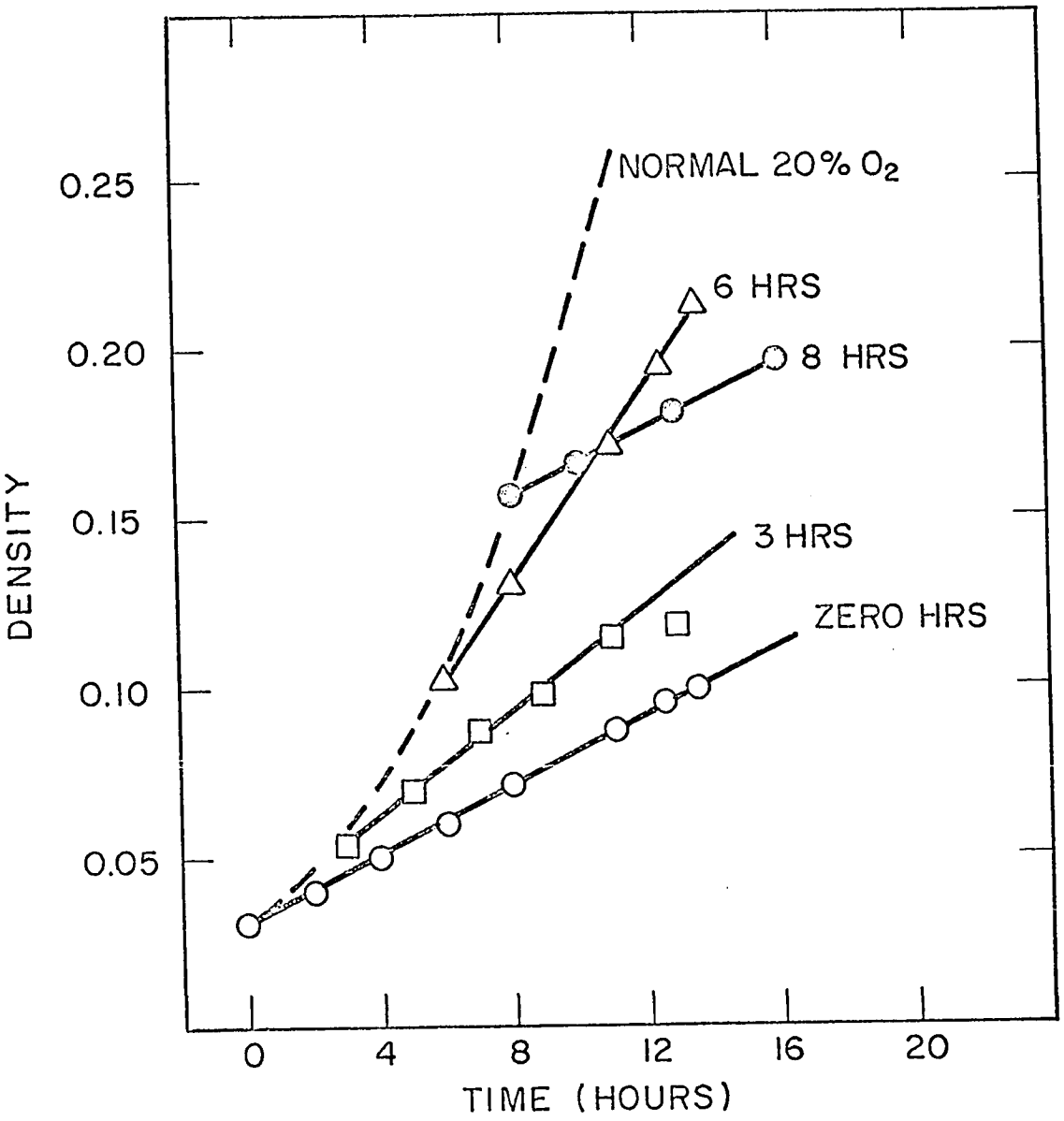

NORMAL 20% O_2
6 HRS
8 HRS
3 HRS
ZERO HRS
DENSITY
0.25
0.20
0.15
0.10
0.05
0
4
8
12
16
20
TIME (HOURS)

Table 3 - Progressive inhibition of photosynthesis in (+) cells under 95% oxygen. Cells were harvested from synchronous culture at the start of the light period and placed under high oxygen.

Culture Age	20% O_2	95% O_2	% Inhib.
0 hours	395 μl O_2/mg hr	240 μl O_2/mg hr	40
2 hours	570 μl O_2/mg hr	130 μl O_2/mg hr	77
4 hours	660 μl O_2/mg hr	100 μl O_2/mg hr	85
6 hours	690 μl O_2/mg hr	10 μl O_2/mg hr	98
8 hours	680 μl O_2/mg hr	—	100

under high oxygen.

All of these structural and physiological data demonstrate the toxic effects of oxygen on (+) cultures and the apparent indifference to high oxygen partial pressure of ORS. The dramatic effects of 95% oxygen on photosynthesis and cell development, particularly division, pointed to these processes as major sites of biochemical interaction between oxygen and cellular constituants. As preliminary to an extensive biochemical analysis of the two strains, the photosynthetic pigments, DNA and RNA content of the cultures were analyzed and compared.

Chlorophylls a and b, total chlorophyll, and total carotenoid content of (+) cultures grown on air level oxygen and ORS cultures grown on both air level and 95% oxygen were assayed spectrophotometrically as described in the methods section. The results show no difference in the pigment content or in the chlorophyll a/chlorophyll b ratios between (+) and ORS when expressed on a dry weight basis. The larger ORS cells contain proportionally more pigment per cell. Typical data are presented in Table 4.

Chromatographic separation of the carotenoid pigments from both strains indicated no difference in the specific pigment content between them, or from those of other Chlorella reported in the literature (Sherma 1970). In order of relative abundance, the carotenoids found were: lutein, α, +β carotenes, violazanthin, and neozanthin. The pigment concentration of the cultures increased in proportion

Table 4 - Comparative pigment composition of two strains on 20% and 95% oxygen. Expressed as percent dry weight.

Culture / Pigment	(+)-20%O_2	ORS-20%O_2	ORS-95%O_2
Chl a	2.5%	2.4%	2.5%
Chl b	0.8%	0.8%	0.8%
Total	3.1%	3.0%	3.1%
Chl a / Chl b	3.2	3.0	3.2
Carotenoids	0.7%	0.7%	0.7%

to dry weight throughout the development of both (+) and ORS.

The DNA and RNA compliment of the cells was determined as outlined in the methods section. Results, presented in Table 5, show no difference in the DNA content between the cultures on a per cell basis. Larger ORS cells had less DNA on a weight basis. The RNA concentration, like the pigments, was similar on a dry weight basis and ORS cultures had a substantially higher RNA/DNA ratio.

Table 5 - Comparative DNA and RNA content of (+)-20% oxygen; ORS-20% oxygen; ORS-95% oxygen.

	(+)- 20% O_2		ORS- 20% O_2		ORS- 95% O_2	
	μg/mg cells	μg/cell	μg/mg cells	μg/cell	μg/mg cells	μg/cell
DNA	3.3×10^{-2}	9.8×10^{-11}	2.0×10^{-2}	9.6×10^{-11}	1.7×10^{-2}	9.3×10^{-11}
RNA	11×10^{-2}	29×10^{-11}	12×10^{-2}	70×10^{-11}	12×10^{-2}	64×10^{-11}
RNA / DNA	3.3	3.0	6.0	7.2	7.0	6.8

DISCUSSION

The cell size distribution studies confirmed the postulated size relationships between the two cultures grown with and without oxygen stress. ORS cells are significantly larger than (+) cells on the average, and the cell size of both strains is increased by oxygen at high partial pressures. Wild type cultures grown under 20% oxygen show a normal size distribution with a slight skew toward the larger sizes. High oxygen, while increasing the mean diameter of (+) cells, has a positive effect on both the smaller and larger ends of the distribution. This corresponds well with the finding that the effects of oxygen are most severe at the early (small) and late (large) stages of (+) development. Thus, a random culture placed under high oxygen tends to accumulate the cells of sizes indicative of oxygen poisoning. The size of ORS cultures is also influenced by the oxygen concentration. In this case, however, the distribution is shifted toward larger cells while retaining a similar shape. This shift points toward a smaller number of large daughter cells produced during division of ORS, a result confirmed by study of synchronous cultures. An understanding of these differences in the sizes of (+) and ORS cultures was important for the valid interpretation of quantitative data based on the amount of cell material involved. These results may also be interpreted as a basic morphological difference when comparing the two strains.

The results of electron microscopic comparison of the ultrastructure of (+) and ORS cultures are highly informative. The micrographs of (+) grown under air level oxygen, (+)-air, show all of the structures and organelles typical of actively growing Chlorella species (Reger and Krauss, 1970; Wanka, 1968; Budd, et. al., 1969). The general appearance of these cells provides the basis for comparative observations. The cells were intact, the cytoplasm appeared normal, and the cell membrane was closely applied to the cell wall which indicated that the fixation and embedding were adequate. The gluteraldehydeosmium fixation provided excellent cell preservation as seen in the mitochondrial and chloroplastic fine structure. The use of a "hard" plastic mixture for embedding the cells prevented distortion of cell shape during sectioning.

There was a pronounced and devastating oxygen effect on the fine structure of (+) cultures. The general impression, obtained from observing numerous grids of (+)-O_2 under the electron microscope, was one of lack of development and structural integrity. The micrographs show a great increase in the amount of vacuolization, and in the type and amount of stored osmiophilic deposits. Widespread effects of this type could originate from membrane damage, or inhibition of membrane formation. This damage could result from lipid peroxidation as suggested by Tappel (1965).

These results provide conclusive evidence that oxygen is a toxic substance, at these concentrations, for wild type

cultures, and it causes severe and dramatic structural damage to the individual cells, and to their compliment of organelles. The effects of oxygen are not limited to any one part of the cell, but are general in nature.

Cells of synchronous (+) cultures exposed to only nine hours of 95% oxygen were gorged with starch grains as well as containing abundant osmiophilic substance. The three day cultures have almost no starch and the chloroplasts are very small and lack fine structure. This provides good evidence that the effects of oxygen occur in stages or that different cell processes are affected at different rates. It is also most probable that the affected processes interact with or influence, through feedback mechanisms, other cell processes which may not be directly affected by oxygen.

The effects of high oxygen on (+) ultrastructure become even more striking when compared with ORS cultures exposed in the same manner. There is very little difference between ORS-air and ORS-O_2 cultures. A slight increase in the size and number of mitochondria and a more pronounced pyrenoid appear to be the only signs of structural alteration in the ORS cultures exposed to 95% oxygen. Both of these changes indicate slight alteration in the respiratory activity of ORS. Comparison of (+)-air and ORS cultures show that the mutant organism lacks the large osmiophilic deposits, maintains a more obvious compliment of mitochondria, and possesses a much more prominant pyrenoid. All

of these features exist in both ORS cultures and are accentuated by increasing the oxygen partial pressure from 20% to 95%.

The origin and function of the pyrenoid is not fully understood, but it is believed that it is concerned primarily with the mobilization of accumulated starch deposits, and is thus involved in the interaction between photosynthesis and respiration (Wanka, 1968). The prominance of this organelle points toward an altered carbohydrate utilization in the ORS cells. The mitochondrial appearance indicates more pronounced activity of this organelle in the oxygen tolerant strain. The osmiophilic storage material, reported in several Chlorella (Reger and Krauss, 1970; Wanka, 1968; and Schmidt, 1966), is present in (+) cultures, increases with exposure to high oxygen in (+), but is completely absent in all cultures of ORS. This is most probably some form of phosphate material (Pulich, personal communication). It decreases or disappears during normal (+) division and may be related to DNA formation in this strain. Its conspicuous absence from ORS indicates that there are definite metabolic differences between the two strains. The larger cell size and lower division number of ORS may well be related to the phosphate supply and/or energy metabolism of the cells.

The electron micrographs reinforce the contention that (+) and ORS are similar, but distinct organisms. They demonstrate the potential toxicity of oxygen in (+) cultures, and

the resistance to structural damage by the mutant strain. The similarity of the two ORS cultures shows that the oxygen resistance demonstrated by this organism is not induced by the presence of high oxygen, but is a permanent aspect of its metabolism.

The comparison of the response of the cultures to temperature change, seen in Figure 21, offered no clues to the physiological or metabolic differences between the two strains, but only served to reinforce their similarity. Optimal growth temperature is an important physiological characteristic in algae, and the 39°C optimum for C. sorokiniana is significantly higher than that for the majority of green algal species. The "retention" of this temperature optimum by ORS is further evidence that it is a mutant of the (+) culture.

The heterotrophic growth experiments presented in this thesis are by no means complete, but were included to indicate that the oxygen resistance of ORS, which is so easily demonstrated autotrophically, may be complicated by the necessity to use an exogenous carbon source. Both cultures grew on glucose with a 20% oxygen atmosphere, and (+) grew at a faster rate than ORS which is similar to the relationship seen in the light. Further comparison of the heterotrophic responses of (+) and ORS to high oxygen are currently in progress in this laboratory.

The presence of glucose in the growth medium of light grown cultures appeared to produce little physiological

response either in 20% or 95% oxygen. Both (+) and ORS displayed typical autotrophic growth rates under these conditions. It appears that photosynthetic activity precludes any use of an exogenous carbon source by these cells. It was also interesting to see that the external supply of glucose in no way decreased the immediate resistance to high oxygen seen in ORS cultures.

The use of DCMU as an inhibitor of photosynthesis allowed for the study of heterotrophic growth in the light. The presence of DCMU, at a concentration of 10^{-5}M, completely inhibited the growth of light grown cultures, but did not affect the ability of either culture to utilize exogenous glucose either in the dark or in the light. Cultures grown on 0.5% glucose in the presence of DCMU in the light were similar to dark grown cells. ORS cultures required an extended "adaptation" period before growth on glucose was evident. These experiments indicate that it is some photosynthetically related process or product, and not light alone, which is responsible and required for ORS to demonstrate the immediate resistance described in the introduction. An additional finding related to these experiments, which deserves a closer and more detailed study, is an apparent long term resistance to the effects of DCMU by ORS. Cultures subjected to growth in medium containing DCMU for many days regain the ability to evolve oxygen in the light. Although the exact site of inhibition by DCMU is not known, it is believed to be in the election transport

of photosystem II (Jackson and Volk, 1970) and directly related to the oxygen evolution phenomenon. It would be very interesting if this oxygen evolution in the presence of DCMU was related to the oxygen resistance of ORS cultures.

Photosynthesis and respiration of the cultures corresponds well with other data describing their physiology. The slightly lower photosynthetic rate demonstrated by ORS-air was expected in the light of its growth rate. The slightly higher rate of photosynthesis seen in ORS-O_2 measured on air level oxygen may be the result of the change in atmosphere and probably does not reflect an increase in photosynthetic capability. The comparative inhibition of the photosynthetic rates by high oxygen was very similar and agrees with that reported by Wagner and Welsh (1969) for (+) for ORS measured at 25°C. However, cultures of ORS are able to maintain this "reduced" photosynthetic rate while (+) shows continued and progressive inhibition of oxygen evolution when exposed to continuous oxygen stress. It is also important to compare the ability of the two strains to recover from the Warburg effect. ORS is able to show complete and immediate recovery of air level photosynthetic rates even after days or weeks of continuous exposure to 95% oxygen, while (+) is "permanently" damaged after several hours of growth at high oxygen partial pressures and is unable to recover its original high rate of photosynthesis over a several hour period. This difference in the ability of the two strains to recover from <u>long term</u> expo-

sure, while showing the same short term response, indicates that the protective mechanism present in ORS is not directly associated with the phenomenon described as the Warburg effect (Turner and Brittain, 1952). These results point toward at least two effects of oxygen on the rate of apparent photosynthesis in green algae. The first is the short term effect which results in immediate and significant drop in oxygen production from which recovery is complete in both (+) and ORS and which fits exactly the description given in the literature for the Warburg effect (Turner and Brittain, 1962; Jackson and Volk, 1970; and Coombs and Whittingham, 1966). The second is a longer term effect which causes progressive damage to the photosynthetic machinery of (+) cells resulting in a permanent loss of photosynthetic capability. ORS cultures are protected from this severe and permanent damage.

The respiration of light grown (+) and ORS cells shows a very rapid increase when first measured after a photosynthetic period at high oxygen concentrations. This corresponds with results reported by others (Jackson and Volk, 1970) in relation to the phenomenon of photorespiration. This oxygen uptake, which accompanies increased oxygen concentration, has been used to explain the Warburg effect as an increase in photorespiration (Ellyard and San Pietro, 1969). The results presented here for (+)-air and ORS-air would seem to corrobrate this opinion since the sum of photosynthesis and respiration (true photosynthesis) are the

same for these cultures when measured either on 20% or 95% oxygen. However, the ORS cultures grown at high oxygen partial pressures show a substantial decrease (about 30%) in this rate of true photosynthesis when compared to the same culture measured on 20% oxygen. This indicates that ORS cultures grown under high oxygen atmospheres have a different mechanism for response to sudden increases in the oxygen concentration. This could be through a lower true photosynthetic rate, which does not appear likely from the data, or a reduced photorespiration rate. Another interesting alternative, which was proposed by Zelitch (1968) as an explanation for reduced photorespiration at high temperatures, is an improved ability for internal recycle of carbon dioxide thus lowering the total gas exchange with the atmosphere. This type of phenomenon could arise from changes in the membrane structure of ORS, a possible effect of oxygen previously mentioned.

The (+) cells, grown on high level oxygen, show "permanent" decreased respiratory activity after several days. This is indicative of oxygen damage to the respiratory apparatus in these cultures. The fact that ORS does not exhibit this same response is good evidence that the protective mechanisms of the mutant strain extend to the respiratory pathways.

A synchronized population of cells is one in which the vast majority of individual cells in the population are at the same stage of their developmental cycle at

the same time. Such a population, once established, can be treated experimentally as one large cell to obtain information concerning the development of the species in question. The usual criterion for the establishment of a synchronized population is that all of the cells divide at about the same time. This can be accomplished, depending on the organism or cell population under study, by a variety of techniques involving manipulation of the physical or chemical environment or sizing.

Chlorella, as well as other green algae, have been studied in synchronous cultures and various physiological and biochemical parameters have been followed through its life cycle (Tamiya, 1963; Sorokin and Krauss, 1959, 1961, 1962; Schmidt, 1966). The (+) strain, has been a popular Chlorella species for this type of study, and the "light-dark" technique developed for its synchronization (Sorokin and Myers, 1954) has been used by many workers and was used for this investigation.

The light-dark regimen required to establish synchrony patterns provides some information concerning the physiology of cell development in the culture under investigation. It has been established (Schmidt, 1966; Tamiya, 1963) that the length of the light period is the critical factor in determining whether division will take place, and the length of time for its completion. The length of the light period is characteristic of the species at any particular light intensity. Thus the differences in the length of the light

period necessary for the establishment of synchrony in (+) and ORS indicate that the development of these cultures is different, and that it is affected by the presence of high partial pressures of oxygen.

The "stability" of synchrony, i.e., the ability of the culture to remain in synchrony as long as the intermittant illumination cycle is continued, depends on the variability of the individual cells in the population. A uniform culture, one in which all the cells have exactly the same development cycle, allows for the best and most stable synchrony. However, individual variation in the time required for any of the developmental stages (growth, ripening, maturing and division) to be completed tends to break down the synchrony of the total population. This variation is present to some extent in all cultures and is the reason complete or 100% synchrony is not possible. ORS grown at air level oxygen appeared to have the greatest amount of variation among the cultures studied. This variation was greatly reduced under the 95% oxygen atmosphere, and made synchronization more complete and more stable for ORS. The synchrony patterns seen in Figures 26, 27, and 28 are included to show the basic differences in the development of (+)-air, ORS-air, and ORS-oxygen.

The number of daughter cells released from the mother cell at division varies from cell to cell, and is another characteristic of the culture or cell type studied. This division number ranges from 2 to 16 for most chlorellas and

is dependent on the growth conditions. A comparison of this number for (+) and ORS cultures grown under the same conditions shows that the mutant strain produces only half as many daughter cells, on the average, as the wild type. This result agrees well with those seen in the cell size distributions and predicted from routine quantitation of cell material which indicated smaller increases in cell number from ORS when compared to (+) cultures.

This increased cell size and lower division number for ORS appears to be characteristic of and resulting from the mutation which provided it with oxygen resistance. These features of the mutant culture pointed toward the developmental process, or to cell division, as holding a clue to the basic difference between the two strains. The synchronized cultures provided an experimental tool for studying this possibility.

The results of a comparative electron microscopic examination of the division process in (+) and ORS revealed that (+) cultures exposed to 95% oxygen were unable to carry out normal division. The primary difficulty appeared to be in the correct placement of the division plane. This progressed to what looks like an aborted division and complete disorganization in the mother cell. The most probable result is cell death. The ORS cultures demonstrated two modes of cell division. The normal autospore release type of division was most prominant and the dividing cells

contained fewer daughter cells. These results agree with those of Wagner and Welsh (1969). The binary mode of division seen in Figures 38b, 39, 42, and 43 results in only two cells, and the retention of the original cell wall material. This type of division has not been reported for any other species of Chlorella. It occurs in ORS both in the presence and absence of high oxygen and must therefore be characteristic of the mutation. We have been unable to explain why it takes place in only a small percentage of the population. It may be an efficient way to reduce cell volume without the necessity of manufacturing large amounts of extra cell wall material which is ordinarily lost during the succeeding division.

This series of micrographs again points out the striking resistance-toxicity phenomenon and, in addition, demonstrates a physical and physiological modification in the mutant strain which results from, or resulted in, the ability to carry out its life cycle under high oxygen partial pressures.

Comparison of the metabolic characteristics of the two strains during their life cycles served to reinforce the findings for random cultures. The parent strain demonstrated superior growth, photosynthesis and respiratory rates, and smaller size on a dry weight basis while ORS, with its larger cells, grew at a slightly lower rate, but had superior photosynthetic and respiratory rates on a cell basis. High oxygen had only slight effects on the ORS cultures, but the

(+) showed oxygen toxicity at all stages of development.

The family of curves presented in Figure 49 shows that the toxic effects of oxygen on (+) are not the same at all of the stages of the life cycle. The "zero" hour cells, which come from 15 hours of darkness, are very susceptable to the effects of oxygen. These young cells are synthesizing chlorophyll and enzymes, and setting up their metabolic machinery during the first few hours of light and it appears that these anabolic processes are severely affected by high oxygen. Cells undergoing division are also severely affected by 95% oxygen. This effect, however, may not be quite as pronounced as it appears in the images because growth normally slows down at this stage of the cycle.

The progressive effect of oxygen on the photosynthetic rate of (+) cells also shows that young cells are more severely and rapidly inhibited than four and six hour cells. These data seem to point toward the synthetic pathways in the cells as primary targets for oxygen toxicity.

The pigment compliment of an algal cell as with most plant species is used as a measure of its photosynthetic capacity, the condition of the photosynthetic apparatus, and as one distinguishing characteristic of the plant type. The comparative analysis of the pigments in (+) and ORS showed no differences between them. The (+) cells, when grown under high oxygen atmosphere, showed a decreased level of pigments but was not constant. The chromatographic separation of the carotenoids was undertaken because of their possible

role as protective agents in photo-oxidation (Krinsky, 1966 and Donohue, et. al, 1966) and the possibility that among them might be the natural antioxidant postulated by Morhardt (1968). The similarity, both in amount and type of pigments found, between (+) and ORS ruled carotenoids out as the protective agents.

The difference in division, the size of the cells, and the convincing evidence for mutation from (+) to ORS suggested possible differences in the amount of DNA present in the two strains. Analysis of random cultures for DNA content indicated that the cultures contained the same DNA compliment on a cell basis (Ward, Pulich, and Hall, 1969). Analysis of the nucleic acid content of synchronous cultures was undertaken to check the division number against the increase in DNA content. The results indicated that each cell contained the full DNA compliment, and that no "extra" nuclein acids were formed which were not incorporated into daughter cells. The DNA content was similar when expressed on a cell basis, but the larger ORS cells contained less DNA on a dry weight basis. The RNA content, like that of the pigments, was similar for the two strains on a weight basis, but was higher for each of the larger ORS cells. The RNA/DNA ratio for ORS was twice that of (+) cultures. This is substantially higher than the weight or size difference between the cultures. Perhaps the requirements for protection against high oxygen require an increased protein

synthesis and thus more or faster RNA turnover compared to other cellular components.

CONCLUSION

A substantial amount of the data on cell size, ultrastructure, growth, photosynthesis and respiratory rates, development and division, and the difference in response to oxygen indicate that (+) and ORS are unique organisms. However, the similarities in temperature optimum, pigment content, DNA compliment and general appearance as well as the conditions of the original isolation provide the basis for a valid comparison between the two organisms in relation to the high oxygen toxicity-resistance phenomenon.

Except for the Warburg effect, very little has been said about the effects of high partial pressures of oxygen on Chlorella. Results of these studies indicate that there are very widespread effects of high oxygen partial pressures on this alga. Its growth rate is substantially decreased at all stages of its development. The photosynthetic capacity of the cells is immediately lowered (Warburg effect), but continued exposure causes progressive permanent damage to the photosynthetic machinery. Respiratory metabolism is eventually destroyed. Cellular integrity is disrupted and division processes are thwarted resulting in cell degeneration. Pigment content declines, nucleic acid synthesis decreases, and it is probable that cell processes in general are adversely affected either directly or indirectly by exposure of (+) to high oxygen.

It is remarkable that an organism so closely related to (+) in almost all of its basic physiological parameters could withstand, completely and immediately, these devastating effects. ORS, however, as has been described, does not show any of these adverse symptoms of oxygen poisoning except for the Warburg effect which reduces apparent photosynthesis by 40%. It does, however, demonstrate subtle structural, physiological, and I am sure biochemical differences.

The cells of ORS are larger, they divide into fewer daughters, they exhibit more sluggish metabolic functions which are reflected in the growth rate, they lack the large deposits of stored phosphate, they have more and better developed mitochondria and pyrenoids and they demonstrate a different mode of division. All of these differences appear to be slightly augmented or exaggerated by the presence of high oxygen partial pressures and can thus be related circumstantially to the mutational event or events which produced the oxygen resistance in this organism. They are in fact, however, present in the organism both in the presence and absence of high oxygen stress and cannot be described as oxygen induced properties of the culture.

Experiments with synchronized cultures indicate that the processes of early metabolic development and autospore formation are severely inhibited by the presence of 95% oxygen. These areas are likely candidates for further biochemical investigation into the mechanisms of oxygen toxicity.

The presence in (+), and absence in (ORS) of osmiophilic storage material indicates a very basic difference in the metabolism of these organisms. All indications point to inorganic polyphosphate as the major component of these deposits. The important role that phosphate plays in energy metabolism and nucleic acid biosynthesis, as both a participant and a regulator of these processes, makes these vital processes primary prospects for the site of an oxygen resistance mechanism. The effects of oxygen on the phosphate content in a green alga have been described by Ullrich (1970), and are in agreement with the results presented here. He reports increased phosphate, and especially inorganic polyphosphate, in high oxygen exposures which can be related to the inhibition of organic phosphate formation from photosynthetically produced ATP.

The Warburg effect is demonstrated by both of the organisms, and can therefore be eliminated as a factor in the resistance-toxicity problem exhibited by them. This also implies that photorespiration, in whatever form it takes in Chlorella, is not involved directly in the resistance phenomenon of ORS. The differences in oxygen response by ORS grown heterotrophically and autotrophically, however, indicate that the resistance mechanism is closely associated with the processes of light metabolism, and requires a prolonged adaptation period before it can function in the dark.

The photosynthetic pigments are protected from oxygen damage in ORS, but the carotenes or zanthophylls do not appear to be involved as the primary protective agents. The fact that the remainder of the cell is also protected from oxygen damage also indicates that the resistance mechanism must be more general than could be explained by carotenoid protection.

All of these comparative data indicate the striking effects of high oxygen on the (+) cells. The damage, evidenced by a loss of detailed fine structure, and apparent difficulty in formation and orientation of daughter cell boundaries, could arise from an attack of the structural lipids by oxygen as proposed by Tappel (1965) and Skrede and Christophersen (1966). This would cause a general breakdown of the membrane systems in the cell and result in loss of photosynthetic and respiratory activity as well as a loss of structural integrity.

Except for the Warburg effect, which seems to be associated with photorespiration and glycolate formation (Jackson and Volk, 1970), and not related to cellular damage, ORS cells show none of the adverse effects of high oxygen seen in (+) cultures. The mutational event(s) which took place in its development provided a broad based protection against the effects of oxygen. Morhardt (1968) postulated the presence of a naturally occuring antioxidant as responsible for this protection. However, it is difficult to conceive of a chemical produced by ORS in sufficient

quantities and distributed to all portions of the cell in a manner which would affect such complete protection.

An alternate, although not mutually exclusive, possibility is indicated by the micrographs and physiological results presented here as well as the data from the literature concerning oxygen toxicity. The mutation which provides such good protection to ORS could have affected the formation and/or structure of its cellular membranes. Such an effect could arise from an alteration in the lipid content or its structural relationship within the cell membranes which would resist peroxidation and thus preserve organelle integrity. This, however, would also change the permeability of the membranes and alter transportation of metabolites both inter and intracellularly.

The end result would be a cell protected from oxygen, but with permeability differences which changed its metabolic balance, required altered energy metabolism, and made reproduction less efficient.

Although this is only one of several possible answers to the toxicity and resistance seen in the comparison of (+) and ORS, it explains both phenomona and the data presented. It is also compatible with the considerable information already in the literature, and provides a direction for future study using this system.

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