RICE UNIVERSITY

METABOLIC AND ULTRASTRUCTURAL CHARACTERISTICS OF HETEROTROPHIC *CHLORELLA SOROKINIANA*, WITH EMPHASIS ON EFFECTS OF HIGH OXYGEN TENSION

by

WARREN M. PULICH, JR.

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C. H. Ward

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INTRODUCTION

Molecular oxygen plays a fundamental role in the biosphere. Without it, most life as we know it, probably would not have evolved. On the physico-chemical level, oxygen enables cells to generate metabolic energy from glucose at a remarkable 40 to 50 percent rate of efficiency. This situation is made possible by a unique thermodynamic property of oxygen, namely its participation in the most positive oxidation-reduction couple (+0.815 v) functioning in biological systems. Its high reactivity towards a large number of elements in the process of oxidation has contributed to its abundance (50-70% by weight) in living matter. From the evolutionary standpoint, oxygen can be considered a major selective agent. Not only has the atmospheric concentration of oxygen controlled evolutionary development of organisms, but the possibility has even been proposed that oxygen is responsible for the use of carbon instead of silicon as the basic component of living matter.

At the same time the effect of this element is somewhat paradoxical. There exists much evidence to prove that oxygen is deleterious to most forms of life at partial pressures greater than air level. Haugaard (1968), Gerschman (1964), and Turner and Brittain (1962) present ample documentation for the toxic effects of oxygen. Many cellular processes and biochemical reactions, both *in vivo* and *in vitro*, are sensitive. Cell division (Brosemer and Rutter, 1961), respiration (Begin-Heick, 1970), glycolysis (Kilburn et al., 1969), protein and nucleic acid metabolism (Berg et al., 1965), and even transport across membranes (Young, 1968),

have all been reported as primary cellular targets of inhibition by high oxygen. Broadly-speaking, oxygen poses the threat of oxidation damage to an organism. The effect may be a direct one, as when enzymes and other macromolecules are altered; or it may take a more subtle form, eg. permanent disruption of oxidation-reduction reactions, so that components are kept in completely oxidized or reduced states.

No single mechanism has been discovered which fully accounts for the severe toxicity of high oxygen, but several hypotheses have considerable merit. (1) Oxygen could easily oxidize free sulfhydryl groups of molecules (eg. proteins, lipoic acid, coenzyme A) and thus cause inactivation. Many such oxygen-sensitive compounds are known and have been studied in model systems. (2) Reducing substances, termed antioxidants, have been isolated from normal cells. Compounds such as glutathione, α-tocopherol, and cysteine could function in counteracting an oxidizing environment (Tappel, 1965). If not abundant enough to maintain -SH groups in reduced form, toxicity symptoms would result. (3) The function of oxygen in formation and propagation of free radicals is well-known from radiation chemistry. Highly unstable free radicals could bring about polymerization of cell components or formation of peroxides (Gerschman, 1964). If the molecules affected were lipids, membranes would be substantially modified and lose their selective permeability. Free peroxide formed could attack intracellular molecules in heavy-metal catalyzed reactions, resulting in drastic damage to enzyme systems and structural molecules. Perhaps enzymes such as peroxidases and catalase, or antioxidants, could prevent this damage.

Haugaard (1968) presents much physiological evidence which indicates that the immediate effect of oxygen is disturbance of the balanced

operation of cellular metabolic processes. Chance et al. (1965) demonstrated that pyridine nucleotides accumulate in the oxidized form upon exposure of cells and mitochondria to hyperbaric oxygen. Specifically their work indicated that one or more sites associated with the electron transport chain are sensitive to oxygen. The net result was reversal of electron flow through the respiratory chain. Disruption of such a basic metabolic pathway would inevitably lead to alterations in total cellular metabolism by feedback controls. Thus oxygen toxicity could be due to defective metabolic control rather than actual structural damage.

This last explanation leads to another point of discussion. Severe toxicity represents only one manifestation of cellular control by oxygen. At concentrations from zero to air level, oxygen may exert only a partially inhibitory effect on cells. Such phenomena as the Pasteur and Warburg effects represent modulating control of metabolic processes by oxygen. The Pasteur effect exemplifies clearly the indirect control by oxygen. Most workers (Lehninger, 1970) attribute it to feedback inhibition of key glycolytic enzymes by the end products of energy metabolism and by various glycolytic intermediates. Phosphofructokinase, the main enzyme involved, is rigidly controlled by the cytoplasmic level of its substrates, ATP and fructose-6-phosphate, and the Kreb's cycle metabolite, citrate. When oxygen is abundant, ATP and citrate levels will be high due to increased respiratory activity. These compounds in turn inhibit phosphofructokinase, and glycolysis is effectively shut off. Under the same conditions, plentiful glucose-6-phosphate would also inhibit hexokinase, another control point. Competition for substrate constitutes a second method of regulation. Mitochondrial enzymes have a higher affinity for ADP than glycolytic enzymes. When the ADP level falls (aerobic situation),

mitochondrial enzymes out-compete glycolytic ones for available ADP, greatly retarding glycolysis. Thus oxygen acts as a throttle on glycolysis.

In many instances, air level oxygen may have a stimulating effect. Consider the tissues of animals which absolutely require oxygen in this range: nerve cells, cardiac muscle cells, and liver cells. Photorespiration in many plants is stimulated by increases in oxygen up to 100%, whereas dark respiration saturates at around 2% oxygen. Cellular morphogenesis in some organisms is induced by increasing the oxygen tension of their environment (Kobr et al, 1967).

In general, at oxygen tensions around air level, oxygen-regulated metabolism operates at a carefully balanced rate. The rate can be expressed as the difference between inhibited and stimulated reactions. Though some reactions may be moderately inhibited, eg. plant cell photosynthesis, other processes, eg. respiration and biosyntheses, are properly integrated to insure that the cell as a unit functions at maximum capacity. Deviations from this rate could be produced by anaerobiosis or high oxygen. Both cases would throttle down metabolism by forcing the cell to rely on less efficient and less coordinated biochemical pathways.

As varied as organisms are, most have developed means to cope with oxygen in the environment. Those that have not remain today as obligate anaerobes. Others can tolerate both the absence and the presence of oxygen, eg. yeasts, bacteria. These facultative aerobes have the ability to switch back and forth from one form of metabolism to the other. Obligate aerobes require oxygen, though usually less than air level, having become adapted to oxidative pathways for growth. Since the oxygen content of the atmosphere has only reached about 21%, most organisms are not prepared for

higher concentrations. As previously stated, such situations are usually toxic.

From the standpoint of evolution, these characteristics are due to the selective pressure of oxygen on developing organismal lines (Gilbert, 1964). Oxygen has acted as a selective agent to the extent that cells capable of oxidative metabolism have had a distinct advantage over cells utilizing less efficient energy pathways. In addition, such organisms have developed protective mechanisms from the increasing oxidizing potential of the atmosphere. This situation has conferred a degree of survival fitness on aerobes. The occurrence of an organism capable of growth under high oxygen tension would be most interesting indeed, as well as a result of selection pressure. It would represent an adaptation to extremely oxidizing conditions, or a further evolutionary step in response to oxygen in the environment.

STATEMENT OF THE PROBLEM

This information forms the background for discussion of a unique organism capable of sustained growth under high oxygen tension. The organism is a mutant strain of the green alga, Chlorella sorokiniana (Shihira and Krauss), known as ORS, and was originally isolated through continuous subculturing on high oxygen by C.H. Ward.

Morhardt and Ward (1968) first described the basic distinction between ORS and the wild type (+) alga, using photosynthetic cultures. ORS grown on both air and high oxygen demonstrates a growth rate just slightly lower than (+) on air. In contrast (+) on high oxygen is severely inhibited, but not killed since growth resumes upon transfer back to air. It has been established that resistance to oxygen is a permanent, stable property. Serial transfer of ORS on air for many generations does not

impair its ability to grow upon re-exposure to high oxygen. When clones are isolated from a culture of ORS plated on agar, each daughter colony tested behaves like the parent culture and grows under high oxygen. These facts provide strong support that a mutation is involved and not merely an adaptation phenomenon. Furthermore our laboratory has been unable to repeat the mutational event by continuously subculturing (+) for several weeks on high oxygen. After this treatment, (+) finally adapts somewhat to oxygen, but its growth rate remains much lower than ORS.

Subsequent investigation has focused on the comparative physiology of (+) and ORS. Both Morhardt (1968) and Richardson et al. (1969) observed that ORS grew under high oxygen on glucose in the dark, though the nature of this heterotrophic response appeared to be more complex than light growth. Richardson et al. (1969) also examined the response of ORS to hyperbaric oxygen pressures and found that oxygen pressures above 1 atmosphere were inhibitory to both autotrophic and dark (heterotrophic) growth. They concluded that photosynthesis was not the oxygen-sensitive site. Wagner and Welch (1969) showed that the photosynthetic activity of ORS, measured as oxygen evolution at 25°C, was moderately inhibited by high oxygen compared to 20% oxygen. They suggested that oxygen resistance was due to a change in non-photosynthetic metabolism rather than a photosynthetic mechanism per se. Morhardt (1968) too agrees with this idea, and hypothesized an intracellular antioxidant mechanism to account for the phenomenon.

Other workers (Ward et al., 1969) have attempted to further characterize resistance and susceptibility to oxygen based on ultrastructural morphology, cell composition, activities of selected enzymes, and response to radiation. Various experimental rationale, based on the previously discussed mechanisms for oxygen toxicity, have been tested. Analysis of

DNA content has revealed that the ploidy of ORS is identical to (+); both have approximately 0.1 picogram DNA per cell. Catalase activity would not appear to account for oxygen resistance, since ORS on oxygen contains half the level of catalase as ORS on air. If ORS were protected by catalase from increased amounts of peroxide produced under high oxygen, a greater amount of catalase would be expected. Carotenoid analyses and preliminary tests of resistance to γ-irradiation would seem to indicate that oxygen resistance is probably not mediated via a radiation-protective mechanism. Metabolic studies have shown significant respiratory differences between (+) and ORS. Glucose or a metabolite of glucose appears to inhibit oxygen uptake in ORS even on air. Simultaneously, the observed levels of succinic dehydrogenase indicate that the entire respiratory machinery of ORS may be regulated differently from (+). In addition, distinctive morphological features have been noted. ORS possesses a larger, heavier cell than (+), under air or high oxygen.

The accumulating evidence suggests that the mutation which produced ORS involves a general metabolic control rather than some specific oxygen-protective mechanism. The number of other morphological and physiological properties associated with oxygen-resistance make it likely that oxygen-resistance is itself merely a secondary result of some altered control mechanism in a fundamental cellular process. If a one step mutation had occurred at a point in a pathway normally subject to strict control by oxygen, then oxygen could conceivably have lost its control over that pathway. Several examples illustrate how oxygen can regulate an entire pathway, which in turn affects the entire cell:

1. The dependence of mitochondrial development on oxygen eg. yeast transferred from anaerobic to aerobic conditions.

2. Synthesis of unsaturated fatty acids (except in bacteria)

3. Synthesis of sterols

4. Oxygen control over glycolysis

A mutation in the basic regulation of such pathways could intrinsically effect the cell's response to oxygen. Such an assumption played a large part in the choice of an experimental system for continued study of the interesting mutant, ORS.

APPROACH TO CHARACTERIZATION OF ORS

The reports that ORS exhibited oxygen resistance in both light- and dark-growth situations suggested that oxygen resistance could be dissociated from photosynthetic light reactions. This fact provided an opportunity to examine more clearly the role of oxygen in ORS metabolism by studying ORS in a heterotrophic system. Such a system appeared to have a distinct advantage over an autotrophic one. In the former case, metabolism operates primarily to degrade exogenous carbon sources, principally glucose. In the latter case, not only must catabolism of glucose (reduced carbon) occur, but also its synthesis by the highly complex reactions of photosynthesis. Furthermore, photosynthetic pathways are not as completely established as heterotrophic ones. Consequently, glycolysis, the Kreb's cycle, and mitochondrial electron transport form a more integrated and comprehensive picture than chloroplastic phosphorylation, dark reactions of CO_2 fixation, and the distribution and use of reduced carbon. With photosynthesis eliminated, the complexity of the system should be reduced.

Investigation of heterotrophic algal cultures was designed around a comparative approach. Initially, routine physiological characterization of (+) and ORS was performed. Studies (Samejima and Myers, 1958; Wiedeman and Bold, 1965) have shown that various chlorellas require different

substrates for dark growth, ranging from numerous sugars to acetate and urea. Often such physiological characteristics provide significant insight into the biochemical aspects of an organism's carbon metabolism. The basic features of dark growth make the heterotrophic system quite promising for partial elucidation of the oxygen-resistance mechanism, as well as invaluable to chlorella taxonomy.

Electron microscopy has developed into the ideal technique for correlating structure and function within cells. Study of heterotrophic (+) and ORS was extended to the ultrastructural level with this tool, representing one of the few times heterotrophic algae have been viewed with EM.

At the biochemical level, the experimental rationale attempted to explain initially the reported differences in cell size and weight between (+) and ORS. Accordingly, analysis of cell constituents was conducted, beginning with general classes of intracellular compounds: total protein, lipid, and starch. Later, analysis of other components became necessary: soluble pools of metabolites, cell phosphorus, and nucleic acids. Subsequently the regulation of specific metabolic reactions was questioned, and activities of selected enzymes were measured. Ward et al. (1969) already had observed peculiarities in oxygen uptake between (+) and ORS. Since respiration can be affected vitally by many of the preceding parameters, it was worthwhile to check this aspect of cell metabolism more thoroughly.

Not only can these data be interpreted and correlated to formulate a reasonable hypothesis on the mechanism of the oxygen-resistance phenomenon in ORS, but also they can help to describe and characterize heterotrophic algae at the physiological, ultrastructural, and biochemical levels. Since these organisms are capable of shifting from photosynthetic

to heterotrophic modes of metabolism, they are most unusual from the standpoint of metabolic control mechanisms. The regulation of heterotrophy in normally photosynthetic algae assumes even more significance when its practical application is considered. Where algae are exposed to high levels of organic nutrients or conditions of severe light limitation (eg. night-time conditions or quiescent bodies of turbid water), heterotrophy is a likely possibility. The mechanism and control of heterotrophic metabolism in these situations can be extrapolated from studies such as the one presented.

MATERIALS AND METHODS

THE ORGANISM AND EXPERIMENTAL CULTURE SYSTEM

The alga used for all studies, Chlorella sorokiniana (Shihira and Krauss), obtained from the Maryland Culture Collection, is a high temperature eukaryote, which exhibits an optimal, autotrophic growth temperature of 39°C. Originally described by Sorokin in 1951 as a strain of Chlorella pyrenoidosa, C. sorokiniana shows remarkable physiological stability in culture due to its growth temperature. Shihira and Krauss (1963) place it at a midpoint in evolution between the obligate autotrophs and those species which are likely to lose their chlorophyll and become completely heterotrophic. Among the properties that have made this species very popular for experimental purposes, its rapid growth, extremely small size, and unicellular condition rank high. Many studies can be done on whole cells with greater accuracy and statistical significance than with larger organisms.

Basic microbiological techniques were employed to handle the organism, enabling axenic cultures to be maintained. Stock cultures of both wild type (+) and ORS strains were kept routinely at room temperature on agar slants containing 1.2% Tryptic Soy Broth. From time to time, stocks were cloned, re-isolated, and checked for physiological characteristics. Contamination was tested for by plating an aliquot of cell suspension on nutrient agar or by microscopic examination.

For dark (=heterotrophic) growth, the following culture system was adequate. Cells were grown in semi-continuous, liquid culture in 2.5

liter "wide-bottom" culture flasks placed inside a New Brunswick Psychrotherm Incubator-Shaker to maintain temperature, darkness, and proper aeration of cells. Temperature was accurate to $\pm$ 0.1°C. The flasks were fitted with a harvesting port for sampling and an inlet port, through which fresh medium or gas mixtures were introduced. Fresh medium was added by means of a siphon tube attached to a feed reservoir. Gases were previously passed through a humidifier and a filter of cotton and glass wool. Altogether the system provided for maintainance of sterile conditions and a continuous supply of cells in exponential growth.

Requirements for autotrophic growth of C. sorokiniana have been investigated and amply described (Sorokin, 1959). Heterotrophic growth has also been reported (Shihira and Krauss, 1963), though its conditions have been less rigorously defined. The basic growth medium, Knops salt solution (Sorokin and Krauss, 1958), supports growth well in both the light and dark. Though Shihira and Krauss (1963) have determined that (+) utilizes ammonia and nitrate equally, it is interesting that nitrate is used as the nitrogen source in the dark. The latter investigators and Morhardt (1968) report that glucose from 0.1-0.5% sustained good dark growth of the organism. Hence 0.3% glucose in Knops medium was initially chosen as the growth medium for these studies. Media were routinely sterilized by autoclaving at 121°C for 15 minutes at 16 psi.

OPTIMIZATION OF HETEROTROPHIC CULTURE CONDITIONS

Preparatory to investigation of heterotrophic cells, it was necessary to optimize culture conditions. This required testing various parameters such as temperature, gas conditions, and substrates for the proper combination promoting maximum logarithmic dark growth.

Preliminary studies at 39°C indicated that this temperature might

not be the optimal temperature for dark growth. Consequently a series of growth experiments were run to determine the temperature requirement. Glucose at 0.3% was used as the substrate, and 1.7% CO_2 in air or 100% O_2 as the gas phase. Growth could be determined in three ways:

1. by dry weight. Samples were filtered through pre-weighed 0.45 μ Millipore filters, then dried at 102°C for 12 hours.

2. by optical density of cultures around 600 nm.

3. by cell numbers. Initially cell counts were made with a Spencer Brightline hemacytometer. Later a Coulter Model B Counter with a 70 μ orifice was employed and gave comparable results.

Growth rates were calculated as doublings per day (d_2), using the formula

$$d_2 = (\log_{10}N_2 - \log_{10}N_1)\ (3.3)\ \left(\frac{24}{t}\right)$$ where

N_1 and N_2 are values for cell mass at times 1 and 2, over the time interval, t, in hours. If logarithms to the base 2 were used, d_2 would be identical to the specific growth rate, K, for exponential growth. Normally dry weight measurements were used. Rates were accepted only if they remained stable for at least two successive runs.

Simultaneously, tests were conducted to determine, roughly, if any special gas requirements were necessary for dark growth on glucose. Gas mixtures tried were:

1. Air only.

2. Air + 1.7% CO_2.

3. 100% O_2.

4. 98% O_2 - 2% CO_2.

Mixtures 3 and 4, as well as all CO_2, were analyzed and supplied by Big Three Industrial Gas and Equipment Company, Houston, Texas.

Selective substrates were screened in order to compare the general nutritional requirements of the two strains. These experiments were conducted by growing static cultures in screw-cap culture tubes in a dark incubator at 37.5°C. Substrates were tested at a final concentration of 0.2% in Knops, pH adjusted to about seven. Most compounds were filter-sterilized through 0.45 μ Millipore filters, except for monosaccharides and acetate which were autoclaved, but separately from the salts. Cultures were pre-adapted to dark conditions on glucose, washed and then exposed to substrates under room air. Growth was checked for 16 days.

On the basis of these preliminary observations, acetate was chosen as a representative example of a non-sugar carbon source supporting growth and the following experiment performed. Cells were grown on 0.25% sodium acetate instead of glucose and their response to high oxygen checked. Since the pH of acetate cultures rose faster and higher than glucose cultures, it was carefully held between 6.5 and 7.0 by addition of 1N HCl as necessary.

DEFINITION OF HETEROTROPHIC CELLS

During the course of these early experiments, ORS demonstrated an extremely long period to completely adapt to heterotrophic conditions. (+) reached maximum log growth very soon (2-3 days) after introduction into the dark, whereas ORS took much longer (about one week). Apparently the transition from autotrophic to heterotrophic metabolism posed a special difficulty to ORS. This situation assumed more significance as time passed, and later became the object of intensive investigation in itself. For the moment, it will suffice to state that cultures were not considered fully "dark-adapted" until they had reached a constant growth rate. Unless otherwise noted, heterotrophic cells refer to these "dark-

adapted" cells. Most of the following experiments were performed with such cells.

Hereafter, all cultures were grown at standard conditions: 37.5°C; 0.3% glucose in Knops medium; and either 1.7% CO_2 in air or 100% O_2.

MORPHOLOGICAL STUDIES

One of the first studies conducted on heterotrophic cells was examination by electron microscopy. The ultrastructure of (+) and ORS seemed likely to provide clues for future biochemical analyses. It should be noted that cells prepared for electron microscopy were grown at 38.5°C, somewhat above the optimal temperature. Log-phase cells were harvested in very dim light, centrifuged in the cold, and washed in cold Knops medium prior to fixation.

The fixative consisted of 6% glutaraldehyde in 0.01 M cacodylate buffer, pH 6.8. Cells were fixed at room temperature for 2 hours, washed four times over a one hour period with 0.01 M cacodylate buffer and post-fixed in 2% OsO_4 in 0.01 M cacodylate buffer for two hours (McLean, 1968). After washing in tap water, cells were dehydrated in a graded ethanol series, transferred to propylene oxide, and finally embedded in Epon 812. Infiltration occurred overnight in the last mixture. Fresh Epon was exchanged the next day, cells were placed in capsules, and then polymerized for two days at 60°C. Thin sections were cut with a diamond knife, stained with 2% uranyl acetate for 15 minutes, post-stained with lead citrate for 7 minutes (Reynolds, 1963), and photographed in an RCA EMU-3F electron microscope.

ANALYSIS OF GENERAL CELL COMPOSITION

After comparing electron micrographs, analysis of cell constituents was suggested by obvious differences in cell inclusions. Initially

total protein, lipid, and starch were determined. Heterotrophic, log cultures of the two strains were harvested on ice, centrifuged in the cold, washed with cold Knops, and frozen for subsequent analyses. Lipid was analyzed by the method of Folch et al. (1957) by homogenizing cells in 2:1 chloroform: methanol. Cells were broken with a Bronwill MSK Mechanical Homogenizer, which yielded 99+ % breakage.

A single sample served for both protein and starch assays. First, cells were homogenized in 100% acetone. Then the sample was divided into two parts, one for protein determination and the other for starch. This procedure had the advantage of removing lipids and pigments, which interfered with the subsequent determinations. Since protein and starch are insoluble in acetone, they were separated as pellets from the homogenate by centrifugation at 10,000 x g.

The protein fraction was dissolved in 0.65 N NaOH overnight and an aliquot assayed by the Lowry method (Lowry et. al., 1951). The standard consisted of bovine serum albumin. The starch fraction was extracted twice with hot 80% ethanol, then twice for 20 minutes each with ice-cold 52% perchloric acid. Protein and cellulose were removed by centrifugation. The hydrolyzed starch was assayed according to McCready et al. (1950), using anthrone-sulfuric acid reagent and glucose as standard. The reaction of live cells to the starch indicator, I_2 - KI, was also tested visually.

A technique was designed to study the initial kinetics of exposure to high oxygen. When log cultures are transferred from air + CO_2 to 100% O_2, a transition occurs as cells respond to oxygen. For (+), these changes should be the primary sequence of events culminating in the pathological symptoms of oxygen toxicity. For ORS, this initial exposure

period should trigger the processes which lead to manifestation of oxygen resistance. Accordingly, studies of changes in various parameters were conducted as a function of exposure time to 100% O_2.

First, changes in pH of culture medium were followed with a Beckman pH Meter.

Second, kinetics of changes in protein and starch content were determined. At various times, samples were collected and analyzed as described above.

As a corollary to electron microscopic evidence and intracellular quantities of starch, glucose assimilation was followed during log growth. Samples were collected at two definite times, usually 4-6 hours apart. Cells were removed from the medium by centrifugation in the cold at 5,000 x g for 15 minutes or by filtration through Millipore filters. Glucose in the supernatant was assayed using the Glucostat reagent (Worthington Biochemicals) with a 10 minute incubation time. Knowing the change in dry weight over the same time period, yield could be computed as mg. dry weight produced per mg. glucose consumed.

ANALYSIS OF CELL PHOSPHORUS CONTENT

From electron micrographs, ORS appeared to lack intracellular deposits of polyphosphate, so-called volutin granules (Hase et al., 1963). To check this, qualitative and quantitative tests were applied. Since volutin granules demonstrate metachromasia, live cells were checked for their reaction to toluidine blue, a dye which stains polyphosphates, as well as other acidic compounds, at alkaline pH.

For direct comparison of phosphorus levels, cell phosphorus was fractionated according to Baker and Schmidt (1963). Cells were harvested in log growth and washed twice with cold 0.01 M Tris-HCl buffer, pH 8.5.

Cold-acid soluble and insoluble fractions were obtained by extracting cells at 0°C once with 10% trichloroacetic acid (TCAc) for 15 minutes and once with 5% TCAc. The soluble phosphorus consists primarily of inorganic phosphate, sugar phosphates, and free nucleotides. The insoluble residue was extracted once with cold 95% methanol, twice with 95% methanol at 50°C, and three times with 3:1 methanol: ether at 50°C. This step removed lipid phosphorus, and left a whitish pellet containing polyphosphate, nucleic acid phosphorus, and phosphoprotein. The lipid fraction was evaporated to dryness. Total phosphorus in these three fractions was digested to orthophosphate (PO_4^{-3}) by heating in concentrated sulfuric and nitric acid (Leloir and Cardini, 1957). The orthophosphate was then determined according to the method of Chen et al. (1956), utilizing ascorbic acid as reagent. Cellular orthophosphate was measured alone in some cases.

The data were sufficient to suggest further examination of the insoluble fraction containing the polyphosphate. For this purpose, it was more convenient to measure RNA and arrive at poly-P by subtraction, assuming phosphoprotein was negligible. RNA was hydrolyzed by treating the lipid-free fraction with 1N KOH at 37°C for 18 hours. Poly-P, DNA and protein were precipitated by adding 6N HCl and 5% TCAc, then centrifuging. The supernatant was analyzed for free ribose by the procedure of Schneider (1957), using the orcinol-ferric chloride reagent. Purified yeast ribonucleic acid (Sigma Type XI) served as the standard. RNA phosphorus was estimated as 10% of the RNA value.

ANALYSIS OF SOLUBLE POOLS OF INTERMEDIARY METABOLITES

Glucose catabolism constitutes a site for oxygen control of metabolism. The flow of carbon through glycolysis and the Krebs cycle is

sensitive to changes in oxygen tension. A basic change in regulation of carbon traffic from glucose could result in apparent "resistance" to oxygen; if so, ORS might contain a significantly different pool of free intermediary metabolites than (+). Therefore free amino acids and organic acids were isolated and measured, essentially as described by Ting and Dugger (1967) and Osretkar and Krauss (1965).

Log phase cells were harvested, kept in the dark, and concentrated by centrifugation at 2-4°C. After decanting the supernatant, 80% methanol was added to the concentrated cell pellet. Time from harvest to addition of methanol was about 45-50 minutes, but cells were kept cold in the dark during this time. Cells were allowed to soak in methanol 18-24 hours in the refrigerator. They were then centrifuged and the methanol extract removed. The pellet was extracted again with boiling 80% methanol for 20-30 minutes. This supernatant was pooled with the previous methanol extract. The total methanol extract was reduced in volume to 10-15 ml by drying under nitrogen gas at room temperature.

Solid sodium chloride was added to the extract to give a 5% salt solution. Lipids were then removed by partitioning the salt phase with 30-45 ml of chloroform. The chloroform layer was washed twice with 2-3 ml of water. The aqueous layer was pooled with the two water washes, giving a total volume of about 20-25 ml.

Amino acids were separated from the aqueous extract by means of ion exchange chromatography. The extract was acidified to about pH 2 and then passed through a cation column of Dowex 50 resin (1 x 10 cm) charged in the H^+ form. Neutral compounds (sugar phosphates) and organic acids do not adsorb to the column, while the amino acids do exchange. The column was washed with 25 ml water which were added to the original col-

umn effluent. The amino acids were then eluted with 20 ml of 3N ammonium hydroxide.

The effluent containing the organic acids was made slightly basic, pH 9, with sodium hydroxide and applied to an anion column of Dowex 1-X8 (1 x 10 cm), which had been charged with 1M sodium formate, pH 7.0. The column was washed with 25 ml water prior to eluting the organic acids with 20 ml of 8N formic acid.

The two purified fractions were evaporated to dryness at 40°C under nitrogen gas. An aliquot of the amino acid fraction, dissolved in 0.1 N HCl, was subjected to ion exchange chromatography on a Technicon AutoAnalyzer with 21 hour elution time.

The organic acids were separated by thin layer partition chromatography according to Myers and Huang (1969) on microcrystalline cellulose plates (Camag, Inc.; Milwaukee, Wis.) in the system: diethyl ether/ formic acid/ water, 7:2:1. Acids could be visualized by spraying with brom cresol green (0.04% in ethanol) or acid ammonium molybdate reagent (Ting and Dugger, 1965). After the latter spray, the plate was air-dried and illuminated with ultraviolet light for at least one hour.

RESPIRATION AND RELATED STUDIES

Glucose respiration had previously been reported to behave differently in autotrophic (+) and ORS (Ward et al., 1969); hence, it was reasonable to assume that oxygen uptake of heterotrophic cells might correlate with data on phosphorus and metabolite pools as well. Accordingly, conventional Warburg manometry was employed to investigate respiratory activity. Dark-grown cells in log growth were harvested under sterile conditions, centrifuged cold, and washed twice with sterile Knops medium lacking nitrate and phosphate (= Knops Special). The cells

were then suspended in Knops Special and starved for 30 hours in the dark at 37.5°C under air + 1.7% CO_2 or 100% O_2 to deplete them of endogenous respiratory reserves. After starvation, cells were washed a final time and suspended in Knops Special. Aliquots were subsequently transferred to the main compartment of Warburg vessels. The sidearms contained either glucose or acetate in phosphate buffer, which when tipped into the main compartment gave a final concentration of 1 mM phosphate and 3.5 mM glucose or 61 mM acetate. Vessels were gassed with either air + CO_2 or 100% O_2, equilibrated, and endogenous oxygen uptake followed before tipping sidearms. Both endogenous and substrate-dependent rates were calculated.

Simultaneously, uptake of inorganic phosphate and glucose was determined. Exactly 15 and 75 minutes after adding glucose-phosphate mixture to cells, an aliquot was harvested in a centrifuge tube and immediately cooled to -3°C with an ice-acetone bath. Cells were removed by centrifugation at 0°C and 10,000 x g. The supernatant medium was saved for orthophosphate and glucose analyses by the methods previously described. Rates were assumed to be linear over this time period, and indirect evidence (oxygen uptake) verifies this.

Many compounds are known to affect respiration at relatively specific target sites. This selective action justifies their use on whole cells. In order to characterize the respiratory pathway *in vivo*, heterotrophic cultures were exposed to various agents; and oxygen uptake was followed with a YSI Oxygen Monitor calibrated against air level oxygen. Cells in maximum log growth were harvested from the main culture flask, 4 ml placed in the chamber of the oxygen monitor, and equilibrated with room air at 37.5°C. During the entire procedure, cells were pro-

tected from light by a black cloth. Substrate for the studies consisted of the glucose in the growth medium which proved to be saturating for the duration of oxygen measurements. Inhibitors were added in a volume of 0.1 or 0.2 ml. Control samples contained water or, in some cases, ethanol in place of the inhibitor. The rate of oxygen uptake was calculated from at least a four minute linear trace obtained on a Beckman Potentiometric Recorder. Inhibition or uncoupling was computed as:

$$\% \text{ inhibition} = 100 - \% \text{ of control.}$$

STUDIES ON THE PROCESS OF ADAPTATION TO DARK GROWTH

In addition to the numerous metabolic differences already noted between ORS and (+), ORS appeared to require a substantial period to adapt to heterotrophic conditions. This time-dependent phenomenon was observed whenever a new culture of ORS was introduced into the dark. Because the acclimation process may be related to one or more of the metabolic differences already investigated, an experimental technique was devised to characterize it.

The adaptation period was studied simply by transferring a log-growing autotrophic culture to glucose medium and placing it in the dark. Standard dark-growth conditions were subsequently maintained. Light-grown cells had been cultured at 39°C, (+) on air + 1.7% CO_2, ORS on 95% O_2 - 5% CO_2. General Electric Cool-White Fluorescent lights provided 1800 ft-c illumination.

Adapting cultures were generally diluted with fresh glucose medium every 24 hours. At various times during the dark exposure, samples were harvested and used for analyses. Several parameters were monitored: growth rate, respiratory rate, RNA, enzyme activities. Growth rate was determined as doublings per day over 12 hour periods based on dry

weight. Oxygen uptake was measured with the Oxygen Monitor on cells straight out of the growth chamber. The method for RNA has already been outlined. Enzymes assayed were alkaline phosphatase, NAD- and NADP-glutamate dehydrogenase, and NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenase.

Alkaline phosphatase was measured by a modification of the method of Fitzgerald and Nelson (1966). Whole cells were washed twice in 0.01 M Tris buffer pH 8.5 and suspended in 0.1 M glycine buffer, pH 10.5. The substrate, p-nitrophenyl phosphate, was added and the suspension incubated with shaking at 38°C. After 30 to 120 minutes, the reaction was stopped by adding 0.02 M sodium hydroxide, and the cells were removed by centrifugation. The colored product was read at 420 nm and the absorbance converted to units of enzyme from a standard curve of p-nitrophenol. A unit of alkaline phosphatase is defined as the amount of enzyme producing 1 micromole of p-nitrophenol per hour.

Other enzymes were assayed in cell homogenates, prepared by grinding washed cells with glass beads in the MSK. All work was done at temperatures of 5°C or lower. The homogenizing medium consisted of 0.05 M potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 0.1 % Triton X-100. The crude homogenate was centrifuged at 750 x g for 10 minutes, then for 20 minutes at 32,000 x g. The resulting supernatant was used for determination of glyceraldehyde-3-phosphate dehydrogenase (GAPD) and glutamate dehydrogenase (GLDH). The spectrophotometric method of Schulman and Gibbs (1968) was used for the GAPD assay, while GLDH was measured according to Joy (1969). A Beckman DB-G Recording Spectrophotometer was used with a temperature-regulated cell compartment.

The GAPD reaction mixture contained in micromoles: pH 8.5 sodium

pyrophosphate, 6; $MgCl_2$, 20; pH 8.0 cysteine, 12; ATP, 5; and NADH or NADPH, 0.4. Twelve micrograms phosphoglycerate phosphokinase (Sigma Type I-C) and the test enzyme preparation were added, and the entire mixture incubated for 7 minutes at 26°C in the spectrophotometer compartment to measure endogenous oxidation of the pyridine nucleotides. After 7 minutes, 10 micromoles of 3-phosphoglycerate (3-PGA) were added, mixed well by inversion, and the reaction followed by decrease in absorbance at 340 nm. Total volume was 2.1 ml and cuvettes had a 1 cm light path. The initial rate remained linear for two or more minutes after addition of 3-PGA. This portion of the curve was used to calculate enzyme activity. Activity was proportional to enzyme concentration.

Likewise, GLDH catalysis was followed in the oxidative direction at 340 nm. NAD-linked activity was determined in 0.2 M Tris-HCl buffer, pH 8.4, and NADP-GLDH in 0.1 M potassium phosphate buffer, pH 7.5. The reaction mixture contained 0.2 ml 0.2 M α-ketoglutarate pH 7.5, 0.2-0.4 micromoles NADH or NADPH, and the test enzyme in 2 ml of the appropriate buffer. Endogenous reaction was measured for 7 minutes at 26°C, at which time, substrate (0.2 ml 1M ammonium sulfate) was added. Initial velocity was calculated from the first 0-2 minutes of the reaction which was linear. Controls without α-ketoglutarate or ammonium were run, as well as several enzyme dilutions.

Decrease in absorbance at 340 nm was converted to moles of substrate, using a molar extinction coefficient of 6.22×10^6 cm^2/ mole for both NADH and NADPH. A unit of GAPD or GLDH activity corresponds to the amount of enzyme which oxidizes 1 micromole of reduced pyridine nucleotide per hour.

RESULTS

BASIC PHYSIOLOGICAL CHARACTERISTICS OF HETEROTROPHIC *Chlorella sorokiniana*

1. Growth on Glucose

Optimal conditions for heterotrophic growth are considered 37.5°C and 1.7% CO_2 in air. Under these conditions, a typical heterotrophic growth curve appears as in Figure 1. The situation is analogous to growth for any organism supplied with saturating amounts of nutrients. When cell mass is plotted versus time, an exponential curve is obtained. All growth curves in this work are plots of $\log_{10}$(cell mass) against time which yield a straight line, the slope of which is equivalent to the growth rate. This type of growth curve was obtained for various culture conditions in order to insure that growth was logarithmic.

Figure 1 also depicts the basic physiological differences between (+) and ORS. (+) on air demonstrates a vigorous growth rate of 7 doublings/day. ORS on air achieves a rate as high as 5.2 doublings/day, though often 4.6 - 4.8 doublings per day is maximum. When exposed to 100% O_2, (+) is severely inhibited by 90%, its growth falling to less than 0.8 doublings/day. ORS however grows on 100% O_2 immediately at a growth rate of 2.1 - 2.5 doublings/day, equivalent to only 50% inhibition. Furthermore, after one day on 100% O_2, ORS has increased its growth rate to 3+ doublings/day. This situation, quantitative rather than qualitative, constitutes resistance to oxygen by ORS.

2. Temperature and Gas Requirements

Examination of the effect of temperature on growth gave the

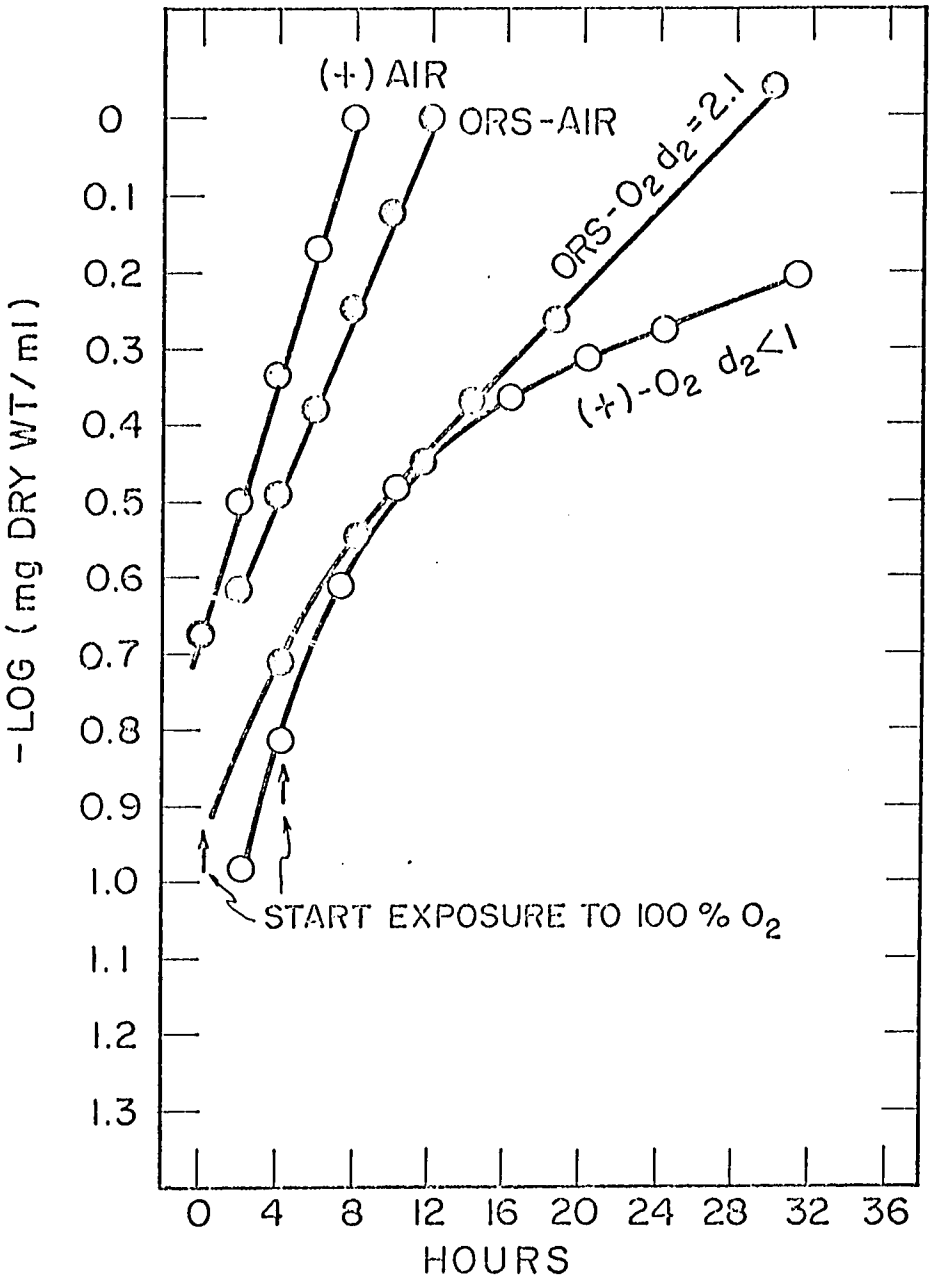


FIG.1 DARK GROWTH OF (+) AND ORS ON 0.3 % GLUCOSE UNDER AIR AND HIGH O_2

results in Figure 2. The optimal temperature lies around 38°C for both (+) and ORS on air. Moreover, the temperature requirement does not differ between air and oxygen, since parallel curves were constructed. The 38°C value is about one degree lower than the autotrophic value of 39°C for (+) published by Sorokin (1959).

Very simple tests of gas requirements showed that (+) was dependent on CO_2 in excess of air level for maximum growth. (+) on air without added CO_2 demonstrated a growth rate of only 5 doublings/day. ORS on air was not affected by lack of CO_2 as there was no difference in growth on air only or air fortified with 1.7% CO_2. However under high oxygen, an interesting CO_2 effect was noted. On 98% O_2 - 2% CO_2, ORS showed a longer lag phase after dilution, and growth was always somewhat slower than on 100% O_2. If CO_2 was increased to 5%, the reduction in growth rate was even more striking. For this reason, 100% O_2 was used for later studies. In summary, CO_2 was required for best growth of (+) on air, but retarded ORS under high oxygen.

3. Substrate Requirements

From analysis of Table 1, it is obvious that C. sorokiniana utilizes carbohydrate exclusively as a C-source for heterotrophic growth. Acetate is the only non-sugar substrate supporting growth. Moreover, it seems hexoses specifically permit growth. There are no apparent substrate differences for (+) and ORS; both grow on the same compounds, though ORS grows slower on glucose. Permeability difficulties could be responsible for the inability to grow on some organic acids. This would not appear to explain the failure of amino acids alone to support growth, since growth on glucose plus amino acids is increased over glucose alone. This observation agrees with the previous work of Shihira and Krauss (1963)

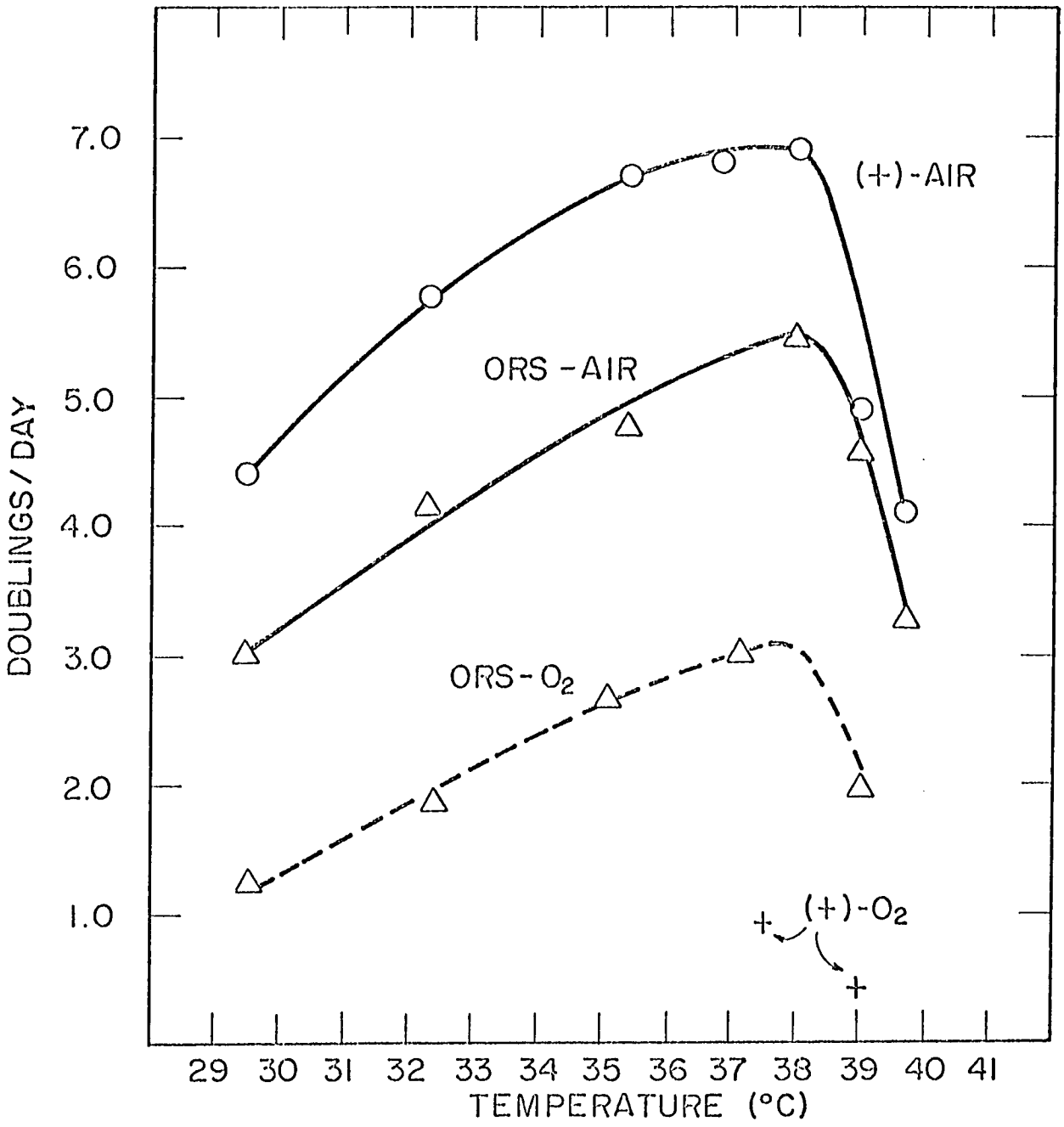


FIG. 2 EFFECT OF TEMP. ON HETEROTROPHIC GROWTH OF Chlorella sorokiniana ,(+) AND ORS STRAINS

Table 1. Substrates Which Support Heterotrophic Growth[1] of (+) and ORS under Air

Substrate	(+)	ORS
Glucose + casein digest	1.0	0.9
Glucose	0.8	0.7
Galactose	0.4	0.4
Acetate (sodium)	0.5	0.5
Casein digest	0	0
Xylose	0	0
Arabinose	0	0
Sucrose	0	0
Glycerol	0	0
D-glucosamine	0	0
Pyruvate (sodium)	0	0
Citric acid	0	0
α-ketoglutarate (sodium)	0	0
Succinate (sodium)	0	0
Glutamine	0	0
Proline	0	0

1. Values = relative growth rates after 10 days.

that amino acid nitrogen stimulates growth of C. sorokiniana over nitrate or ammonium nitrogen.

4. Growth on Acetate

In view of the results in Figure 3, ORS evidently does not manifest resistance to high oxygen if grown on acetate, and thus responds identically to (+). Growth ceases completely within 16 hours after initial exposure to high oxygen, even though both strains grow well (3 doublings/day) on acetate under air. This characteristic of ORS contrasts with the response noted by Begin-Heick and Blum (1967) for another organism. The achloric euglenoid, Astasia longa, was found to grow on acetate under high oxygen, though oxygen did inhibit its growth by 50%. It is likely then that oxygen resistance here in Chlorella operates via a different mechanism from the Astasia system.

ULTRASTRUCTURAL CHARACTERISTICS OF HETEROTROPHIC C. sorokiniana

All electron photomicrographs are located at the end of the paper.

1. Wild Type on Air

Typical dark-grown Chlorella sorokiniana are represented by the electron micrographs of (+) on air (Figures 4-6). Many morphological features are similar to those of autotrophic Chlorella (Reger and Krauss, 1970) and other eukaryotic microbes grown on reduced organic carbon eg. some protozoans, unicellular fungi and algae (Lesemann and Fuchs, 1970; Neal, 1969; Budd et al., 1969). Other features are relatively specialized for the organism, and have not generally been described in other systems.

Cells are basically spherical (2.5 to 6 micra) and are always surrounded by a dense cell wall, characteristic of plant cells. The wall does not reveal layers, rather it appears amorphous in texture. Generally the space between the cell wall and the underlying plasmalemma contains

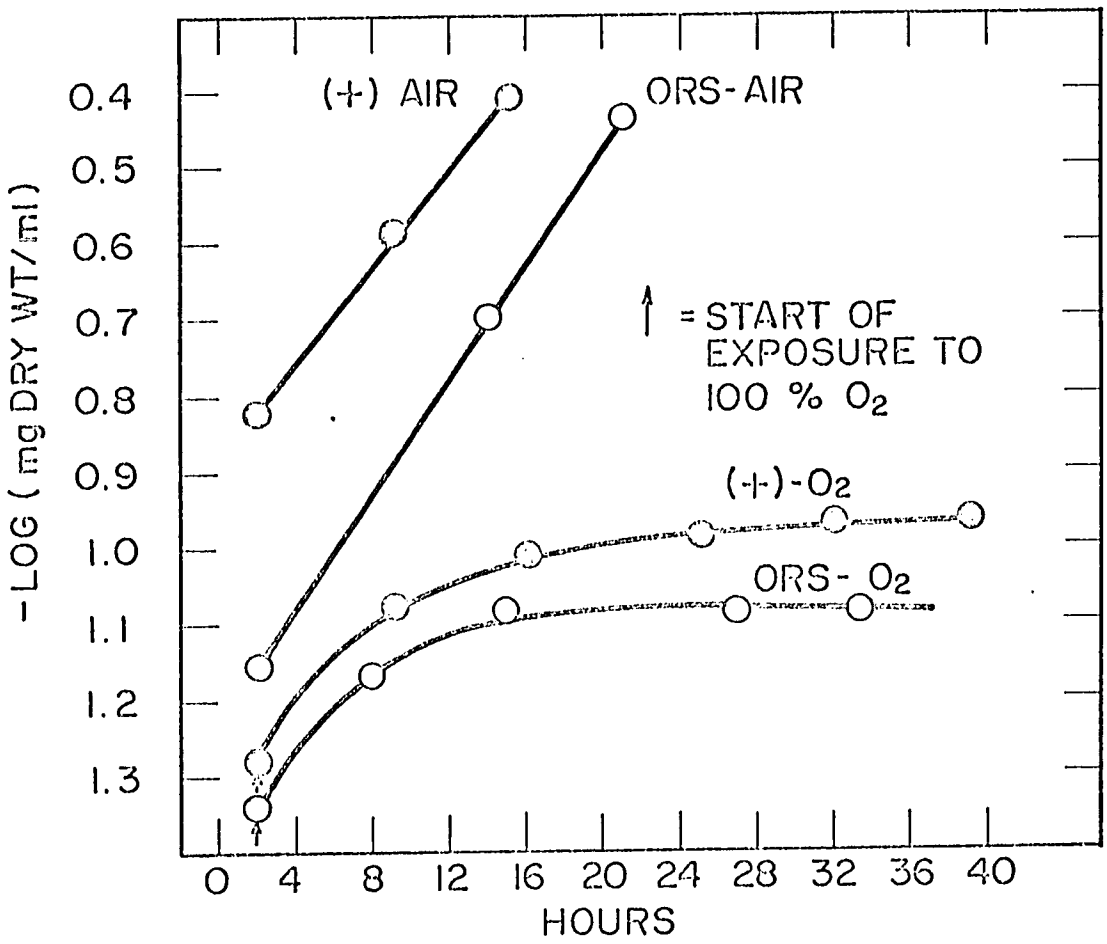


FIG. 3 DARK GROWTH OF (+) AND ORS ON 0.25 % SODIUM ACETATE UNDER AIR AND 100 % O_2

flocculent material, which may be especially prominent in the invaginations commonly seen in the cell surface.

The cytoplasmic ground substance is interspersed with normal organelles. The nucleus, containing a denser core of nucleolar material, is usually situated acentrically. Numerous ribosomes appear free in the cytoplasm, as very little rough endoplasmic reticulum is visible. Occasionally polyribosomes are evident. Smooth ER is moderately abundant, often close to expanded vacuoles and vesicles. Large membrane-bounded vacuoles may contain particulate material (Figure 4A). Apparently these vacuoles may open to the cell surface and release their contents. Dictyosomes are seen frequently in close proximity to the nucleus.

Several well-developed mitochondria are present in each cell and are often seen in close association with the single chloroplast of the cell. Their shapes are variable (Figure 6B), but most often they appear elongated and "sausage-like." They possess prominent cristae which form laminar or retiform networks in the mitochondrial matrix.

Due to lack of light, the chloroplast is found in a fairly dedifferentiated condition, as noted by Budd et al. (1969). The chloroplast lamellae, or thylakoids as they are also called, are extremely degenerate, existing as dark-staining pre-thylakoidal "ribbons" running longitudinally in the lighter-staining stroma. However these membranes do not disorganize in the dark as occurred in the Chlorella mutant examined by Bryan et al. (1967).

Since the chloroplast in C. sorokiniana is a cup-shaped structure, it occupies considerable space on one side of the cell. Under heterotrophic conditions, the chloroplast becomes reticulate, as it dedifferentiates. This occasionally causes "pockets" of cytoplasm to appear in

it and also explains why it may have several parts in some sections (Figure 4B). The swirl of membranes often noticed is a section through part of the chloroplast, consisting of a flat sheet of stroma sandwiched between the double outer membranes (Figures 5,6).

Typical starch grains are frequently observed, always within the stroma of the chloroplast, never in the cytoplasm. Even in the heterotrophic state, the chloroplast contains noticeable osmiophilic droplets; while apparently the pyrenoid, quite prominent in autotrophic cells, disappears in the dark.

One of the characteristic features of heterotrophic (+) C. sorokiniana are dense electron-opaque deposits, often of a granular nature, occurring in the cytoplasm (Figures 4A,6). They do not appear membrane-bounded and frequently develop holes in them under the electron beam. Though often randomly distributed in the cell, the material sometimes is in close proximity to mitochondria or the chloroplast, in the in the latter instance especially in the swirls. Previous investigators describe similar-looking material in Chlorella sp. and Myxococcus sp. and identify it as insoluble deposits of polyphosphate (Oschman, 1967; Voelz, 1966) or volutin. Later studies will establish that the material is probably poly-P.

A rather non-descript inclusion, evident in Figure 4A, 4B has not been definitely identified. Similar structures pictured in the literature (Matile, 1968) are known to be lysosomes or peroxisomes (Tolbert et al., 1968). However, these microbodies have never been described in algae such as Chlorella, a fact which might correlate with their specialized mechanism for photorespiration (Bruin et al., 1970). Since the present work involves dark-grown organisms, this may still leave open the possi-

bility. For now it must suffice to state that this inclusion bears a strong resemblance to microbodies in higher plants.

2. ORS on Air (Figures 7-10)

ORS on air presents overall the same cell morphology with several outstanding differences. ORS cells generally are more variable in size and shape than (+) (Figures 7,8). The osmiophilic (= poly-P) deposits, so abundant in the cytoplasm of (+), are conspicuously lacking here in ORS. However osmiophilic globules, probably lipid, are visible within the chloroplast. In many cells, the chloroplast shows a more compact, spindle or disc shape, and appears in a more differentiated condition (Figure 8). Thylakoids retain a more characteristic stacked appearance. Often starch occupies much of the volume of the chloroplast in the form of large grains.

ORS cells are typically more vacuolated and vesiculated than (+) (Figures 7A,9A). The area adjacent to the nucleus and the chloroplast often contains a large vacuole within which abundant flocculent material (possibly carbohydrate) is present. Smooth ER too, as well as dictyosomes, appear to be abundant in this region of the cell (Figure 9B).

Mitochondria show much the same configuration as (+), and often are closely appressed to the chloroplast (Figures 8B,E,D). Cristae are well-developed, sometimes appearing closely-stacked and shelf-like.

A major difference between (+) and ORS pertains to cell division. Heterotrophic (+) undergoes typical Chlorella division by formation and liberation of autospores from a mother cell. It appears that the number of daughter cells per division in heterotrophic cells is lower than in light-grown cells, as two to four progeny were constantly observed rather than higher numbers. However ORS routinely demonstrated a division figure

almost never seen in (+), involving a type of binary division in which one cell gives rise to two daughter cells, which remain joined by a common portion of the cell wall. The resulting "dumb-bell" cells (Figure 10B) comprised roughly 5-10% of ORS cultures at all times. It has not been definitely established whether this phenomenon constitutes true binary fission or whether it is incomplete autospore formation. The latter seems more likely.

Figures 9B, 8A, 10A, 10B show the probable sequence of events for the division. After the nucleus, chloroplast, and mitochondria divide (Figures 8A,9B), these organelles separate and are distributed to opposite poles of the mother cell (Figure 10A). Cleavage of the cell occurs along a median cleavage furrow. Formation of the cell wall seems to occur by indentation rather than outgrowth. Since the cell wall does not completely form in many cases, a "dumb-bell" cell is produced (Figure 10B).

3. Wild type on 100% oxygen

(+) cells exposed to 100% O_2 manifest symptoms of extreme oxygen toxicity. Intact cells are scarce. Many cell ghosts and cells in various stages of degeneration are present. Figures 11, 12 show some of the typical intact cells. It is evident that the chloroplast undergoes disintegration most rapidly. The pre-thylakoidal strands become totally disoriented and the chloroplast loses its normal cup-shape. Starch appears to increase as well.

Of the remaining organelles, mitochondria and dictyosomes increase in numbers. Mitochondrial structure remains similar to that of (+) on air. The fact that Chlorella mitochondria increase due to high partial pressures of oxygen agrees with the observations of Schaffner

and Felig (1965), who obtained similar results for rat liver cells. The abundance of dictyosomes and vesicles produced by them indicates that membrane may be rapidly turning over or needed to package material such as lipid, hydrolytic enzymes, or carbohydrate. Numerous vesicles can be seen adjacent to the dictyosomes in Figure 12. These vesicles possibly coalesce, becoming the large vesicles containing medium density material. There is also the possibility that some material is being excreted from the cell, as evidenced by the scalloped appearance of the cell membrane in places (not shown). The osmiophilic deposits show an increase over air level and assume more of an amorphous rather than granular texture (Figure 11A).

4. ORS on 100% oxygen (Figures 13-15)

In contrast to (+) on high oxygen, the majority of ORS cells on 100% oxygen are intact. But they differ vastly from their air-grown counterparts. Metabolism appears to have shifted or slowed down substantially, causing correspondingly striking changes in morphology. The cell definitely responds to high oxygen by some general cellular phenomenon, as practically all features are modified.

Cells can be described as large and bulky. Many structures have enlarged physically. The cell wall may appear thicker, in some cases to the point of being sporelike (Figure 14B). Much of the increased cell mass can be accounted for by the deposits of reserve or storage materials in evidence. Most cells contain increased amounts of starch, always in the remnant of the chloroplast. Some cells are packed with it (Figure 13B). In many cells, the cytoplasm contains numerous membrane-bound vesicles filled with a material of medium density, possibly lipid or carbohydrate (Figures 13,14A,15). This extrachloroplastic material some-

times appears similar to amorphous patches within the chloroplast (Figure 13A).

The chloroplast itself loses much of its integrity and becomes highly disorganized. The thylakoidal strands often separate and spaces (vacuoles) form between them where starch would normally be situated. In some cases the lamellae dedifferentiate to the extent that tubules of the pro-lamellar body are present (Figure 14B).

The cytoplasmic ground substance occupies limited space due to the space occupied by the chloroplast, and copious vesicular deposits. However around the edges of the cell, a large quantity of atypical rough endoplasmic reticulum is prominent (Figure 13B). In cross-section, this ER(?), studded with ribosomes, forms a tubular network of cisternae, which may contain visible material (Figure 14B). Small vesicles and dictyosomes are also present.

Mitochondria show one of the most pronounced changes in ORS on high oxygen. A cursory inspection reveals hardly any typical mitochondria. Upon careful examination, a moderate number of small rudimentary mitochondria are discernible, especially around the cell periphery (Figure 15). These mitochondria, consisting primarily of matrix and very few cristae, resemble the repressed, inactive mitochondria of yeasts grown on high concentrations of fermentable sugars (Neal, 1969). Thus a major response of ORS to high oxygen is reflected in the dedifferentiation of its mitochondria and its atypical mitochondrial morphology.

METABOLIC CHARACTERISTICS OF HETEROTROPHIC (+) AND ORS

1. General Cell Composition

Examination of cell constituents on the basis of dry weight confirms striking differences between (+) and ORS (Table 2). ORS on air

Table 2. Lipid, Protein, and Starch Composition as Percent Dry Weight

	(+)		ORS	
Component	Air	100% O_2	Air	100% O_2
Lipid	11	11	10	10
Protein	34	26	21	18
Starch	26	37	45	60

contains a sizeable portion of dry weight as starch (45%), nearly twice as much as (+) under similar conditions. Under high oxygen this component increases to 60%, still twice as high as (+) on high oxygen. Possibly this abundance of starch causes the percent protein for ORS (air and high oxygen) to be correspondingly lower than (+) on air and high oxygen. Lipid appears relatively equal for both strains (ca. 10%).

These data can be analyzed further on a per cell basis (Table 3). It is apparent that ORS cells are heavier than (+) cells. In air this increased weight can be accounted for as starch, since ORS cells contain over 100% more starch than (+). Protein per cell is lower (ca. 25%), while lipid is somewhat greater (ca. 10%) than (+). Under high oxygen, starch is again responsible for the bulk of ORS cell weight, while protein is only moderately elevated over air level. A major change has occurred in the lipid level of ORS on high oxygen which is almost 60% greater than (+).

The conclusion would appear to be that ORS preferentially accumulates starch under both oxygen conditions, but does not synthesize as much protein as compared to (+). ORS on air does however maintain a normal complement of lipid, which increases proportionately on high oxygen. These measurements correlate strongly with several ultrastructural observations:

1) Starch - ORS has more than (+), especially on high oxygen.

2) Lipid - Since (+) does not contain greater quantities than ORS, the osmiophilic deposits seen in (+) would not appear to be lipid.

2. Kinetics of Response to 100% Oxygen

Though ORS manifests quantitative resistance to 100% oxygen on the basis of growth rate, there are qualitative metabolic differences

Table 3. Lipid, Protein, and Starch Composition as Picograms per Cell

	(+)		ORS	
Component	Air	100% O_2	Air	100% O_2
Weight	11.6	11.5	14.3	21.4
Lipid	1.3	1.3	1.4	1.9
Protein	3.9	3.0	3.0	3.4
Starch	3.0	4.3	6.4	11.5

between cultures of (+) and ORS exposed to high oxygen. Figure 16 demonstrates the kinetics of pH change for such cultures. ORS shows only a transient change in pH due to oxygen, whereas pH changes drastically in (+) cultures. This is due possibly to acids liberated into the medium as cells die and lyse; or it could be an indication of active excretion of organic acids. The latter would result from a definite shift in metabolism. Such a situation has been documented for C. vulgaris by Karlander and Krauss (1966), who found acetic and formic acids being excreted in the dark. Weis and Mukerjee (1958) also reported that various chlorellas excreted acetic acid, ethanol, or lactic acid as anaerobic dark fermentation products. Obviously ORS is immune to this aspect of metabolic control exerted by oxygen.

Starch and protein change in response to high oxygen as seen in Figures 17 and 18. The stability of ORS cultures is once again evident. For the first 10 hours on oxygen, both starch and protein remain constant in ORS. During this same period in (+), starch has risen some 50% and protein has dropped 35%. Subsequently, starch increases suddenly in ORS to the typical high oxygen level, while in (+) starch peaks out around 18 hours and then starts to drop. Protein parallels starch changes in an inverse manner.

3. Culture Yield Data

When yield data are compared for heterotrophic growth, (+) on air and ORS on air do not differ markedly, though ORS does achieve a slightly higher value (Table 4). It should be pointed out, however, that yield does not depend on rate of glucose utilization nor the end products of glucose metabolism. Though (+) may not store up as much assimilated glucose in starch as ORS does, it could conceivably take up as much

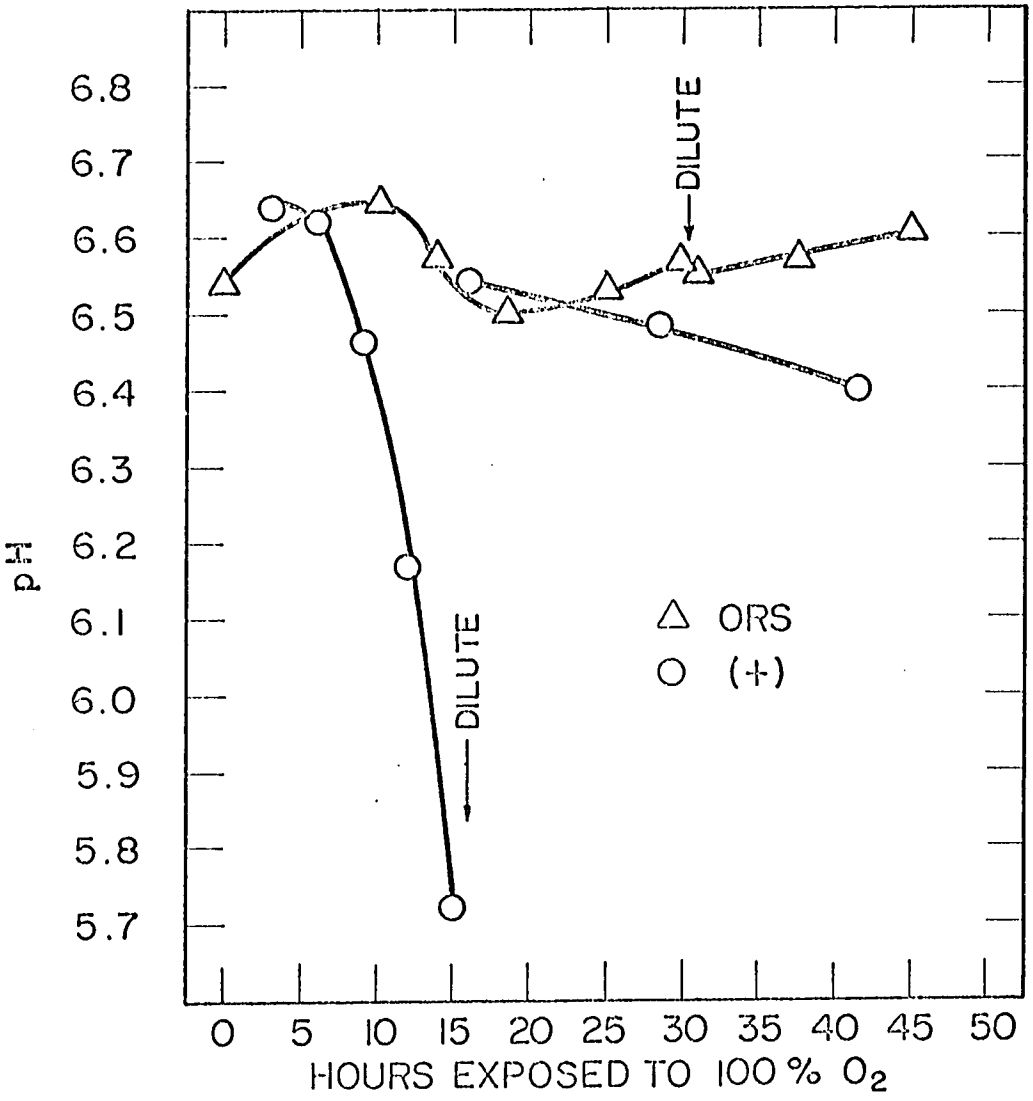


FIG. 16 KINETICS OF CULTURE MEDIUM pH CHANGE WITH EXPOSURE TO 100 % O_2

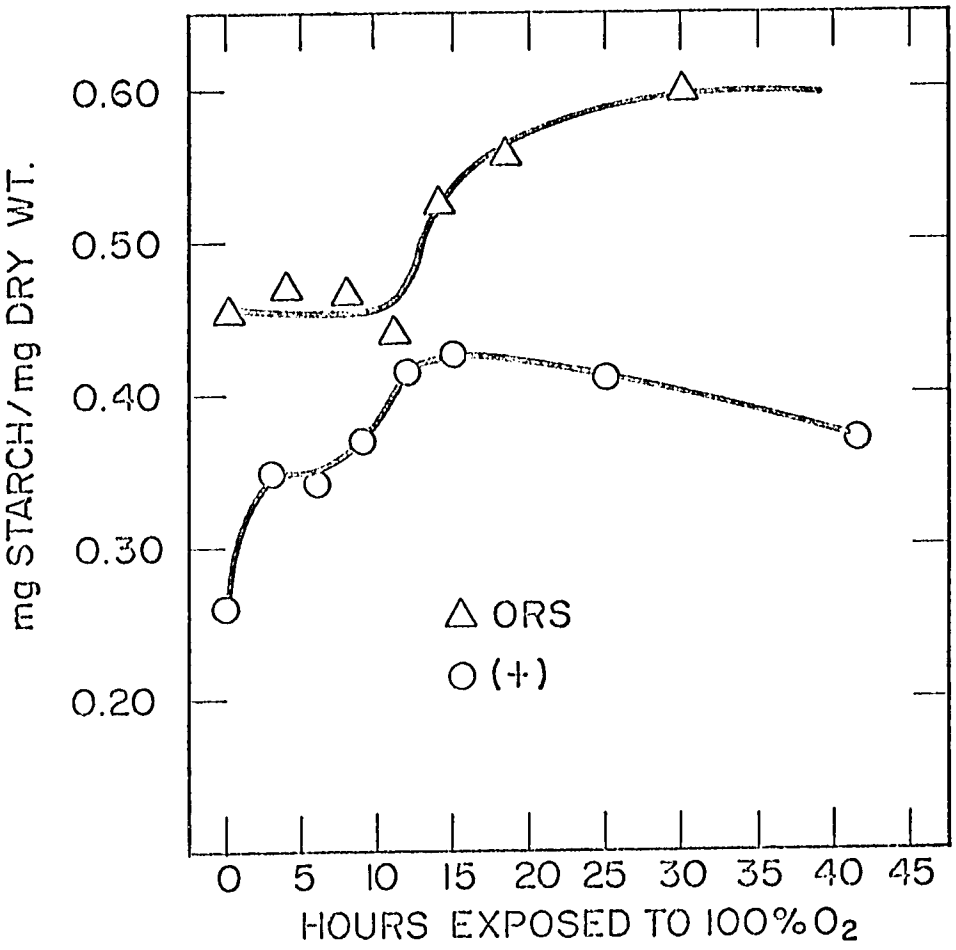


FIG. 17 KINETICS OF STARCH CHANGE WITH EXPOSURE TO 100 % O_2

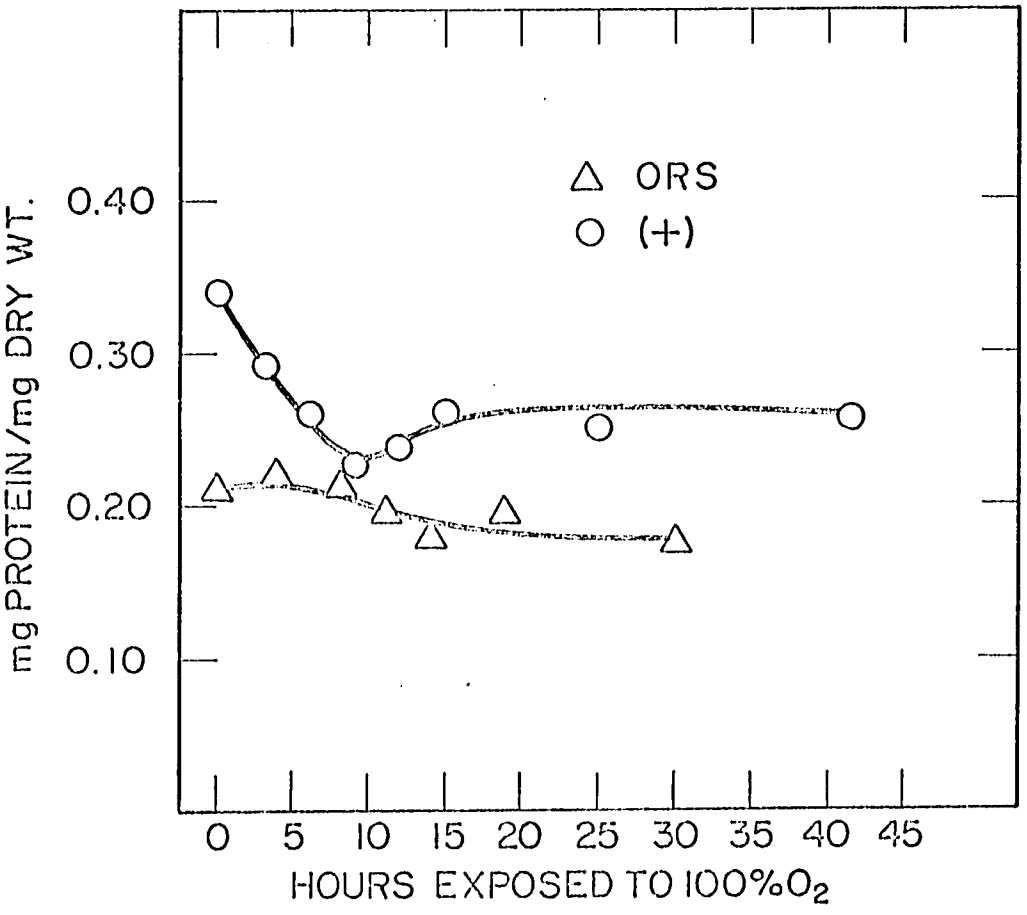


FIG. 18 KINETICS OF PROTEIN CHANGE WITH EXPOSURE TO 100% O_2

Table 4. Culture Yield[1] Data for Heterotrophic Growth under Air and 100% Oxygen

Culture	Air	100% O_2
(+)	.505	.243
ORS	.545	.452

1. $$\text{Yield} = \frac{\Delta \text{ (mg dry wt/ml)}}{\Delta \text{ (mg glucose/ml)}}$$

glucose and use it for other purposes, eg. energy metabolism, protein synthesis, or structural material. Moreover starch formation in ORS could be a slower process than in (+).

On the other hand, the yield of cultures under high oxygen definitely correlates with the large size and healthier condition of ORS compared to (+). Since yield is a measure of efficiency of a system, oxygen resistance in ORS does seem to be characterized by efficient conversion of glucose to dry weight.

4. Fractions of Cell Phosphorus

Analysis of Table 5 indicates the necessity for examining subcellular fractions of phosphorus (= P). Based on total cell-P, ORS contains around 20% less P than (+) under both air and high oxygen. The significance of this is equivocal, in view of the diversity and numerous functions of intracellular phosphorus compounds.

Two major fractions are responsible for lower total cell-P in ORS: cold-acid soluble (= sol. P) and insoluble (= insol-P) phosphorus fractions. ORS on air has 30% less sol-P and 20% less insol-P than (+) on air. Under high oxygen the contributions of the two fractions become unequal: ORS now has 48% less sol-P, and only 5% less insol-P, than (+). Hence total cell-P does not reflect the true phosphorus characteristics of ORS and (+).

If these two fractions are analyzed further, the phosphorus situation assumes even more complexity. The main difference in the sol-P is due to the level of inorganic phosphate (= Pi). ORS on air and high oxygen has less than 50% of the Pi found in (+) on air and high oxygen respectively. The remainder of the sol-P fraction does not vary at all between (+) air and ORS air, whereas it is grossly different for (+) and

Table 5. Micrograms Phosphorus[1] in Various Cell Fractions of Heterotrophic (+) and ORS under Air and High Oxygen

Fraction	(+)-Air	ORS-Air	(+)- O_2	ORS- O_2
PO_4-P	52	25	66	30
Other Sol-P	25	28	103	57
RNA-P	160	300	130	420
Poly-P	225	0	323	0
Lipid-P	20	24	20	31
Total Cell-P	487	383	662	542

1. Values = micrograms phosphorus per 10^9 cells

ORS on high oxygen. The composition of insol-P reveals a very strange situation indeed. ORS on air contains twice as much RNA as (+) on air, but no poly-P! Under high oxygen, ORS now has over 200% more RNA than (+), and still no poly-P. It appears that ORS lacks poly-P entirely, and, perhaps consequentially, contains a massive quantity of RNA.

These phosphorus differences can be characterized another way. Values in Table 5 were used to calculate the ratio of air level to high oxygen level for each P fraction. The resulting Table 6 reveals the percent increase in a fraction due to high oxygen. From these data emerges a pattern of opposite trends for (+) and ORS, at the level of their phosphorus metabolism. In general, high oxygen causes total cell phosphorus of both strains to rise roughly 25-30%. For (+), this increase is mainly in Pi, other sol-P, and poly-P, where increases of 21, 76 and 30% respectively, are noted over air levels. Outstanding is the 20% reduction in RNA content of (+). In contrast, increases are noted for all ORS fractions except poly-P. RNA and lipid-P have risen 30 and 23% respectively, while other sol-P is up 50%. The increases in ORS are in keeping with its tendency to enlarge in response to high oxygen, while the decreases in (+) could easily denote perturbations in phosphorus metabolism by high oxygen, especially RNA metabolism and sol-P.

Phosphorus in each fraction can be expressed as percent of the total cell P. Table 7 reviews the subcellular distribution of phosphorus on this basis. Once again, phosphorus metabolism of (+), when compared to ORS, shows these prominent features:

1. higher Pi - under air and high oxygen
2. higher other sol-P - under high oxygen
3. lower RNA - under air and high oxygen

Table 6. Percent Change[1] in Phosphorus Fractions due to 100% Oxygen for Heterotrophic (+) and ORS

Fraction	(+)	ORS
PO_4-P	+21	+17
Other Sol-P	+76	+50
RNA-P	-19	+29
Poly-P	+30	0
Lipid-P	0	+23
Total Cell-P	+26	+29

1. Values = $\frac{\text{Oxygen Level - Air Level}}{\text{Air Level}}$ X 100

Table 7. Phosphorus Fractions as Percent of Total Cell Phosphorus for Heterotrophic (+) and ORS

Fraction	(+)-Air	ORS-Air	(+)- O_2	ORS- O_2
PO_4-P	10.7	6.5	10.0	5.5
Other Sol-P	5.1	7.3	15.6	10.5
RNA-P	32.8	78.3	19.6	77.5
Poly-P	46.2	0	48.8	0
Lipid-P	4.1	6.3	3.0	5.7

4. high levels of poly-P present - under both air and high oxygen

5. lower lipid-P - under air and high oxygen

5. Metabolite Pools

Mounting evidence indicates that control of physiological processes by oxygen acts at the level of carbon metabolism.

Ellyard and Gibbs (1969) proposed that the Warburg effect results from depletion of intermediates in the photosynthetic carbon cycle. In the presence of oxygen, carbon is drained out of the dark cycle in the form of glycolate, which is probably formed from the glycolaldehyde moiety in the transketolase reaction. As a consequence the level of ribulose-5-phosphate falls and photosynthesis is inhibited.

Kilburn et al. (1969) observed that in mouse LS fibroblasts cultured at high oxygen (320 mm Hg), lactate production from glucose was inhibited. Only 60% of the glucose consumed by the cells could be accounted for as lactate and CO_2. They concluded that oxygen interfered with normal carbohydrate metabolism and destroyed the homeostatic equilibrium between glycolysis and respiratory pathways.

Hogetsu and Miyachi (1970), using Chlorella sp., determined that the flow of fixed CO_2 was preferential, depending on the presence or absence of oxygen during the period of dark CO_2 fixation. Under anaerobic conditions, it went into alanine, whereas under aerobic conditions the main products were aspartate and glutamate.

In all cases, the oxygen effect was manifested by significant shifts in metabolic carbon pathways.

Originally, free pools of metabolites were examined to provide an overall comparative view of intermediary metabolism in (+) and ORS.

Resistance to oxygen could conceivably occur if a normally sensitive step in carbon metabolism had been altered and now was insensitive to oxygen regulation. Defects in regulatory enzymes, whether constitutive enzymes (eg. glycolytic sequence) or adaptive enzymes (eg. TCA cycle, amino acid biosynthesis), are often reflected in the levels of metabolites produced. Of the numerous carbon pathways in a cell, an amphibolic sequence (eg. glycolysis or TCA cycle) would afford a likely location for such a basic control mechanism.

Soluble amino acids. Intracellular levels of free amino acids are listed in Table 8. Comparison of (+) and ORS on air indicates that the two strains vary substantially in metabolism of only a few amino acids. Overall, (+) has a larger total cell pool than ORS, by 26%. Two acids particularly contribute to this increased pool in (+): alanine and serine, which are 1.6 and 3.3 times higher, respectively than ORS. Other minor differences exist for proline, lysine, and γ-amino-butyrate; (+) shows larger amounts of these too.

Simultaneously, ORS contains somewhat higher levels of glutamate, glutamine, aspartate, and threonine (1.3, 7.1, 1.5, and 2.1 times respectively). If (+) and ORS differ in amino acid metabolism, it most certainly is in the area of glutamate metabolism. Interestingly, a large fraction of glutamine occurs in ORS on air (at 8% of total pool), whereas (+) on air lacks any trace of this glutamate derivative. In no other aspect do the amino acid pools of (+) and ORS on air differ as greatly.

Both alanine and serine are synthesized from glycolytic intermediates, alanine from pyruvate and serine via phosphoglycerate. Their abundance in (+) indicates that a substantial amount of carbon is being degraded by glycolysis and that the TCA cycle is operating at rate-lim-

Table 8. Soluble Amino Acid Pools[1] of Heterotrophic (+) and ORS under Air and 100% Oxygen

Amino Acid	(+)-Air	ORS-Air	(+)- O_2	ORS- O_2
Asp	0.73	1.07	0.37	2.37
Thr	0.86	1.82	0.81	3.20
Ser	13.95	4.25	5.41	12.63
Gln	-[2]	7.26	3.35	23.30
Pro	6.20	1.98	-	4.25
Glu	21.74	28.59	3.28	39.07
Gly	2.19	2.23	4.51	4.19
Ala	59.16	36.10	12.64	37.67
Val	1.86	1.59	-	1.44
Ile	-	-	-	0.46
Leu	0.82	0.68	-	1.26
Lys	5.12	2.94	7.16	3.20
Trp	-	0.76	-	-
Phe	-	-	0.48	-
GAB[3]	0.32	-	1.49	-
Total Pool	113.00	89.82	39.52	133.04

1. Values = micromoles per 10^{11} cells

2. - indicates less than 0.1 micromole per 10^{11} cells

3. GAB = γ-aminobutyric acid

iting activity. That is, glycolysis can provide the mitochondrial machinery with fuel (as pyruvate) faster than the cycle can burn this substrate. Since alanine is the major amino acid in both (+) and ORS on air (52 and 40% of total pool), this conclusion applies to both strains.

Glutamate, glutamine, proline, aspartate, and threonine are derived mainly from TCA cycle intermediates, namely α-ketoglutarate and oxaloacetate, and comprise a so-called "mitochondrial pool." At first glance, the quantity of mitochondrial pool amino acids shows an obvious disparity between (+) and ORS. These metabolites constitute 45% of the total cell pool in ORS on air, and only 26% in (+) on air. Of these mitochondrial pool acids, glutamate and glutamine combined account for 88% in ORS, while in (+) they total only 73%. Hence in ORS, glutamine and its precursor glutamate, are chiefly responsible for the high content of mitochondrial amino acids. This suggests that glutamine is drained away from the TCA cycle in ORS perhaps in order to satisfy a metabolic requirement for it elsewhere.

These facts could be interpreted as evidence that in ORS the TCA cycle operates at a higher rate of activity than (+). However the data are not sufficient to deduce the precise rate. Indeed, the lower respiratory rate of ORS on glucose contradicts a higher rate (Ward et al., 1969a). Moreover the TCA cycle *in toto* need not function in ORS at a higher rate than (+), but only the reactions leading to glutamate and glutamine.

Under high oxygen, amino acid metabolism of (+) is considerably depressed, while ORS maintains a highly active metabolic state. The total cell pool of (+) on high oxygen is reduced to about 30% of the (+) air value, whereas ORS on high oxygen has a larger cell pool (48%) than ORS on air.

Comparison of (+) on high oxygen to (+) on air reveals interesting metabolic shifts, indicative of oxygen toxicity symptoms. The amino acids derived from glycolytic intermediates, alanine, serine, glycine, and lysine, are most abundant. Strikingly, glutamate metabolism is most affected by high oxygen. Glutamate and proline have dropped immensely, while a large amount of glutamine has formed. The breakdown product of glutamate, γ-aminobutyrate, is greatly increased.

In contrast, the amino acid profile of ORS on high oxygen remains quite similar to ORS on air, especially glutamate and derivatives. Glutamate, proline and glutamine have all increased over air level, glutamine the most (about three times). No γ-aminobutyrate can be detected. Alanine shows no change, while serine, glycine, aspartate, and threonine, all appear in greater quantities.

Soluble organic acids. Inspection of organic acid pools, as initially stated, was qualitative. Table 9 contains a summary of the relative amounts of organic acids observed on thin layer chromatograms. The acids listed were detected in extracts equivalent to 200 mg dry weight of cells. Only gross differences are considered significant, but several differences were readily discerned.

(+) on air shows the greatest number and amounts of acidic compounds, followed by ORS on air, ORS on high oxygen and (+) on high oxygen. (+) cultures are most unique because they contain a considerable quantity of lactic acid, while ORS cultures show only a trace. This is reminiscent of the results determined by Weis and Mukerjee (1958); they found that only strains of C. pyrenoidosa produced lactic acid upon dark fermentation.

In (+) on air, malate constitutes the most abundant acid, followed

Table 9. Relative Amounts of Organic Acids per cell in Heterotrophic (+) and ORS under Air and 100% Oxygen

Acid	(+)-Air	ORS-Air	(+)- O_2	ORS- O_2
lactate	+++	TR	++	TR
fumarate	+	TR	TR	TR
succinate	+	TR	+	TR
α-ketoglutarate	TR[2]	++	TR	+
malate	++++	++	++	++++++
unknown 1	++	TR	0[3]	0
unknown 2	++	TR	0	0

1. Relative amounts determined by visible inspection of intensity of spots on thin layer chromatograms. Number of +'s is proportional to spot intensity.

2. TR= trace amount; very faint spot.

3. 0 = no visible spot.

by lactate, two unknown compounds, succinate, and fumarate. In contrast, ORS on air shows equal amounts of malate and α-ketoglutarate most prominently. Its level of malate is much lower than (+), while other acids are present in smaller amounts.

Under high oxygen, (+) contains abundant malate and lactate, a sizeable amount of succinate and very little of the others. ORS on high oxygen accumulates a huge pool of malate, more than any other sample. Some α-ketoglutarate also is noted. All other acids are reduced to trace amounts.

In summary, the following major differences were noted in organic acid pools:

1. Lactate - accumulates only in (+); less in (+) on high oxygen, than (+) on air.

2. Succinate - more in (+) on air and high oxygen than ORS.

3. α-ketoglutarate - substantial quantities only in ORS.

4. Malate - most abundant acid in all samples; order of abundance: ORS-O_2 > (+)-air > ORS-air = (+)-O_2

Lactate accumulation agrees with the previous suggestion that glycolysis is proceeding faster than the TCA cycle. (+) evidently reoxidizes NADH via lactate dehydrogenase. ORS would appear to utilize pyruvate exclusively by other pathways, eg. mitochondrial reactions. This is supported by the level of malate and α-ketoglutarate in ORS. The α-ketoglutarate level also correlates with the glutamate and glutamine content from amino acid analysis, while the build-up of malate in ORS on high oxygen is somewhat difficult to interpret.

6. Respiratory Characteristics of Log Cultures

Some basic respiratory characteristics are compared for the two

strains in Table 10. Several relevant points should be reiterated. These are respiratory characteristics of cells directly from the growth chamber, unstarved and using glucose as the exogenous substrate. All rates are for air-grown cells under air, except for 100% O_2 values which were obtained on 100% O_2 grown cells. These primarily qualitative tests, then, were designed to reveal the sensitivity of cultures to various agents under the same conditions as growth.

Two types of agents had identical effects on both (+) and ORS. One group inhibited respiration to approximately the same extent (cyanide, ethanol). Another group (glucose, fluoride, dinitrophenol) either had no effect on or stimulated oxygen uptake equally in (+) and ORS.

However several compounds were strain-specific in their effects. High oxygen was very inhibitory to (+), whereas ORS was almost insensitive to it. This, of course, is one of the physiological manifestations of oxygen resistance. A surprising respiratory response occurred when malonate and rotenone were administered. ORS demonstrated remarkable sensitivity to malonate whereas (+) is totally insensitive to malonate, even at concentrations twice as great as those used for ORS inhibition. Likewise ORS can be inhibited severely by 1 mM rotenone, while (+) is only inhibited half as much as ORS by 2 mM rotenone.

The significance of these observations is not of course unequivocal. But they do imply a basic difference in operation of respiratory systems between the two strains. (+) could be less permeable to malonate and rotenone than ORS. But the other data do not make this very likely. If permeability differences are discounted, two other permissible explanations invoke the specificity of malonate and rotenone, as inhibitors of mitochondrial enzymes (Webb, 1966). First, it could be argued that ORS

Table 10. Effect of Various Agents on the Respiratory Activity of Heterotrophic (+) and ORS under Air (see Legend)

Agent	(+)	ORS
KCN, 1 mM	+	+
NaF, 20 mM	-	-
100% O_2	80	20
Ethanol, 0.5 M	30	30
Malonate	- (at 25 mM)	+ (at 12 mM)
Rotenone	29 (at 2 mM)	59 (at 1 mM)
2,4-Dinitrophenol, 1 mM	$+^u$	$+^u$
Glucose, 0.35-28 mM	-	-

Legend: + = nearly complete inhibition
- = no noticeable inhibition
Numbers = percent inhibition
$+^u$ = strongly uncoupled (ca 67% stimulation)

relies more on mitochondrial pathways for oxygen uptake than (+), and that this metabolism is not sensitive to oxygen control. In this case, (+) might use respiratory pathways not involving succinic dehydrogenase or a rotenone-sensitive site. Second, and this appears more likely, ORS' respiratory metabolism could be more delicately balanced and sensitive to rate limitations. Since malonate is a competitive inhibitor, it would effect a cell with a limited amount of succinate more severely than a cell with an abundance of succinate. If ORS drains TCA cycle intermediates off as glutamate, its succinate could be rate-limiting. As seen in the organic acid analysis, succinate does appear to be lower in ORS than (+). The inverse situation in (+), namely higher substrate levels, might account for malonate and rotenone insensitivity.

7. Respiration of Starved Cells

The results from Warburg studies of glucose respiration are presented in Figure 19. The cells in this case had been starved for 30 hours without phosphate or nitrate, so that no new synthesis of protein could occur. It was hoped that this would reveal the basic endogenous respiratory ability of heterotrophic cells. The data show that without phosphate, the endogenous rate of heterotrophic ORS is 60% less than (+). This contrasts with previous work (Ward et al., 1969) showing that the endogenous rate of light-grown ORS is similar to (+). Upon exposure to glucose, (+) on air and high oxygen both show a sudden increase in QO_2 within 20 minutes. They reach a maximum QO_2 of 80. (+) on high oxygen begins to show inhibition by oxygen within two hours after addition of glucose.

ORS on air and high oxygen also attain a maximum QO_2 within 20 minutes after addition of glucose and phosphate. However this rate of 30

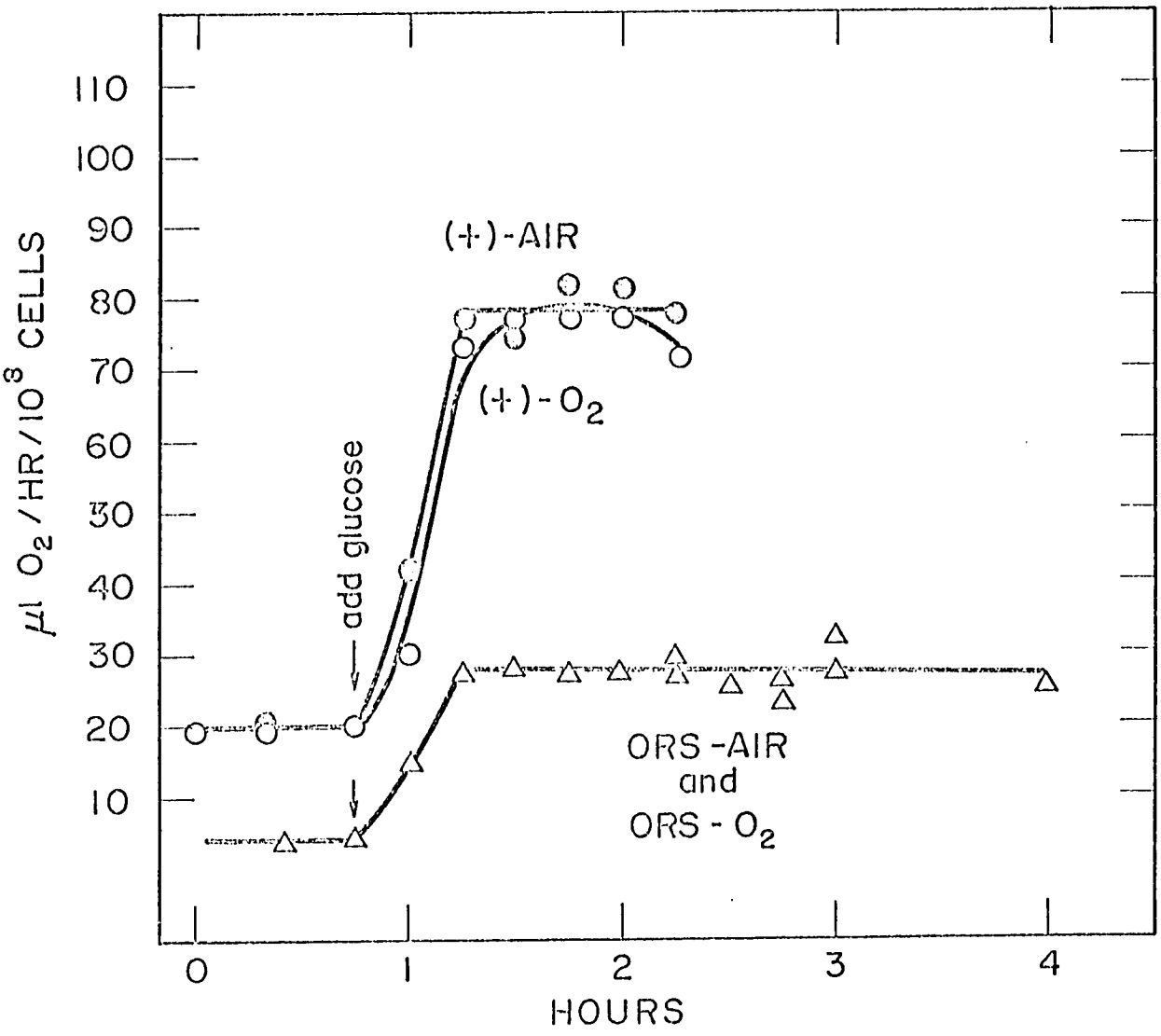


FIG. 19 RESPIRATION OF STARVED CELLS ON GLUCOSE

is 60% less than the (+) rate. There is no inhibition by oxygen though.

Figure 20 presents oxygen uptake on acetate and phosphate. It is evident that rates on acetate are higher for both strains than on glucose. (+) on air reaches a maximum QO_2 of 120, while (+) on high oxygen is somewhat lower, 110. The QO_2 for ORS on air finally reaches 40 after two hours, but is still climbing; while the QO_2 for ORS on high oxygen parallels ORS on air, but is somewhat lower. Malonate completely inhibits respiration of ORS on acetate, reducing the QO_2 to the endogenous level.

Thus, these data confirm that the rate of respiration in ORS is lower than (+). In conjunction with metabolite pool data, as well as ultrastructural evidence of mitochondria, low respiration is probably due to abnormal metabolic control, rather than an entirely different pathway from (+). This defective metabolic control could operate at the mitochondrial level to make ORS respiration insensitive to oxygen, but concomitantly more sensitive to other inhibitory compounds.

8. Rates of Uptake of Respiratory Substrates

Uptake of oxygen, glucose and phosphate occurs at the rates listed in Table 11. The ability to assimilate these substrates varies greatly from (+) to ORS, being much slower in ORS. Oxygen and phosphate uptake for (+) on air are about three times higher than ORS, while glucose uptake is about five times higher. This situation provides some evidence that glucose assimilation by ORS is most abnormal compared to (+), moreso than phosphate or oxygen uptake. Uptake of phosphate and glucose seem to reflect directly an overall lower capacity for energy metabolism by ORS.

High oxygen depresses uptake rates in (+) cultures only. Both glucose and phosphate assimilation by (+) are much lower than in air. In contrast, consumption of all three substrates by ORS on high oxygen is

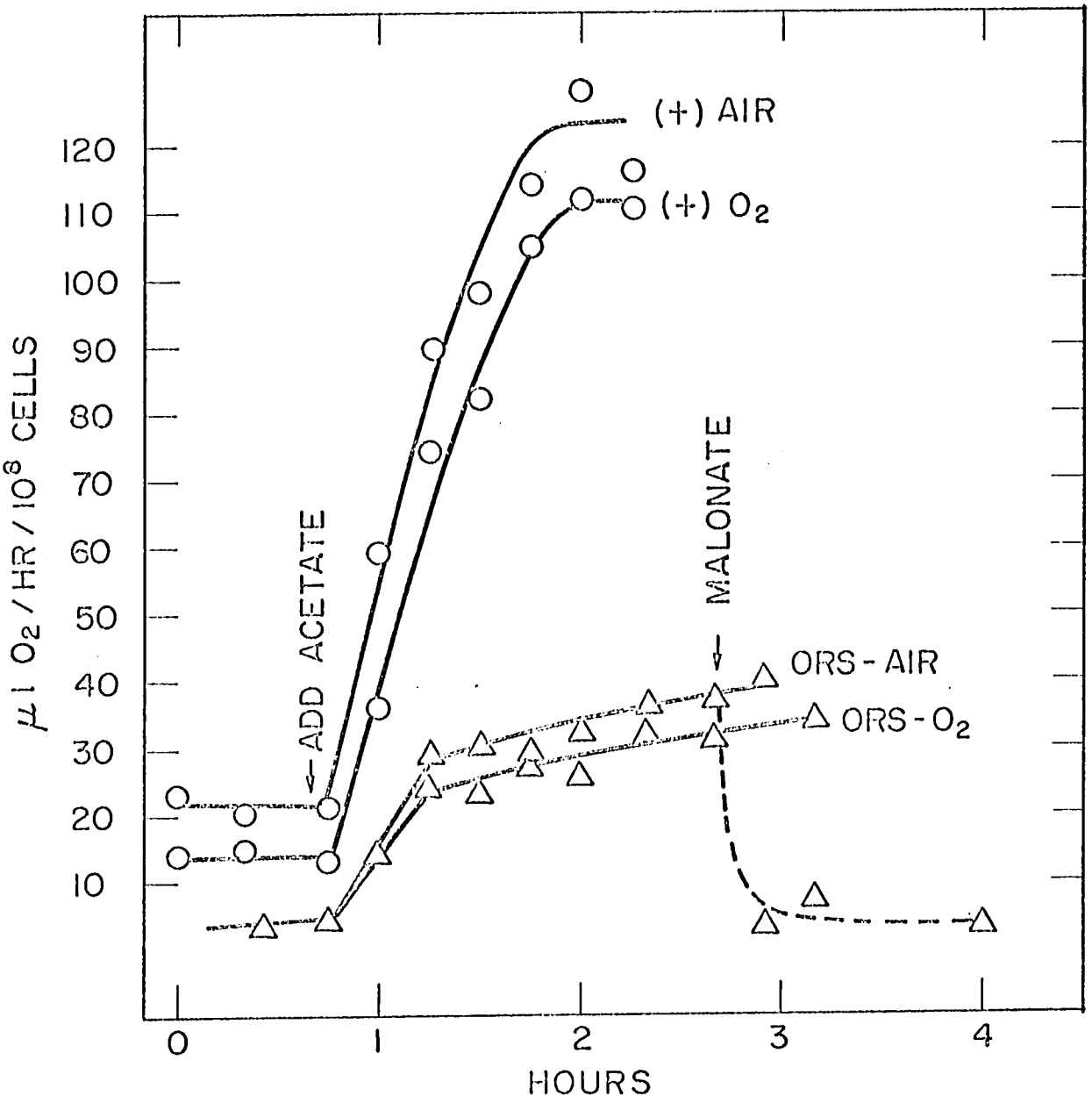


FIG. 20 RESPIRATION OF STARVED CELLS ON SODIUM ACETATE

Table 11. Uptake Rates[1] of Oxygen, Glucose, and Phosphate by previously-starved Heterotrophic (+) and ORS Cells under Air and 100% Oxygen

	(+)		ORS	
Substrate	Air	O_2	Air	O_2
Oxygen	34.7	34.0	11.9	12.3
Glucose	34.7	24.2	6.2	6.4
Phosphate	0.61	0.35	0.23	0.27

1. Values = $\frac{\text{micromoles substrate per hour}}{10^9 \text{ cells}}$

very similar to ORS on air, with even a slight stimulation noticeable. The effect of high oxygen on (+) uptake supports the conclusion of Young (1968) that high oxygen specifically inhibits transport of exogenous materials across the cell membrane.

CHARACTERISTICS OF THE ADAPTATION PROCESS

Previous workers have investigated changes occurring when photosynthetic organisms are transferred into heterotrophic, dark conditions. No one single pattern describes the responses of all organisms. Some species (especially lower plants) show relatively slight change, while others undergo extensive alteration of metabolic characteristics. In many cases, normally photosynthetic organisms are unable to grow in darkness. Naturally, the principal organelle effected by the transition is the chloroplast.

Budd et al. (1969) investigated the cellular morphology of Chlorella pyrenoidosa (van Niel's strain) during dark adaptation. The transition to heterotrophy was marked by regression of the chloroplast to the proplastid stage, with gradual reduction in thylakoid number. In the most rudimentary condition, a prolamellar body was noted. Starch was observed to accumulate within the regressing chloroplast, the cell wall thickened, and cytoplasmic material of variable density was sometimes seen.

Adaptation to dark conditions can result in fluctuations of entire enzyme systems. Ohad et al. (1967), studying dark-grown Chlamydomonas sp., substantiated this for various chloroplastic and mitochondrial enzymes, which showed opposite responses in light or to exogenous carbon sources. Fuller and Hudock (1967) have documented relative changes in chloroplastic and cytoplasmic metabolism by following the levels of NAD- and NADP- linked glyceraldehyde-3-phosphate dehydrogenase in algal cells.

In general they found that the chloroplast enzyme (NADP-GAPD) was inversely proportional to the glycolytic enzyme in the cytoplasm (NAD-GAPD) and directly related to the chlorophyll content of the cell. Moreover, the shifts in metabolic pathways occurred more rapidly in an organism with very few stringent growth requirements (ie. a wild type organism) than in mutant strains.

1. Change in Growth Rate

Figure 21 presents (+) and ORS adaptation to dark conditions based on growth rate. It is evident that (+) adapts rapidly to darkness by the second day. In contrast, ORS grows at a constant rate of less than 3 doublings/day until day six, when its growth rate starts to increase. By day eight ORS has attained a maximal dark growth rate of about 5 doublings/day.

Apparently ORS lacks the capacity for immediate dark growth shown by (+). Such a long lag period in response to new culture conditions can be characteristic of an induction or derepression period. In this case, glucose and/or darkness itself could be the agent to which ORS is responding. In all probability, the adaptation period is directly related to the basic genetic difference between (+) and ORS.

2. Change in Respiratory Rate

Over the same time period, respiratory rate increases as shown in Figure 22. Adaptation of (+) is accompanied by a parallel increase in QO_2, which amounts to 200% over the light level. Oxygen uptake by ORS increases moderately (only 50%) over a five day period. Hence ORS is already respiring vigorously in the light, and does not respond to dark stimulation to the same extent of (+). This correlates with observations of Ward et al. (1969) that succinic dehydrogenase is higher in ORS light-

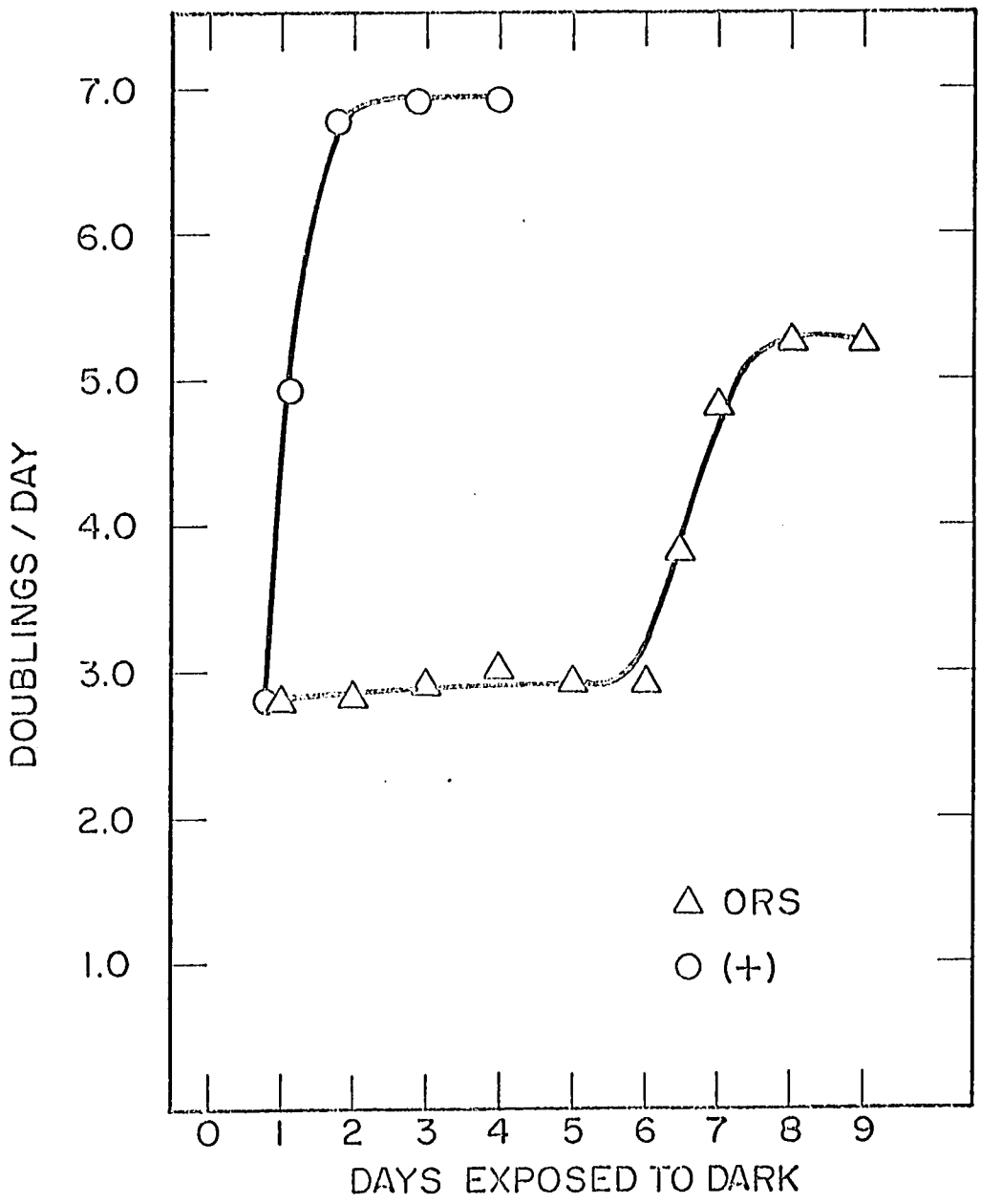


FIG. 21 KINETICS OF ADAPTATION TO DARK GROWTH BASED ON GROWTH RATE

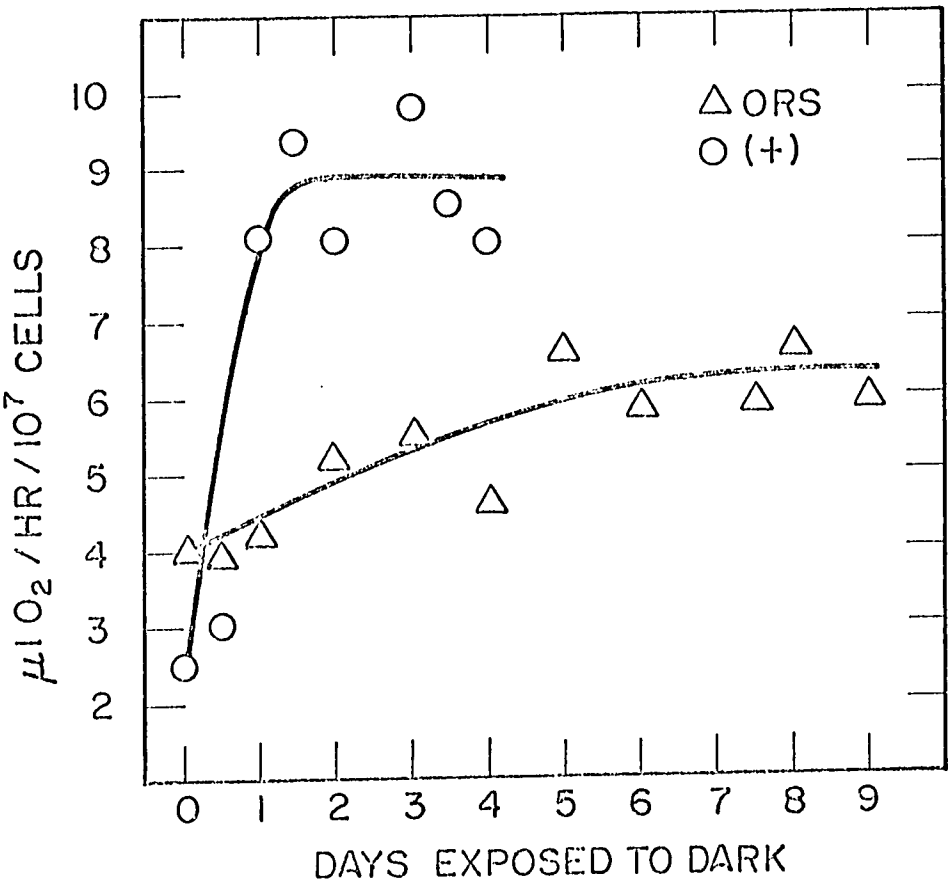


FIG. 22 KINETICS OF ADAPTATION TO DARK GROWTH FOR RESPIRATORY RATE

grown than (+) light-grown, but is somewhat lower in ORS dark-grown than (+) dark-grown.

3. Change in RNA Content

Total cellular RNA shows the adaptation kinetics in Figure 23. There appears to be no striking correlation between transition period and total RNA level, although ORS has much more RNA that (+) in the dark. RNA of both strains rises rapidly to the dark level, (+) increasing by 100% and ORS by 200% over the light level. This amount per cell reflects somewhat the size relationship of dark-grown cells.

4. Change in Alkaline Phosphatase Activity

Elevated alkaline phosphatase activity has been found in organisms subjected to limiting inorganic phosphorus concentrations. Studies by Torriani (1960), Kuenzler and Perras(1965), and Fitzgerald and Nelson (1966) indicate that the level of this enzyme correlates with P-limited growth in many bacteria and algae. Since ORS on air contains only about half as much Pi as (+), and no polyphosphate, the possibility appeared likely that ORS mimics, if indeed it does not suffer from, P deprivation.

Alkaline phosphatase data appear in Figure 24. Over the adaptation period, ORS shows a substantial decrease (50%) in the enzyme, whereas (+) retains a constant level. ORS however contains significantly more of the enzyme than (+), over five times more in the light, dropping to three times more in the dark. This situation for ORS is consistent with the contention that higher alkaline phosphatase represents a mechanism to recycle a restricted amount of intracellular phosphate among low molecular weight metabolites, eg. nucleotides and sugar phosphates. It is noteworthy that the ORS enzyme level seems to stabilize around the sixth day, precisely when adaptation starts for the growth rate.

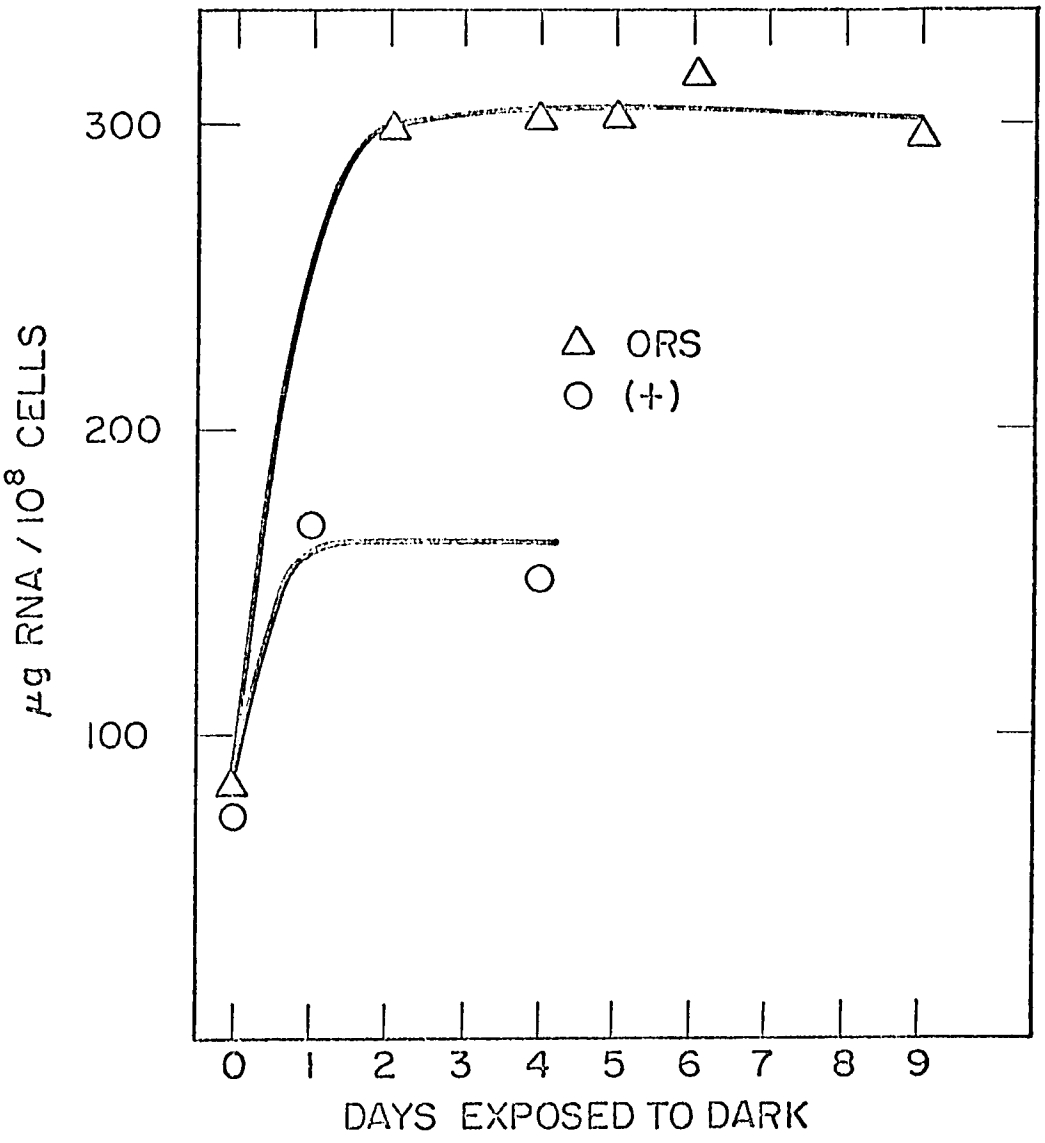


FIG. 23 KINETICS OF ADAPTATION TO DARK GROWTH FOR RNA CONTENT

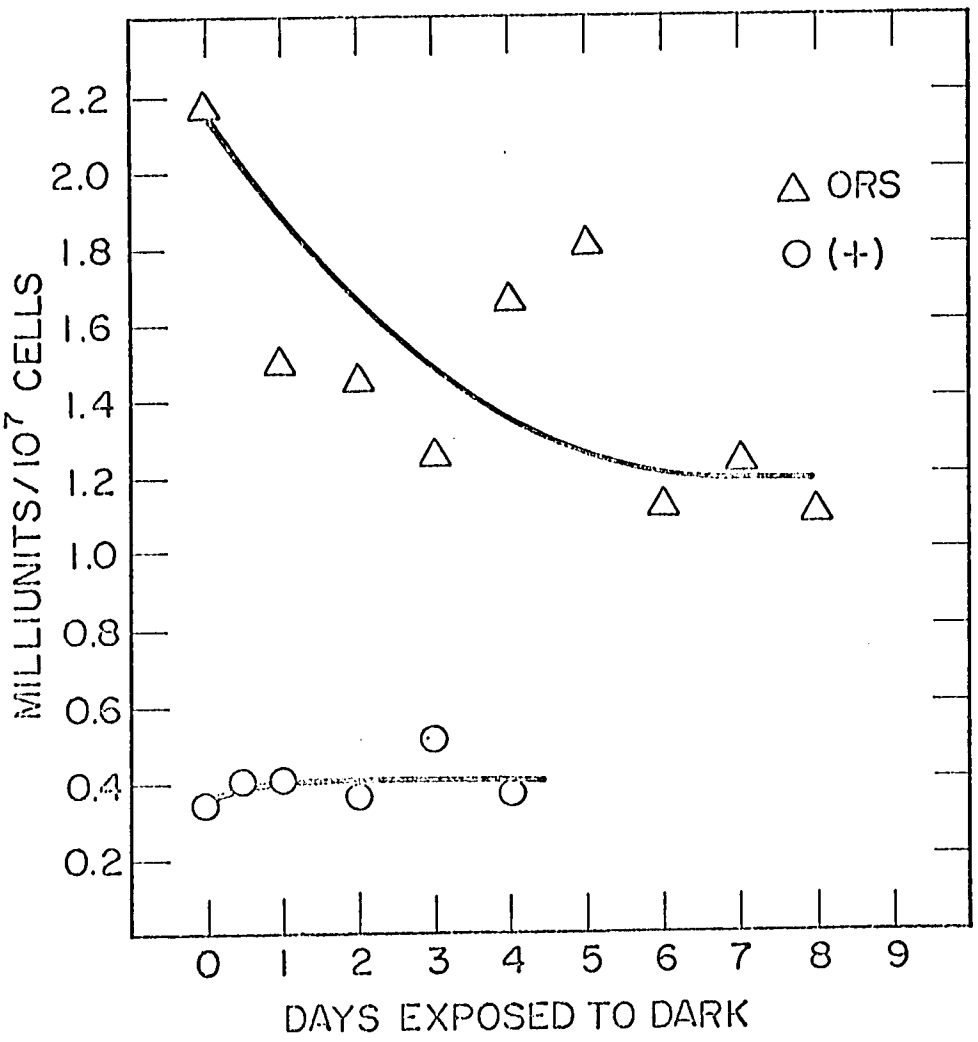


FIG. 24 KINETICS OF ADAPTATION TO DARK GROWTH FOR ALKALINE PHOSPHATASE

5. Change in Triose Phosphate Dehydrogenase Activities

NADP-GAPD kinetics are presented in Figure 25. The enzyme, localized in the chloroplast of algal cells, behaves as expected, decreasing in darkness to a constant level. (+) enzyme activity appears to be less-influenced by dark than ORS. ORS activity shows a dramatic drop to less than 20% of its light value, while (+) retains about 50% of its light level. With respect to cell concentrations, ORS has more NADP-GAPD than (+) in the light, reflecting its larger cell size. In the dark, ORS has less and this causes a slight paradox. Since dark-grown ORS retain the ability to photosynthesize immediately out of the dark, this low level of NADP-GAPD does not seem to indicate a defective chloroplast. An exact explanation is rather difficult, though the dark level is certainly sufficient for photosynthetic metabolism. The NADP-GAPD kinetics do support the general conclusion that (+) and ORS chloroplast metabolism is reduced by darkness, but that it still continues at an adequate level.

More crucial to the problem are the data presented in Figure 26. The outstanding feature is that ORS contains a high level of the cytoplasmic enzyme, NAD-GAPD, already in the light. (+) conversely has a very low level in the light (only 33% of the ORS level), but rapidly forms it upon adaptation to dark. Both strains contain about the same amount of the enzyme in the dark. This would suggest that ORS is actively glycolysing in the light, indeed almost at its maximum rate, since its NAD-GAPD level barely increases in the dark.

NADP- and NAD-GAPD are considered to have separate functions, though they may be the same enzyme in two different active conformations. The NADP-linked enzyme supposedly participates in biosynthetic reactions in the chloroplast, resulting in the production of reduced carbon com-

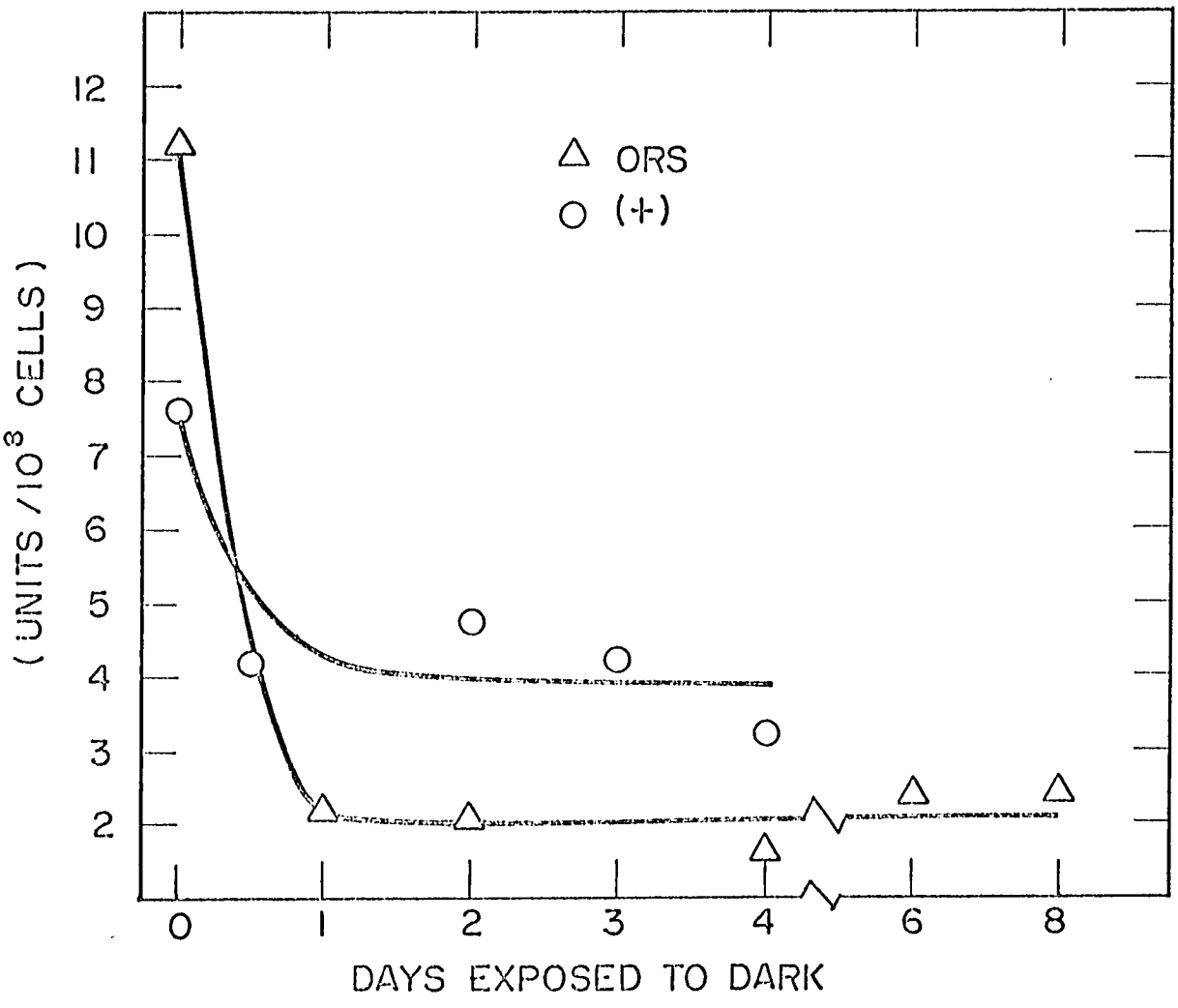


FIG. 25 KINETICS OF ADAPTATION TO DARK GROWTH FOR NADP - GLYCERALDEHYDE - 3 - PHOSPHATE DEHYDROGENASE

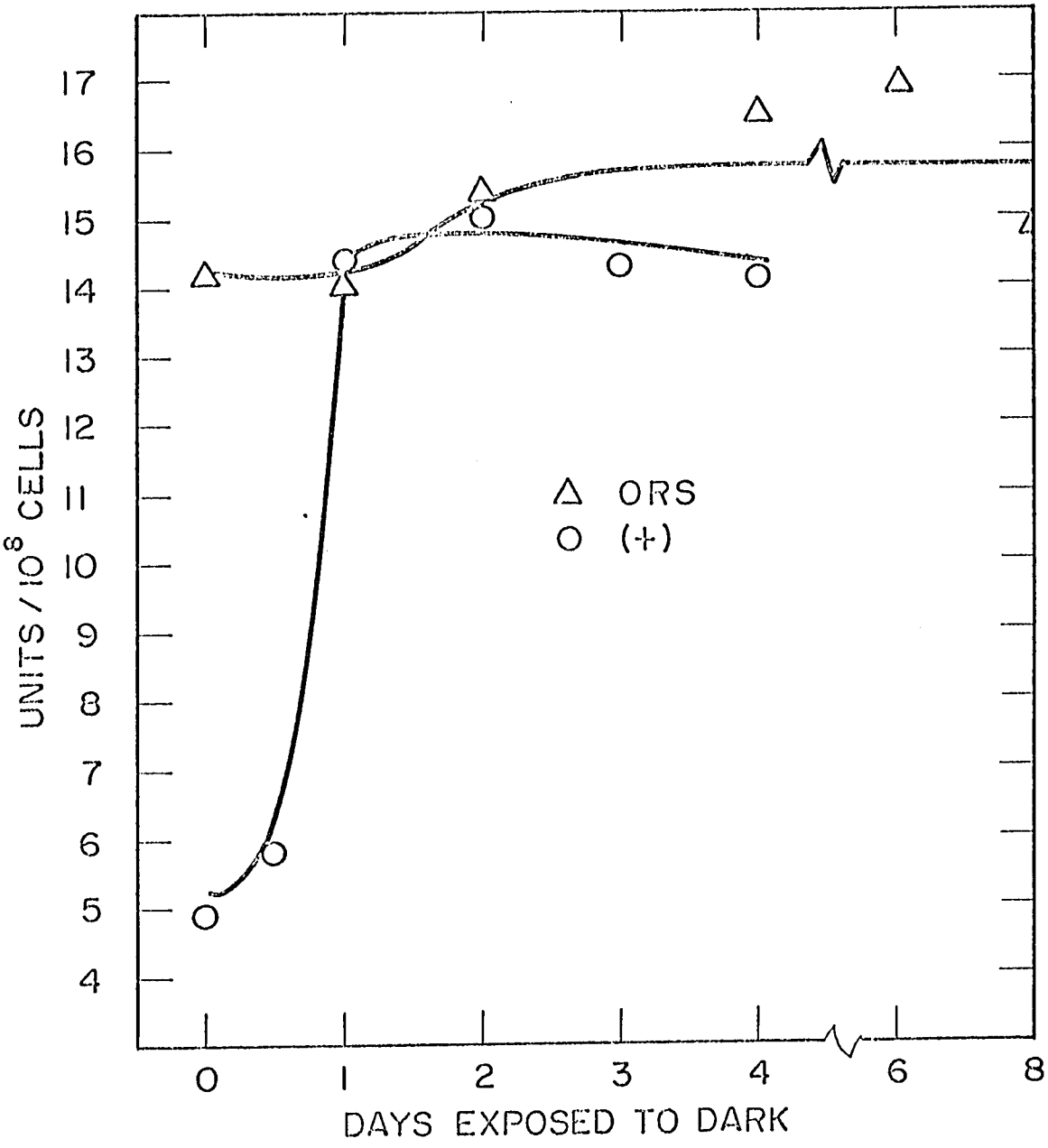


FIG-26 KINETICS OF ADAPTATION TO DARK GROWTH FOR NAD-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

pounds. The NAD requiring enzyme participates in the catabolic, degradative process of glycolysis in the cytoplasm.

The levels of the two GAPD's suggest that in ORS cytoplasmic metabolism operates at a constant rate, unaffected by chloroplast, and perhaps other types of cellular, metabolism. This apparent lack of coordination between the cytoplasm and chloroplast implies that ORS uses glycolytic metabolism continuously to provide energy for endergonic reactions and to provide carbon for cell synthesis. In order to sustain these processes unabated under a variety of conditions, glycolysis in ORS must be sufficiently insensitive to normal control by the many substrates, products and allosteric regulators present in (+), at the enzyme or the gene level.

One of the disadvantages to constant flow of carbon through glycolysis might be a decrease in metabolic efficiency. However, a cell could increase its survival by such a mechanism under adverse conditions like high oxygen. The results in Table 12 are consistent with this idea. ORS under high oxygen maintains almost the same activity of NAD-GAPD as under air (maybe even slightly higher), whereas (+) on high oxygen shows a 52% reduction in activity. Simultaneously NADP-GAPD shows a slight increase (about 30%) in both strains under high oxygen. Hence resistance to high oxygen occurs only in the organism with a high (= normal) glycolytic rate, rather than with active chloroplast metabolism.

6. Change in Glutamate Dehydrogenase Activities

NAD- and NADP-glutamate dehydrogenases (GLDH) represent another case where the "division of labor" hypothesis of Kaplan (1963) may extend to ORS. Studies with yeast (Kaplan, 1963) and Neurospora (Sanwal and Lata, 1962) GLDH provide evidence that the NAD-enzyme is involved in catabolism

Table 12. Comparison of Glyceraldehyde-3-phosphate dehydrogenase (GAPD) and Glutamate dehydrogenase (GLDH) Activities (both NAD- and NADP-linked) of Heterotrophic (+) and ORS grown on Air and 100% Oxygen

	GAPD[1]		GLDH[1]	
Culture	NAD	NADP	NAD	NADP
(+)-Air	14.1	3.1	0.71	0.09
(+)- O_2	6.8	4.2	0.90	0.20
ORS-Air	14.8	2.4	1.01	0.23
ORS- O_2	16.1	3.7	1.31	0.32

1. Values = $\frac{\text{micromoles substrate per hour}}{10^8 \text{ cells}}$

of glutamate, while the NADP- enzyme participates in glutamate synthesis. Both enzymes, probably localized in the mitochondrial matrix, are subject to regulation by the intracellular level of glutamate. At 0.01 M glutamate in the external medium, Neurospora contains equal levels of the two enzymes. Higher concentrations of glutamate repress the NADP- enzyme and induce NAD-activity, while lower glutamate has the opposite effect.

Table 12 also shows the relative activities of the two GLDH proteins in heterotrophic (+) and ORS. Both strains contain a higher level of NAD-GLDH than the NADP-enzyme. However in (+) on air the ratio of NAD-GLDH to NADP-GLDH is 7:1 (0.127), while in ORS on air the ratio is about 4:1 (0.228). Therefore the NADP enzyme constitutes a much higher percentage (almost 100%) of the total cellular GLDH activity in ORS than in (+). This correlates with the higher level of glutamate-related amino acids (particularly glutamine) in ORS.

Under high oxygen, the two enzymes increase in both strains. But only (+) shows a change in ratio of the two activities, the ratio of NAD enzyme to NADP enzyme now being 4.5 to 1. ORS sustains a ratio of about 4:1.

The kinetics of dark adaptation are presented for NADP-GLDH in Figure 27 and for NAD-GLDH in Figure 28. Based on these data, it appears that the role of GLDH in metabolism may be directly related to ORS dark adaptation. The levels of both NADP and NAD enzyme rise over 100% upon transfer of ORS to dark conditions. They remain at that level until about day five when they begin to drop. By day six, they have reached the level characteristic of fully dark-adapted ORS. In contrast, (+) demonstrates practically no change for either enzyme during or after adaptation.

It is significant that both light and dark-grown ORS have over

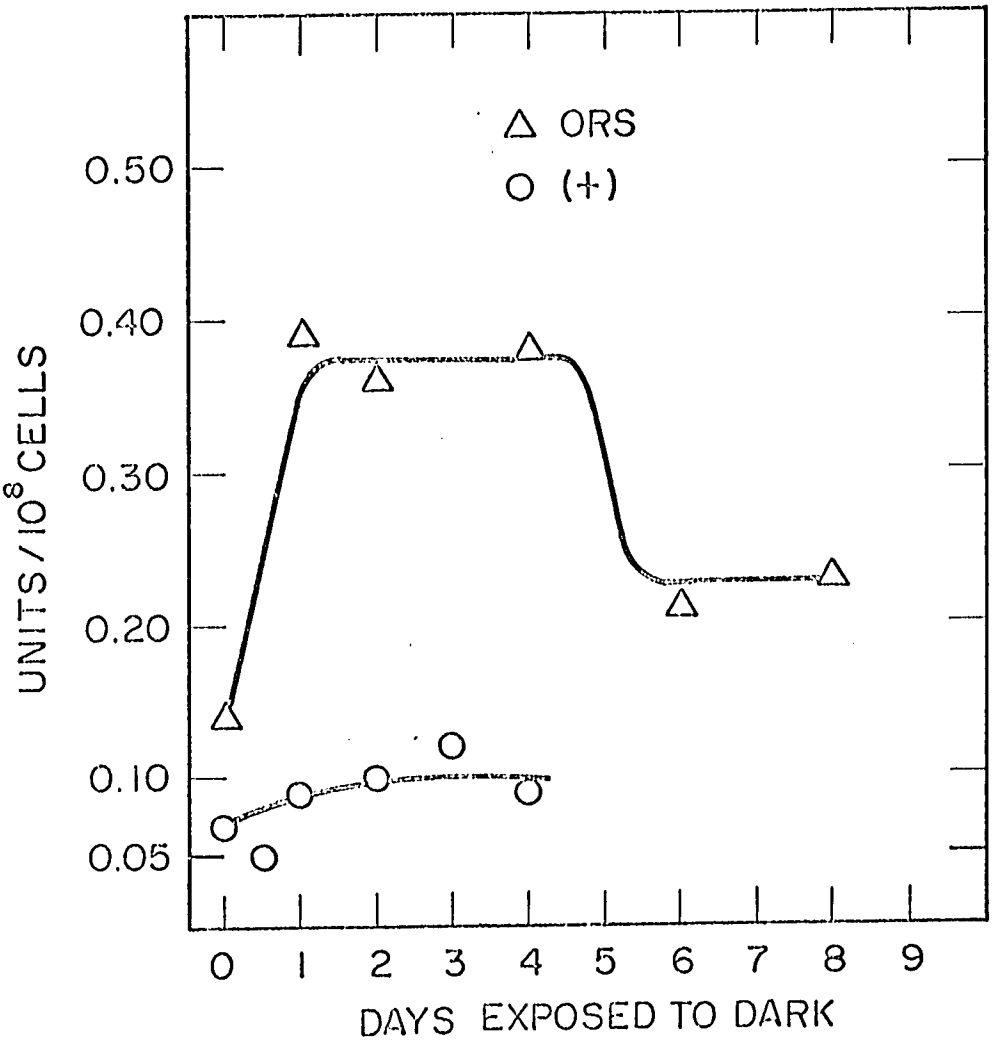


FIG. 27 KINETICS OF ADAPTATION TO DARK GROWTH FOR NADP-GLUTAMATE DEHYDROGENASE

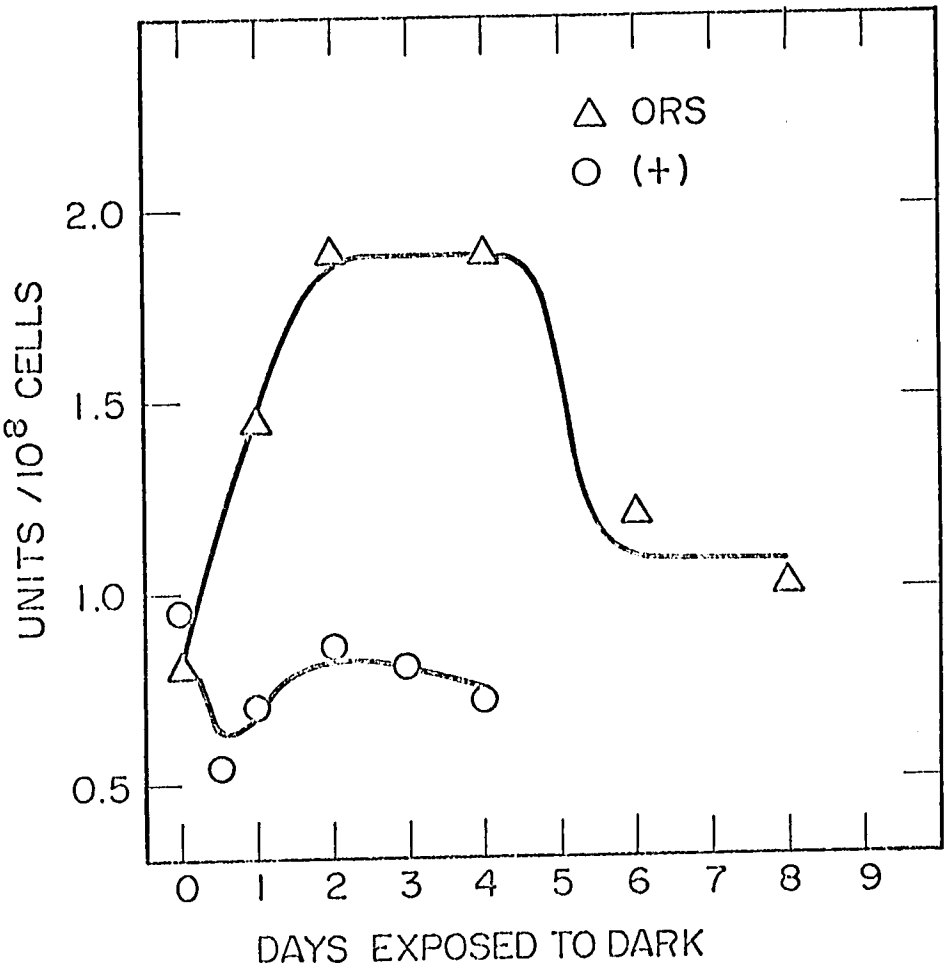


FIG. 28 KINETICS OF ADAPTATION TO DARK GROWTH FOR NAD-GLUTAMATE DEHYDROGENASE

twice as much NADP-GLDH as corresponding light and dark-grown (+). This is not the case for NAD-GLDH. In fact (+) has more of the latter in the light than ORS. Even in the dark, (+) contains only about 25% less than ORS. This strengthens the argument that it is the biosynthesis of glutamate rather than its degradation which primarily differs between (+) and ORS.

In view of these enzyme data plus the soluble metabolite pools, there appears to be a cellular stimulus for glutamate and/or glutamine synthesis in ORS. Though they contribute to this objective, mitochondrial and cytoplasmic (ie. glycolytic) metabolism possess a high degree of independence from normal control mechanisms operative in (+). Dark adaptation, however, represents a regulatory mechanism, inconsequential to (+), which does control ORS metabolism, perhaps at the oxygen-resistance site. The number of characteristics involved (growth rate, respiration, cytoplasmic enzymes, glutamate enzymes, and phosphorus metabolism) suggest that a rather fundamental cell phenomenon occurs during dark adaptation of ORS.

DISCUSSION

Based on this study, wild type C. sorokiniana is considered a very limited heterotroph. Its simple, but selective, dark-growth requirements form a pattern which suggests that the species relies on relatively unspecialized metabolic pathways. Similar to primarily autotrophic organisms (Karlander and Krauss, 1966), C. sorokiniana apparently uses a minimal repertoire of heterotrophic pathways, being restricted to acetate and hexose substrates for growth.

Many organic compounds, especially undissociated molecules, seem to be absorbed by Chlorella through passive processes (Stadelmann, 1969; Krauss, 1958). Moreover, experiments have determined that growth of Chlorella on TCA cycle intermediates is often precluded because of metabolic disturbances and not by failure of substrate to permeate (Oaks, 1962). In these situations, the supply of energy or reducing power is often a rate-limiting factor.

Investigators have demonstrated that many heterotrophic chlorellas utilize the familiar carbohydrate pathways of the Embden-Meyerhof-Parnas (EMP) sequence, the pentose phosphate shunt, and the TCA cycle (Devlin, 1963; Millbank, 1957; Oaks, 1962; and Tjostem, 1968). Several observations from this study imply that normal pathways of glucose metabolism are operative: organelles in the electron micrographs, intermediates from organic and amino acid analyses, enzyme assays, and respiratory data. Hence the assumption seems warranted that (+) C. sorokiniana metabolized glucose carbon by established pathways of carbohydrate and intermediary

metabolism. Hereafter "glycolysis" will refer to carbohydrate catabolism in a general sense, and not merely the EMP sequence, since no estimate is available for the relative activities of the EMP or pentose phosphate pathways in heterotrophic C. sorokiniana.

The ability to degrade carbohydrates has been retained exclusively as the only mode of catabolism sustaining growth except for acetate oxidation. This situation testifies to the importance of carbohydrate pathways in the organism's metabolism. Concomitantly, it suggests that metabolic controls are most likely related to regulation of this pathway(s). In order to interpret many of the results of this study, it is necessary to consider the general question of regulation of glucose metabolism. For example: Glucose enters the metabolic pool at the level of glucose-6-phosphate. The significance of various amino acid pools must then take into account that the amino acids are derived ultimately from glucose through the intervening steps of carbohydrate catabolism. Therefore this study actually examined characteristics of a green alga's carbohydrate metabolism under heterotrophic conditions.

Comparison of ORS and (+) reveals that the two strains vary primarily in the metabolic control of carbohydrate metabolism. One of numerous metabolic controls, oxygen has a selectively different effect only when glycolytic pathways are used. ORS tolerates high oxygen only when grown on glucose. When grown on acetate, both strains appear subject to inhibitory metabolic control by oxygen; and consequently neither demonstrates oxygen resistance.

Growth on acetate is unique because it requires the participation of the glyoxylate cycle. Syrett et al. (1963) showed that only Chlorella grown in the dark on acetate, in contrast to autotrophic or dark,

glucose-grown cells, have isocitritase activity. Further studies showed that the activities of the requisite glyoxylate cycle enzymes were adequate to account for growth of the alga on acetate. This pathway operates at the level of the TCA cycle, within a subcellular compartment (either the mitochondrion or glyoxysome), physically and chemically separated from the cytoplasm. A novel sequence of anaplerotic reactions are involved which are not required in glycolysis-mediated growth. Thus oxygen exerts very stringent and inhibitory regulatory control over the metabolism of this non-cytoplasmic compartment.

Though ORS manifests oxygen resistance only on glucose, this does not mean that glycolysis alone functions under high oxygen. Some of the data (amino acid and organic acid pools, respiratory activity, and electron micrographs) clearly indicate that oxidative metabolism also occurs. The dependence on carbohydrate metabolism does, however, suggest that mitochondrial oxidative pathways by themselves are severely rate-limited by high oxygen in both ORS and (+). This is consistent with the observation of Begin-Heick (1970) that high oxygen specifically inhibits mitochondrial NADH:cytochrome c oxidoreductase in Astasia. In addition, glycolysis in ORS seems to provide the buffering capacity needed to prevent total inhibition of oxidative metabolism. ORS carbohydrate metabolism has the potential for adjusting to high oxygen, a modulating mechanism that (+) lacks. This shift in carbohydrate metabolism thereby enables the following mitochondrial pathways to continue functioning, albeit at a lower rate, under high oxygen.

A fundamental difference in control of (+) and ORS carbohydrate metabolism is evident at the level of polysaccharide biosynthesis. The starch content of (+) is considered to represent the normal amount of

glycolytic activity in the anabolic direction. Instead of glucose being completely degraded in the cytoplasm, intermediates are transported into the chloroplast where the normal Calvin cycle enzymes aid in polymerizing it into starch. This activity reflects the cellular capacity to utilize glucose carbon (Rodriguez-Lopez, 1966), and is carefully coordinated to the energy state of the cell. The tendency for ORS to store increased quantities of starch indicates that feedback between carbohydrate degradation and synthesis has triggered net storage of glucose. More simply-stated, starch synthesis in ORS is apparently less inhibited or more stimulated than in (+).

In Chlorella and other plant systems, two facts suggest that formation of ADP-glucose is the limiting reaction in starch synthesis: 1) ADP-glucose α-1, 4-glucantransferase activity is not stimulated by any glycolytic intermediates and 2) the level of this transglucosylase is higher than ADP-glucose pyrophosphorylase (Sanwal and Preiss, 1967).

The synthesis of ADP-glucose by Chlorella ADP-glucose pyrophosphorylase is regulated by various allosteric effectors. 3-phosphoglycerate is the strongest activator, followed by other glycolytic intermediates in order of decreasing activation: fructose-6-phosphate, fructose-1, 6-diphosphate, and phosphoenolpyruvate. Inorganic phosphate is the only potent inhibitor known for plant enzymes, although most bacterial enzymes are also inhibited by ADP and AMP. 3-PGA can completely reverse the Pi inhibition. A decrease in Pi (below 1 mM in vitro) or an increase in 3-PGA (or fructose intermediates) leads to an increase in the rate of ADP-glucose and starch synthesis.

Many nonphotosynthetic plant tissues are known to contain ADP-glucose pyrophosphorylase and to synthesize starch: etiolated peas and

mung beans, potato tubers, carrot roots, and avocado mesocarp. These systems have the same regulatory properties as photosynthetic ones (Preiss, 1969).

Sanwal (1970) distinguishes between the regulatory signals utilized for control of biosynthetic and catabolic pathways. Inhibition by specific end products and precursor activation are most important in anabolic pathways. Catabolic sequences are most sensitive to an ultimate product (or easily interconvertible product) of energy metabolism. Amphibolic routes (ie. those which function both in anabolism and catabolism) are controlled by a combination of these mechanisms, but may show a preponderance of one type of control under certain conditions. This applies especially to situations where compartmentation separates the anabolic and catabolic functions. The present system of heterotrophic C. sorokiniana seems to fall into this category. With starch synthesis relegated to the chloroplast, cytoplasmic glycolysis probably functions mainly in a degradative capacity.

Compounds which control catabolism, either energy donors or energy acceptors, serve as indicators of the energy state of the cell. Compounds with such properties are inorganic phosphate, pyrophosphate, and adenine or other nucleotide phosphates. In addition to glucose catabolism, oxidative respiratory pathways are modulated by the same compounds. Thus ADP or Pi activates phosphofructokinase, and mitochondrial NAD-isocitrate dehydrogenase. AMP activates pyruvate dehydrogenase, but inhibits fructose diphosphatase. Recognizing the regulatory properties of the adenylate pool in the cell, Atkinson (1968) has even formulated a concept of metabolic control known as "energy charge." This theory envisions reciprocal allosteric effects for ATP and ADP (or 5'-AMP) on all enzymes which con-

sume or produce ATP. Many of these enzymes are extremely sensitive to the balance between the intracellular concentrations of adenylates, particularly AMP (or ADP).

The glycolytic pathway contains a number of key enzymes affected by energy compounds (Rose and Rose, 1969). Hexokinase is known to be inhibited by glucose-6-phosphate; however this inhibition may be relieved by inorganic phosphate. Pyruvate kinase from liver and yeast responds rapidly to certain energy metabolites. ATP inhibits the reaction and the ATP/ADP ratio is important in determining the rate of the reaction. Fructose diphosphate stimulates the enzyme, though ATP can negate this increase in activity.

However, the rate of degradation of hexose monophosphate in glycolysis depends chiefly on the ability of phosphofructokinase to phosphorylate fructose-6-phosphate. Essentially irreversible, this reaction can be considered the branch point to the glycolytic sequence. As such, it is extremely sensitive to steady-state concentrations of intracellular metabolites. In many systems, particularly bacterial and animal, ATP and citrate are potent feedback inhibitors. Again it is worth noting that this may be the key mechanism of the Pasteur effect. Other nucleotides, especially UTP, substitute for ATP in various systems. The list of activators includes Pi, AMP, and ADP. These molecules also reverse ATP inhibition.

Plant systems, and possibly Chlorella, show minor variations from this regulatory control. Dennis and Coultate (1967) reported that leaf phosphofructokinase was not activated by ADP or AMP, but instead Pi alone was the positive activator at concentrations greater than 1 mM in vitro. Citrate functioned as a strong inhibitor. In all cases, though,

phosphofructokinase speeds up or slows down in response to the energy state of the cell.

Competition for Pi and ADP acts as a throttle on glycolysis and oxidative phosphorylation. Under aerobic conditions glycolysis may operate at a submaximal rate, because its enzymes do not have as high an affinity for ADP + Pi as mitochondrial enzymes. Thus coordinated stimulation of glycolysis by Pi has been observed by Uyeda and Racker (1965) in yeast, tumor, and muscle cell systems. Glucose utilization in reconstructed glycolytic systems was found to be dependent on the concentration of Pi. Pi could be assigned a regulatory role at three sites in glycolysis. It was concluded that Pi directly counteracted the inhibition of hexokinase and phosphofructokinase by glucose-6-phosphate and ATP, respectively, due to specific allosteric effects of Pi on these enzymes. Stimulation of glyceraldehyde-3-phosphate dehydrogenase was also noted.

At the level of the TCA cycle, Pi effects have been noted in only a few specific instances, though the general effect of Pi on mitochondrial activity has been widely accepted (Chance and Maitra, 1963). The NAD linked isocitrate dehydrogenase from several sources (Aspergillus, rat heart, and ascites tumor cells) has been reported to be stimulated by and possibly require Pi. This same enzyme from other sources, though insensitive to Pi, is strongly activated by ADP, while ATP is a potent negative effector. This is most significant as a metabolic control measure, because isocitrate dehydrogenase is present in the lowest amounts of any of the TCA cycle enzymes. Thus isocitrate oxidation is considered the rate-limiting step of the TCA cycle. The rate of the cycle is also modulated at the citrate synthetase step, and surprisingly, at the fumarase reaction. Both of these enzymes from a variety of sources are

sensitive to the ATP/ADP ratio, being inhibited by high ATP levels.

These mechanisms all insure that the rate of electron transport is determined mainly by the relative concentrations of ADP, ATP, and Pi and not by the concentration of respiratory substrate. As demonstrated by the phenomenon of respiratory control, maximal oxygen uptake by mitochondria occurs when ADP and Pi are abundant and ATP is low. It is not clear however what role active transport of Pi into mitochondria plays in the regulation of respiration (Hanson and Miller, 1967).

Examination of the phosphorus fractions in (+) and ORS suggests significant differences in their energy metabolism. Two of these fractions, the soluble P and poly-P, are most intriguing from the standpoint of metabolic regulators. Preiss (1969) goes so far as to state that Pi levels in the plant cell might be indicative of the "energy charge" of the cell, just as 5'-AMP and/or ADP are in mammalian and bacterial cells. This is based on the aforementioned observations that in plants, so far as is known, Pi alone inhibits ADP-glucose pyrophosphorylase and activates phosphofructokinase, in contrast to bacteria where AMP and/or ADP in addition to Pi effect these enzymes. Based on the high-energy phosphate bond of ATP, cellular bioenergetics requires a quasi-intracellular equilibrium between Pi and ATP. The reaction ADP + Pi $\longrightarrow$ ATP implies a reciprocal relationship between Pi and ATP under steady-state conditions, a low concentration of Pi reflecting a high concentration of ATP and vice versa. Thus the low Pi in ORS could denote a higher ATP/ADP ratio than in (+). Such a situation would have significant influence on Pi-sensitive reactions in the glycolytic and respiratory pathways, as previously described.

Several pieces of data argue for stringently-regulated glycolysis

in ORS. First, the respiratory rate of starved ORS supplied with glucose is much lower than corresponding (+). Since glucose must pass through the glycolytic sequence before reaching the respiratory enzymes, the low rate of oxygen uptake could certainly result from limited substrate reaching the respiratory machinery. Second, the rate of glucose uptake by starved ORS is much lower than comparable (+). Compare the ratio of ORS glucose uptake to (+) glucose uptake with the ratio of ORS oxygen or phosphate uptake to (+) oxygen or phosphate uptake. This comparison suggests slower initial metabolism of glucose. Third, ORS lacks the large quantity of lactate demonstrated by (+), and also contains less alanine. This supports the idea that there is less excess carbon (= pyruvate) produced from glycolysis in ORS, and that the pyruvate synthesized is used rapidly elsewhere. Fourth, the level of the constitutive enzyme, NAD-linked glyceraldehyde-3-phosphate dehydrogenase, indicates that glycolytic enzymes are present in normal amounts in heterotrophic (+) and ORS. If ORS glycolytic activity is reduced, it is probably due to regulation of existing enzymes and not to lack of enzymes. Fifth, much more glucose is stored as starch in ORS, whereas more glucose is degraded in (+). A decrease in ORS glycolytic activity could account for this. Sixth, Ward et al. (1969) reported that ORS contained more succinic dehydrogenase per cell than (+) in the light, and somewhat less in the dark. Since this enzyme is diagnostic of mitochondrial activity, it was concluded that ORS oxidative respiratory machinery was competent. The typical well-developed mitochondria seen in the electron micrographs of ORS on air agree with this.

These facts suggest that ORS degrades glucose specifically at a slower rate than (+). The metabolic control imposed on ORS acts as a

throttle primarily on glycolysis, and secondarily on respiration. However, the regulatory mechanism insures that glycolysis operates constantly, even under a stress condition such as high oxygen. Moreover, there would appear to be more than just a coincidental relationship between the metabolic control and the inorganic phosphate level.

Inorganic polyphosphate represents a biochemical enigma despite much information on its chemistry. Technically poly-P consists of pentavalent phosphorus compounds in which tetrahedral phosphate groups are linked together by oxygen bridges. It is very stable to alkali but labile to hot acid. Hydrolysis yields orthophosphate. Two basic classes are known from analysis: metaphosphates, which are cyclic ring structures, and linear condensed polyphosphates, which are unbranched chains of phosphate. The latter predominates in biological systems.

Within the cell, poly-P is often deposited in the form of granules, termed volutin. Beyond the fact that they contain poly-P, the exact nature of these metachromatic granules is not certain. Various workers report that traces of other materials, such as RNA, lipid, protein, and Mg, are associated with intracellular poly-P. Other workers suggest that the poly-P exists <u>in vivo</u> essentially free in the cytoplasm.

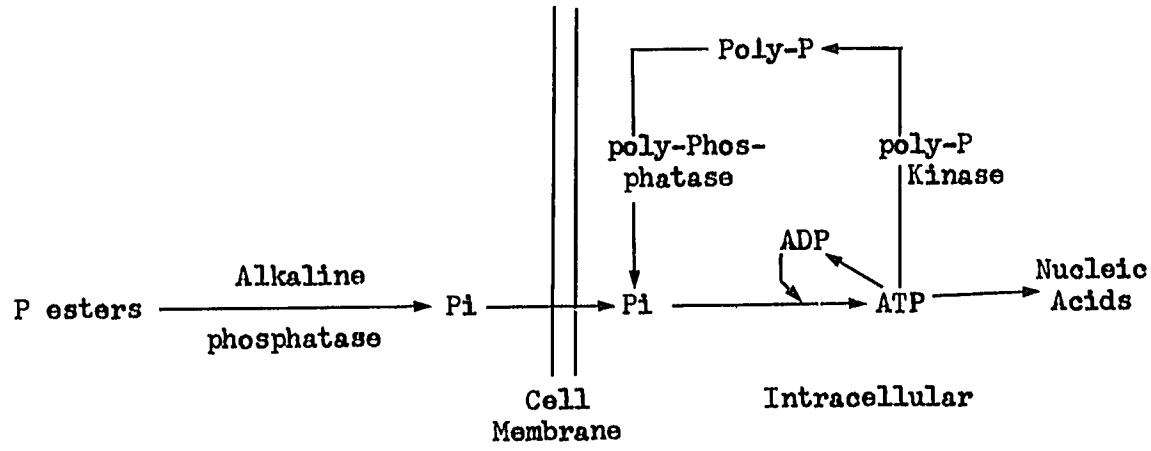
Considerable quantities of poly-P occur in <u>Chlorella</u> and other photosynthetic algae under normal growth conditions. This situation contrasts with poly-P metabolism in other organisms, where it is a function of the growth phase. Bacteria and yeasts accumulate poly-P primarily in the stationary phase, under conditions of nutritional imbalance unfavorable to growth, such as pH changes, sulfur and nitrogen starvation, or after P starvation as an overplus phenomenon. In all organisms studied, however, a reciprocal relationship seems to exist between RNA and poly-P

(Harold and Harold, 1965). Nucleic acid synthesis blocks poly-P synthesis, while poly-P synthesis occurs if RNA synthesis has stopped or slowed down.

Harold (1966) has discussed the various physiological roles of poly-P in cell metabolism. All hypotheses imply a regulatory function for poly-P. First, energy storage has been attributed to poly-P. Because some phosphate can be transferred from poly-P to ADP, poly-P could serve as a microbial phosphagen. However, this high energy role receives little support, since very little ATP is made from poly-P degradation. Second, obscure roles have been attributed to poly-P specifically in the processes of cell division and active transport across membranes. Third, poly-P could represent a phosphorus reserve for such intracellular molecules as nucleic acids or phospholipids. Fourth, poly-P is concerned with, not just one of the specific functions described above, but with the regulation of phosphorus metabolism in general. In this capacity, poly-P participates in a cell phosphorus cycle which involves nucleic acids and phospholipids as "phosphate sinks," and energy compounds, eg. Pi, ADP, and ATP, in constant flux. Poly-P serves as a metabolic buffer enabling the cell to control closely the steady-state concentrations of these phosphorus compounds. Hence, poly-P would constitute a "shunt product" of the cycle, functioning in the cell phosphate balance.

This last role appears most tenable. It also forms a basis for far-reaching consequences of a single alteration in the phosphorus cycle. Feedback relationships between phosphorus compounds may pertain to clarification of the ORS and (+) situation. Several important aspects of ORS phosphorus metabolism show major changes, namely Pi, poly-P, and RNA. These facts suggest that the entire phosphorus cycle in ORS may no longer operate normally compared to (+).

Harold and Harold (1965) have proposed a possible scheme for the phosphorus cycle based on studies with mutants of Aerobacter aerogenes defective in polyphosphate metabolism. The model includes phosphorus compounds interrelated as follows:



All three enzymes in this scheme are derepressed when the Pi supply falls to a low level, indicating that Pi represses poly-P synthesis. Furthermore, evidence suggests that all three enzymes share a common regulator gene, but are not located on the same operon.

The higher alkaline phosphatase activity in ORS might be a result of derepression by lower intracellular Pi. Kuenzler and Perras (1965) have shown that this is a widespread response among numerous algae. Concomitantly, this enzyme may serve to maintain other soluble P at a sufficiently high level.

Biosynthesis of poly-P is apparently catalyzed only by poly-P kinase, which transfers the terminal phosphoryl group of ATP to a poly-P primer. It is strongly inhibited by ADP at low ATP/ADP ratios. Degradation is accomplished by several reactions, the chief one involving a specific polyphosphatase. However, in some organisms, alternate routes

have been demonstrated: a hexokinase which uses poly-P to phosphorylate glucose, and a polyphosphate-AMP phosphotransferase which synthesizes ADP from AMP and poly-P.

The relationship of nucleic acids to poly-P metabolism is very ill-defined. By inhibiting RNA synthesis, Harold (1963) observed that RNA synthesis was antagonistic to poly-P accumulation. RNA synthesis could inhibit poly-P synthesis, and stimulate poly-P degradation, perhaps by competition for ATP. Some evidence indicated that poly-P was actually converted to RNA during these experiments.

One of the mutants (Pn-2) investigated by Harold (1964) was unable to synthesize poly-P. Analysis revealed that it lacked poly-P kinase, but contained fairly normal amounts of the poly-P degradative enzymes. The striking resemblance between this mutant and ORS is probably coincidental, inasmuch as Pn-2 contained a normal amount of RNA, in contrast to ORS. However, examination of some polyphosphate-related enzymes in ORS, eg. poly-P kinase and phosphatase, is certainly a logical step and might be very informative.

In reference to the previously-presented diagram, the phosphorus cycle in ORS would seem quite abnormal at the site coupling RNA metabolism to poly-P metabolism. ORS may use available energy to synthesize RNA but not poly-P. Whether low Pi is a consequence or a cause of low poly-P and high RNA levels remains speculative. The possibility exists that ATP in ORS represents a common denominator between poly-P and RNA. Though Harold's scheme does not elaborate on the segments between Pi, ATP, and ADP, this area is probably susceptible to substantial regulation by metabolic controls mentioned earlier.

Glutamine metabolism is known to play an integral role in the

control of cellular nitrogen metabolism (Umbarger, 1969). The products usually derived from this amino acid receive either a N atom or an amino/amide group, the carbon being incorporated sometimes into proteins. The size of the glutamate-glutamine pool is a function of several factors: the supply of ammonia or amino nitrogen, the amount of citrate synthesis through the TCA cycle, and the use of these amino acids. The higher glutamine content of ORS would seem to indicate therefore more storage of N than (+), as a result perhaps of a basic difference in steady-state N metabolism.

There are a number of physiological mechanisms which could account for the increased level of glutamine:

1) Rate of conversion of nitrate to ammonia

2) Rate of protein turnover

3) Synthetic uses of glutamine

1) With respect to the first possibility, ORS might convert nitrate to ammonia at a faster rate than the ammonia can be used in cellular biosyntheses. Since ORS has a slower growth rate than (+), this suggests that biosynthesis, especially protein synthesis, is slower than (+). The lower protein content of ORS compared to (+) provides additional evidence for this. Continued production of ammonia with low protein synthesis would perhaps stimulate glutamate production. Growth on nitrate is known to induce nitrate and nitrite reductase in algae, the enzymes which together reduce nitrate to ammonia (Eppley and Rogers, 1970).

2) In combination with lower protein content and a slower growth rate, higher glutamine could indicate a lower rate of protein turnover than (+). This would be especially likely if carbon skeletons for protein amino acids were a limiting factor, and not nitrogen. In such a case,

amino acid N available for transamination would increase. That glutamine, with two N groups, is used to store nitrogen has been observed by Reisner et al. (1960) and Baker and Thompson (1962) with N-starved Chlorella vulgaris supplied with nitrate, ammonia, and urea.

Reisner et al. (1960) also indicate that glutamate is normally a major amino acid in Chlorella, whether grown on nitrate, ammonia, or urea. The present study confirms this for (+) and ORS on nitrate. If TCA cycle activity in ORS were depressed, α-ketoglutarate (α-KG) carbon might be shunted off to glutamate at an increased rate, thus decreasing carbon for synthesis. Some evidence for this can be construed from the relative pools of organic acids, of which ORS on air shows less succinate and malate than (+) on air. The higher concentration of α-KG in ORS suggests that the TCA cycle is particularly rate-limited after α-KG oxidation.

3) Lynch and Gilmore (1966) report that glutamine N alone can support growth of C. pyrenoidosa. This is significant because of the critical role played by glutamine in the synthesis of essential cell components. Increased "demand" for one or more of these components could represent a stimulus that triggers glutamine synthesis in ORS. Conversely, decreased synthesis of constituents containing glutamine or its N would lead to an increase in the free glutamine pool.

Glutamine synthesis itself is a very stringently-regulated reaction. The enzyme, glutamine synthetase, from a variety of microbes (including C. pyrenoidosa), is subject to cumulative feedback inhibition by a number of compounds among which are alanine, glycine, carbamyl phosphate, tryptophan, glucosamine-6-phosphate, and the ribonucleotides AMP and CTP (Cohen, 1968; Woolfolk and Stadtman, 1967). Moreover, E. coli glutamine synthetase is strongly repressed by ammonium ions. In order for

glutamine synthesis to continue, these metabolites in ORS must be removed from the vicinity of the enzyme. This could be accomplished by compartmentation or rapid metabolism of the effector molecule.

It is tempting to speculate on the involvement of glutamine in RNA metabolism in (+) and ORS. Whether fortuitous or not, the increased level of glutamine in ORS is paralleled by increased RNA. Because glutamine participates as an amino N donor in purine (ATP, GTP) synthesis directly, RNA synthesis could be related to glutamine level.

There is evidence that an increase in glutamine can contribute to increased RNA constituents. In the biosynthesis de novo of purine ribonucleotides, the rate-limiting reaction unique to the pathway is the first step: the formation of phosphoribosylamine from phosphoribosyl pyrophosphate (PRPP) and glutamine (Raivio and Seegmiller, 1970). The activity of the enzyme, PRPP amidotransferase, is limited by 1) the availability of substrate and 2) feedback inhibition by purine ribonucleotide products (AMP, GMP, but not ATP). If the purine products were polymerized into RNA, then perhaps increased glutamine would sustain a higher level of RNA synthesis.

Certain bacteria (eg. Aerobacter aerogenes) show an increase in organic carbon content under phosphorus-limited conditions, due to an increase in a poly-glucose polymer similar to glycogen (Strange et al., 1961). Marinos (1963) observed the accumulation of starch in P-deficient barley shoots. Fuhs (1969) has also reported this same occurrence in P-limited diatoms. At least in the bacterial systems, those cells which accumulate carbohydrate did not degrade N-containing constituents (proteins and nucleic acids). Consequently ammonia was not liberated during P-limitation. It is believed that the polysaccharide reserve has a sparing

effect on degradation of N-containing components. Catabolism slows down to preserve cell integrity.

Reid and Bieleski (1970) confirmed these data for P-deficient Spirodela oligorhiza (duckweed). In addition to starch, glutamine and asparagine increased markedly, while other free amino acids remained relatively constant. They concluded that P-deficiency imposes a slower growth rate on the organism, without a corresponding decrease in N uptake, resulting in increased soluble N in the cell.

Experiments by Fuhs (1969) indicate that phosphorus assimilation is closely coupled to cell growth in a complicated manner. In certain diatoms, the specific growth rate varies if the cell phosphorus content varies. Growth rate versus P per cell follows a saturation curve. Below a certain minimal concentration of P, cell growth decreases in a non-linear manner.

Any change in cell P significantly alters cell composition, especially subcellular P fractions. "Structural" P components required to maintain integrity and viability of the cell (ie. DNA-P and membrane lipid-P) show no change right up until death. But the "functional or synthetic" fraction, consisting of phosphorylated intermediates, chloroplast lipid-P, and RNA-P, is closely proportional to growth rate, decreasing with decrease in growth rate. Storage or reserve compounds (poly-P) appear only when P supply is restricted.

According to Mandelstam (1963), the stability of protein generally reflects that of the cellular RNA. When cell growth stops, or when the cell is deprived of some nutrient, protein and RNA turnover are simultaneously triggered. The properties of ORS, however, namely accumulation of polysaccharide reserve and retention of N in the form of glutamine,

correlate with a lower rate of turnover compared to (+). This is necessary for ORS to survive since its growth takes longer than (+). Moreover the relationship between protein and RNA turnover in ORS must be unusual. With a higher ratio of RNA/protein than (+), ORS would appear to have lost the close coupling between RNA and protein metabolism that normally prevails in (+), and which may be necessary for vigorous growth.

The adaptation experiments for (+) clearly describe the behavior of a facultative heterotroph (Parker et al., 1961). Though C. sorokiniana may be limited in its range of heterotrophic growth substrates, the immediate growth upon placing (+) into the dark indicates that its non-chloroplastic glucose metabolism is readily activated. With respect to GAPD adaptation, (+) is similar to the Chlamydomonas systems of Hudock and Levine (1964) and Ohad et al. (1967). Unlike higher plants and various mutant algae, eg. Euglena (Ben-Shaul et al, 1963), the chloroplast of (+) does not dedifferentiate completely in the dark; (+) retains a deep green color plus the ability to photosynthesize immediately upon removal from the dark. In particular, heterotrophic C. sorokiniana contrasts with C. variegata, studied by Fuller and Gibbs (1959), where the mere presence of glucose caused degeneration of the chloroplast and loss of its enzymic complement.

The kinetics of ORS adaptation show two basic differences from (+).

1) Dark conditions per se prevent immediate maximum growth of ORS. Apparently an induction or adaptation period is necessary before ORS can use or develop pathways of heterotrophic metabolism supporting maximum growth. This lag period does not correspond to the repression phenomenon noted for C. protothecoides when placed into the dark on a sugar substrate (Aoki et al., 1965). In the case of this thiamin auxotroph, glucose

causes the chloroplast to "bleach" and the cells turn white. ORS in contrast maintains a dark green pigmentation, and shows no substrate specificity during this time. The lag could not be eliminated by replacement of glucose with acetate, nitrate N by casein digest (amino acid N), or by addition of a vitamin supplement.

Since glutamate dehydrogenase was essentially the only parameter which showed any correspondence to adaptation, it is conceivable that N metabolism is somehow involved. NAD-GLDH is known to be induced under high glutamate concentrations and NADP-GLDH under high ammonia levels (Woolfolk et al, 1966). These conditions might exist if protein synthesis were decreased in ORS moreso during, than after, the adaptation period.

The results of Kivic et al. (1969) suggest that glutamate dehydrogenase in green algae may be controlled by either light or heterotrophic conditions, somewhat like GAPD. These workers reported that Chlamydomonas reinhardi contains two electrophoretically distinct forms of GLDH, depending on whether cells are grown in the light or dark. The most abundant form is found in both the light and dark, while a second type is found only in the dark. The y-2 mutant strain of C. reinhardi, which cannot grow in continued darkenss, lacks the dark type GLDH. Perhaps the adaptation data reflect an analogous form of control for ORS GLDH.

2) The limited data indicate that ORS may have a higher level of oxidative metabolism during light growth than (+). The higher light respiratory rate is consistent with the higher light level of succinic dehydrogenase in ORS reported by Ward et al. (1969). Furthermore the extremely high level of NAD-linked GAPD in light ORS sustains this pattern.

Comparison of dark metabolism reveals almost equally contrasting

behavior. The rise in QO_2 for (+) is indicative of the normal increase in mitochondrial metabolism which occurs upon transfer of a photosynthetic organism to the dark (Ohad et al., 1967). Again this corresponds with the three fold increase in succinic dehydrogenase activity reported by Ward et al. (1969) for (+) in the dark. The sharp increase in (+) NAD-GAPD is as expected (Fuller and Hudock, 1967).

But ORS seems unable to increase its metabolic output in the dark to any great extent. Thus its QO_2 and succinic dehydrogenase are now lower than (+), and its NAD-GAPD remains comparatively the same as in the light.

Though the significance of these observations remains speculative, they are consistent with the hypothesis that carbohydrate metabolism in ORS is under more stringent metabolic control than (+). The control causes ORS to exhibit a higher rate of oxidative metabolism in the light, but throttles down this same metabolism in the dark. Nitrogen metabolism (specifically the incorporation of ammonium N) does not appear to decrease in proportion to carbohydrate and respiratory metabolism.

CONCLUSION

ORS bears a striking resemblance to phosphorus-deficient organisms in many respects. The following characteristics of ORS are consistent with intracellular P-limitation: slower growth rate, increased starch, N storage in glutamine, increased alkaline phosphatase activity, and the lower levels of soluble orthophosphate and poly-P. Concomitantly, other facts suggest that the ORS condition is one of controlled or regulated limitation of orthophosphate and not a lethal deprivation per se. RNA, if not normal, is abnormally high. ORS cells appear to assimilate Pi from the medium at a reasonable rate, as well as maintain a constant growth rate. It is conceivable that an alteration in the control of phosphorus metabolism has occurred in ORS which restricts the intracellular Pi available to many metabolic pathways. Despite this control defect, ORS can maintain a sufficient level of P-related metabolites to sustain steady-state growth.

As a consequence of altered P metabolism, ORS has become highly insensitive to the primary cellular control exerted by oxygen. The response of (+) indicates that high oxygen typically disrupts the P cycle, specifically that part related to energy metabolism (ie. phosphorylation). High oxygen could distort the normal equilibria between those P-metabolites which are crucial to energy pathways. This might "short-circuit" energy metabolism by some mechanism such as too high an ATP/ADP ratio, which would inhibit a number of enzymes susceptible to "energy charge" regulation. The result would be profound metabolic

disturbances, characteristic of the symptoms of oxygen toxicity. In ORS, the energy pathways (eg. glycolysis and respiration) would be rigidly throttled by a defective P control mechanism, thus negating normal control by high oxygen.

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LABELS ON ELECTRON MICROGRAPHS

N = Nucleus

C = Chloroplast

S = Starch grain

CW = Cell wall

PP = Polyphosphate deposit or granule

V = Vacuole in cytoplasm

mb = Microbody

m = Mitochondrion

d = Dictyosome

CV = Cytoplasmic vesicle

er = Endoplasmic reticulum

PB = Prolamellar body

PV = Chloroplast vacuole

o = Osmiophilic droplet

st = Stroma or matrix of chloroplast

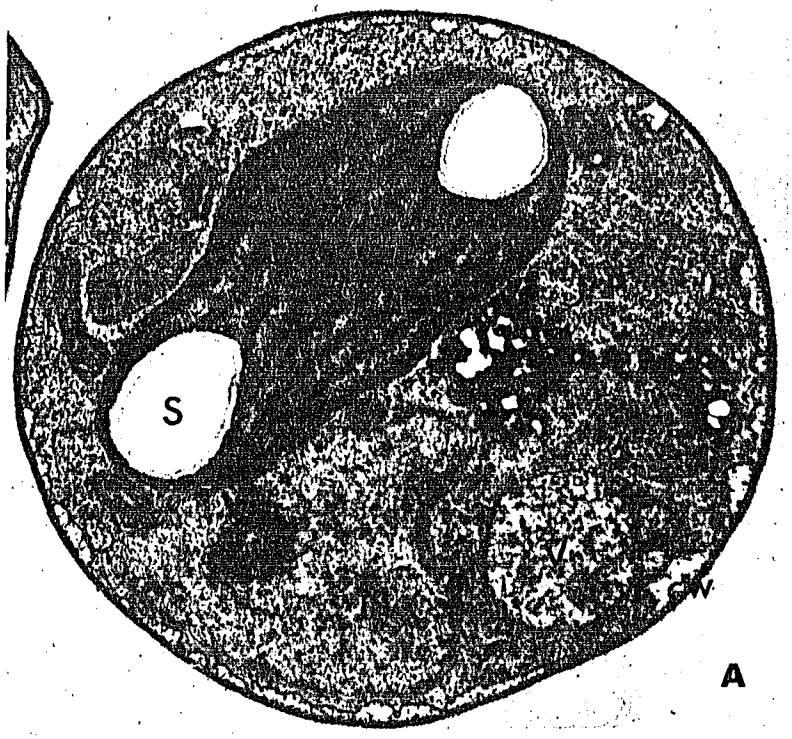
nu = Nucleolus

t = Thylakoid (=lamella) of chloroplast

Figure 4

Typical (+) C. sorokiniana grown on air, showing normal cell morphology for heterotrophic conditions. A) Structures visible: nucleus, chloroplast, polyphosphate deposit, vacuole, microbody, starch, cell wall. about 27,500 X

B) Structures visible: microbody, mitochondrion, dictyosome. Note the appearance of the chloroplast in two parts. about 27,500 X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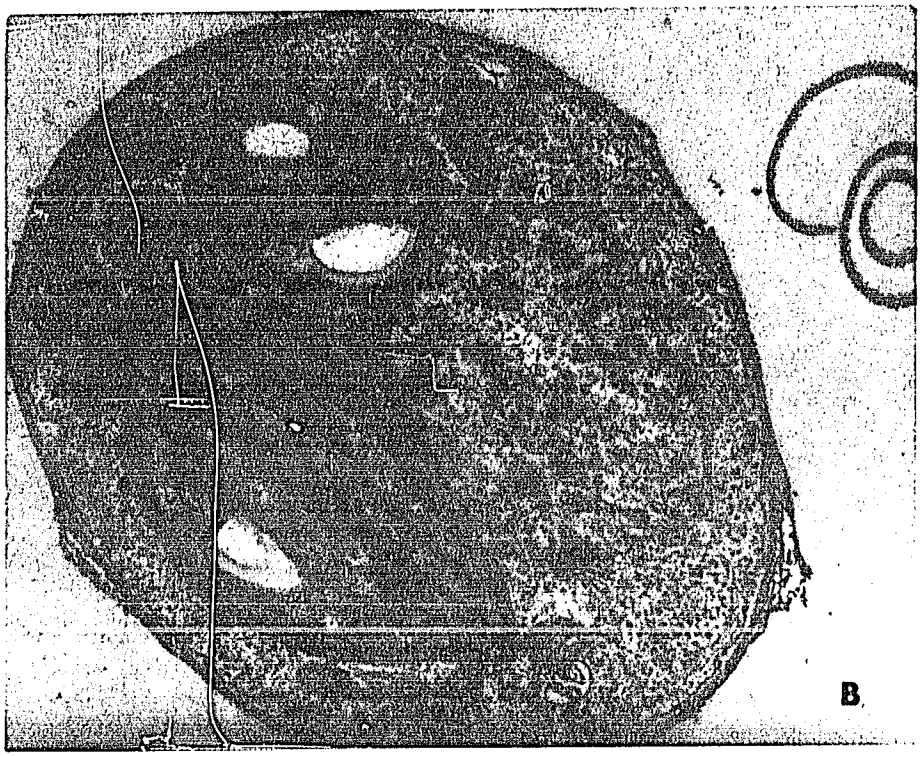

B

Figure 5

Wild type cells grown on air, showing typical cell organelles, and the abundance of polyphosphate.

about 27,500 X

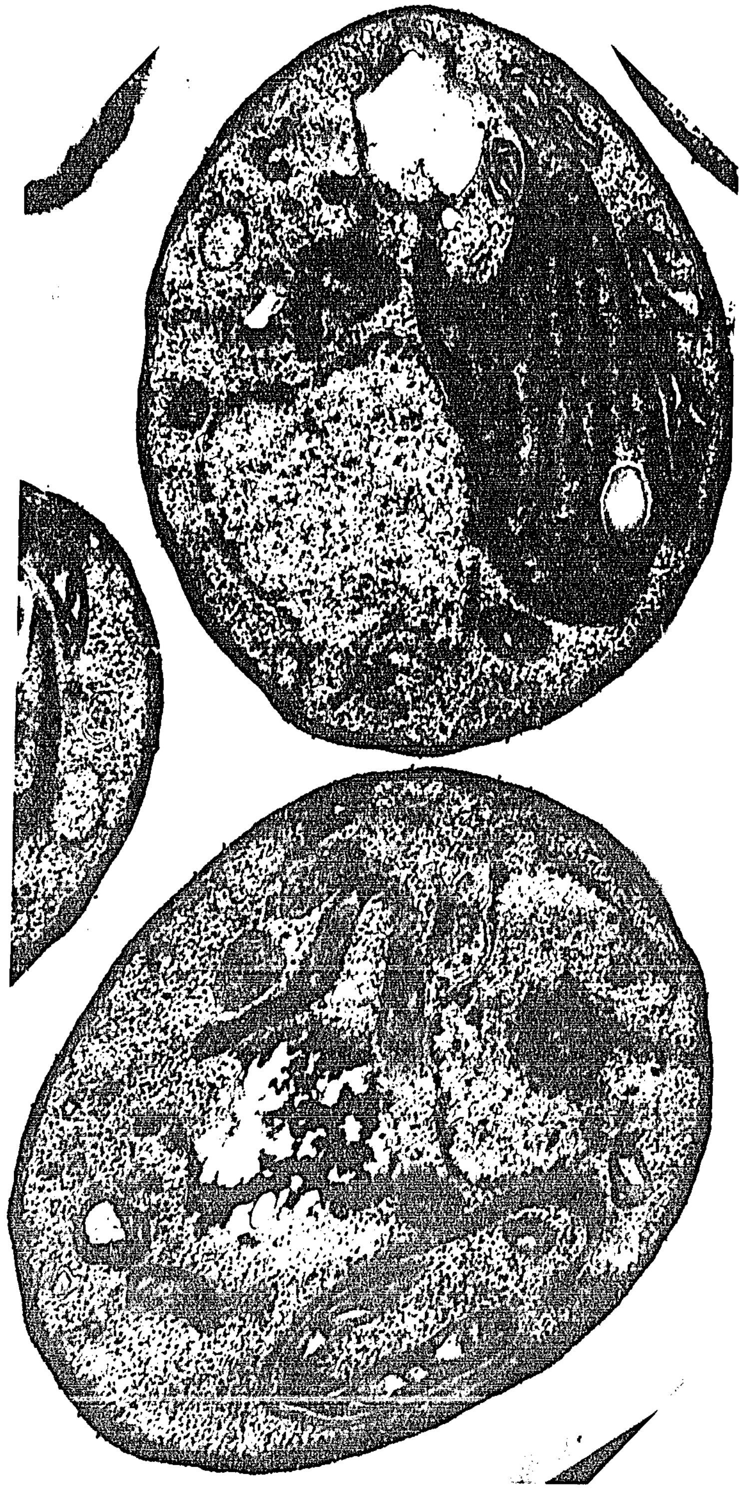
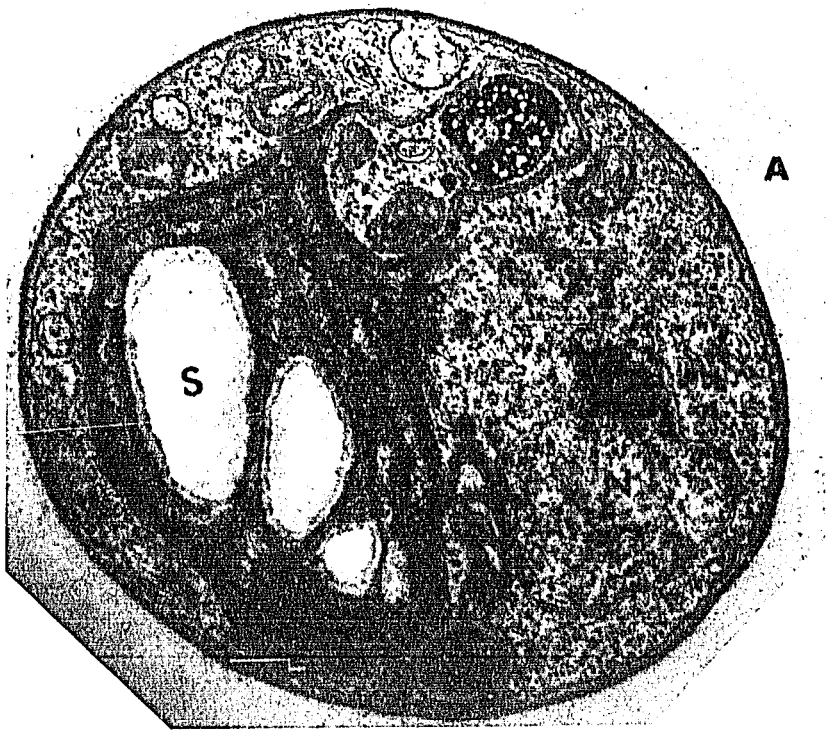


Figure 6

Wild type grown on air.

A) Note the reticulated nature of the chloroplast. A polyphosphate granule appears within a "pocket" in the chloroplast. (See text) about 27,500 X

B) Note mitochondrial structure and the close proximity of mitochondria to chloroplast and polyphosphate. about 27,500 X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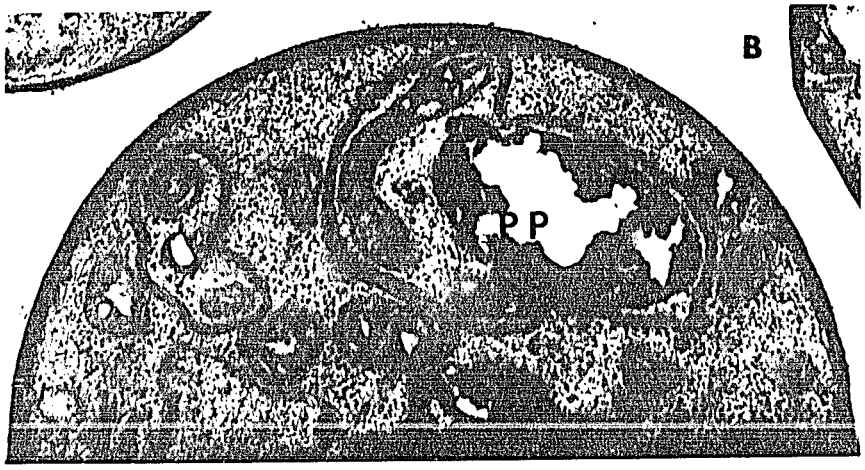
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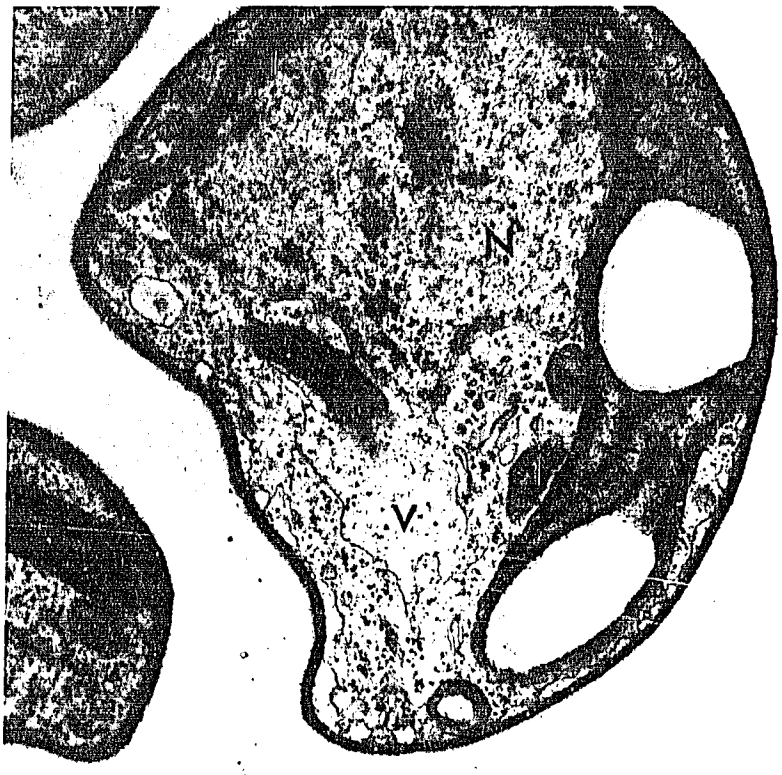
Figure 7

Typical ORS C. sorokiniana grown on air, showing general cell morphology for heterotrophic conditions. A) Structures visible: chloroplast containing starch, nucleus, mitochondrion. Note irregular shape of cell. about 27,500 X

B) A large amount of starch is found in the chloroplast. about 30,000 X


N
V
A

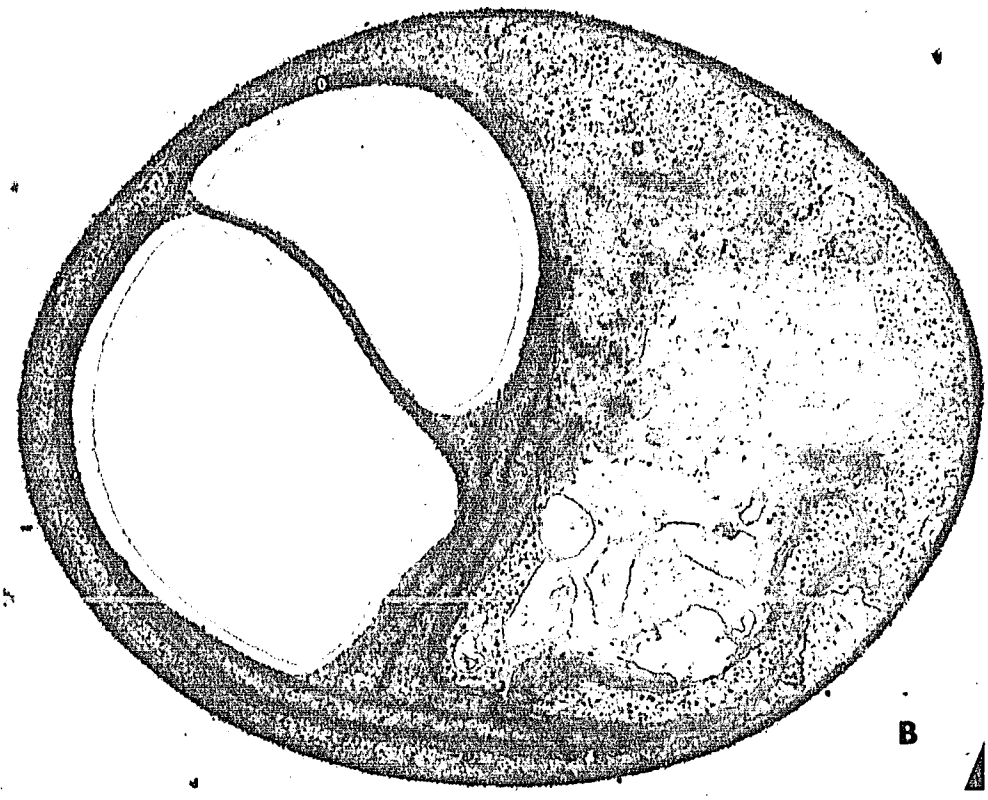

B

Figure 8

ORS grown on air. All cells display variations of mitochondrial morphology, and close apposition of mitochondria to chloroplast. Mitochondria appear to align themselves along the length of the chloroplast. Lamination of the chloroplast is also noticeable in all cells. about 27,500 X

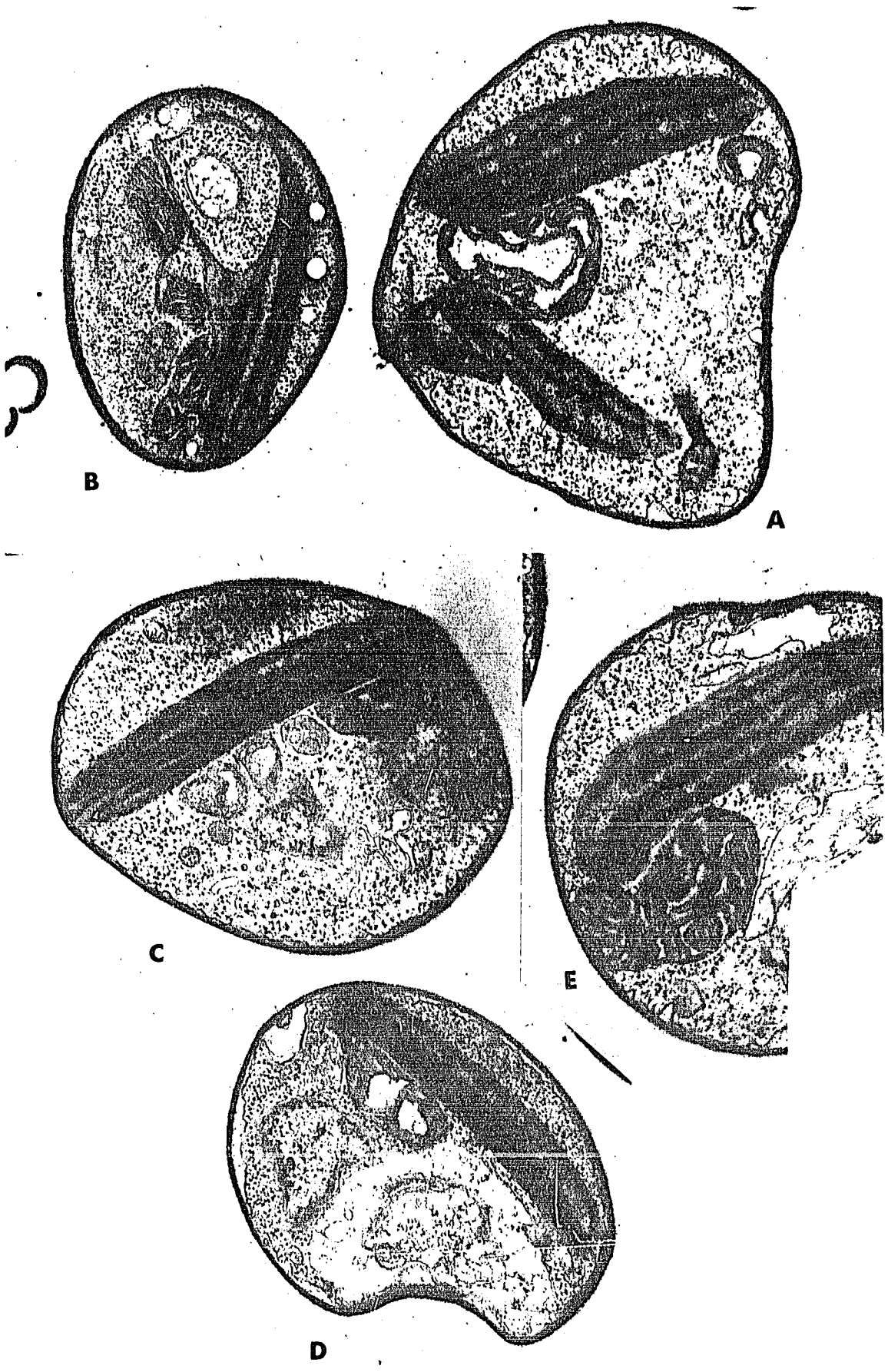

B
A
C
E
D

Figure 9

ORS grown on air.

A) A large amount of vacuolation is evident.

about 27,500 X

B) ORS cell possibly preparing to divide. Note dictyosomes, starch within chloroplast, and vacuolation. about 27,500 X

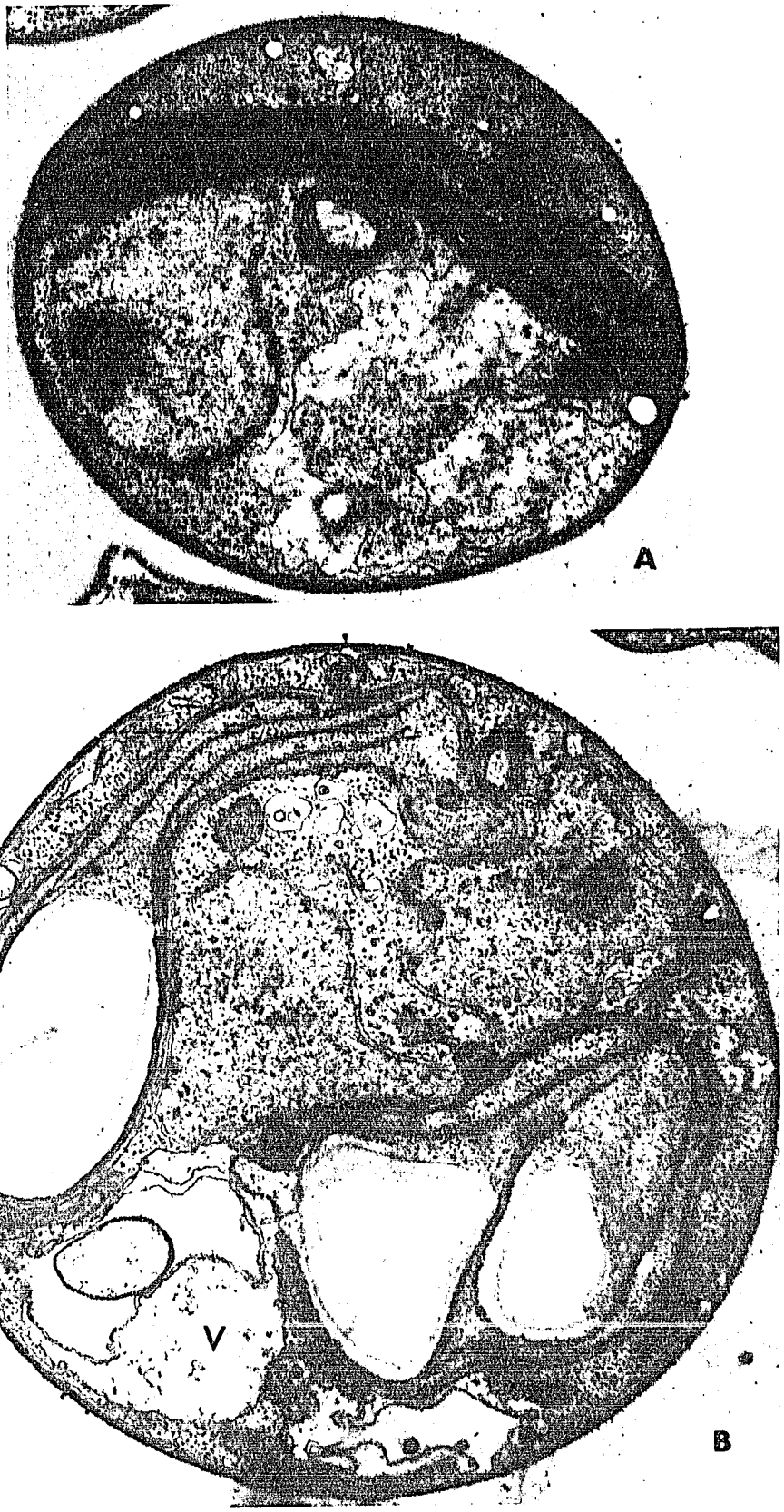
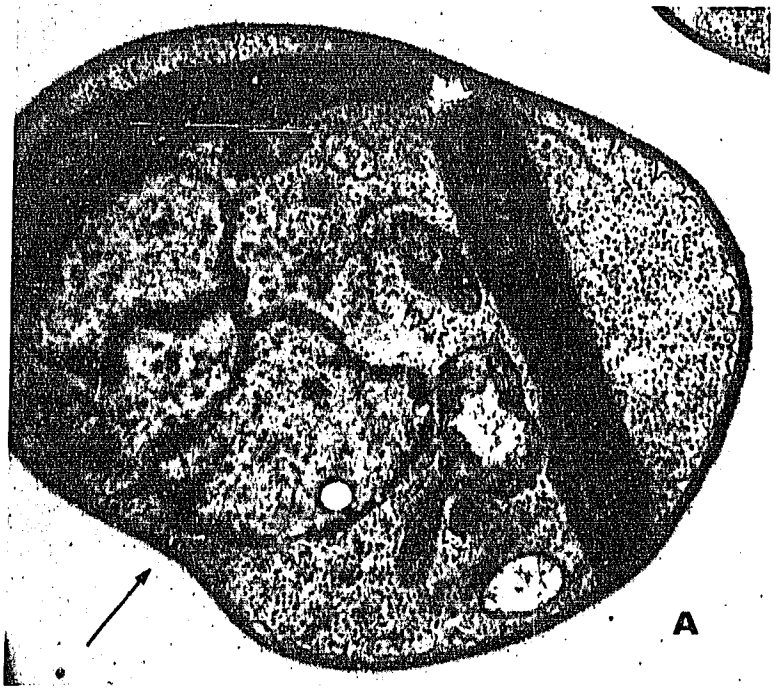

A
V
B

Figure 10

ORS grown on air, showing stages in cell division. A) Nucleus and chloroplast appear to have divided and separated to opposite poles of the cell. Note slight indentation (at arrow) of cell wall, which occurs early in ORS "binary" division cycle. about 27,500 X

B) "Dumb-bell" cell, characteristic of very late stage in ORS "binary" division. Arrows point to common portion of cell wall. Bottom cell has been cut in half. about 27,500 X


A

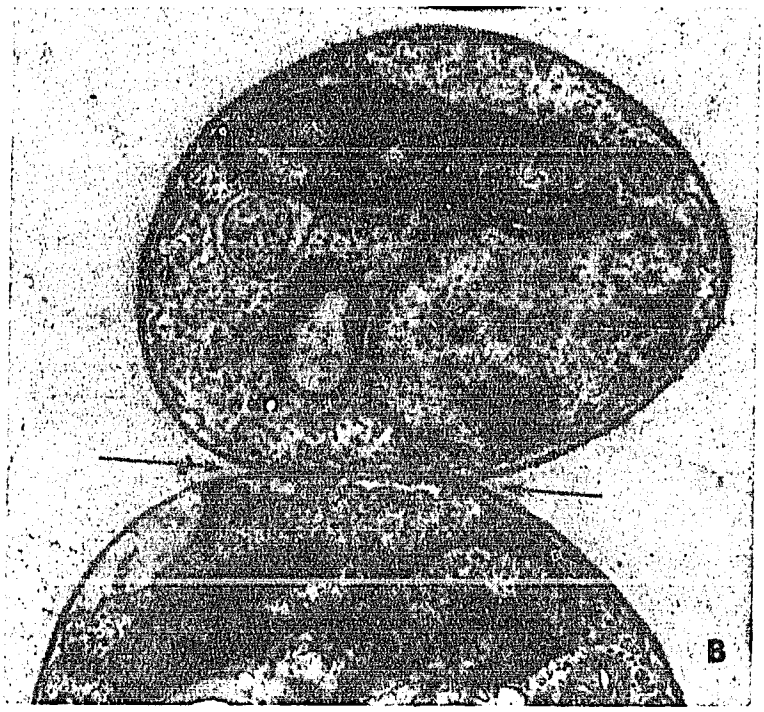
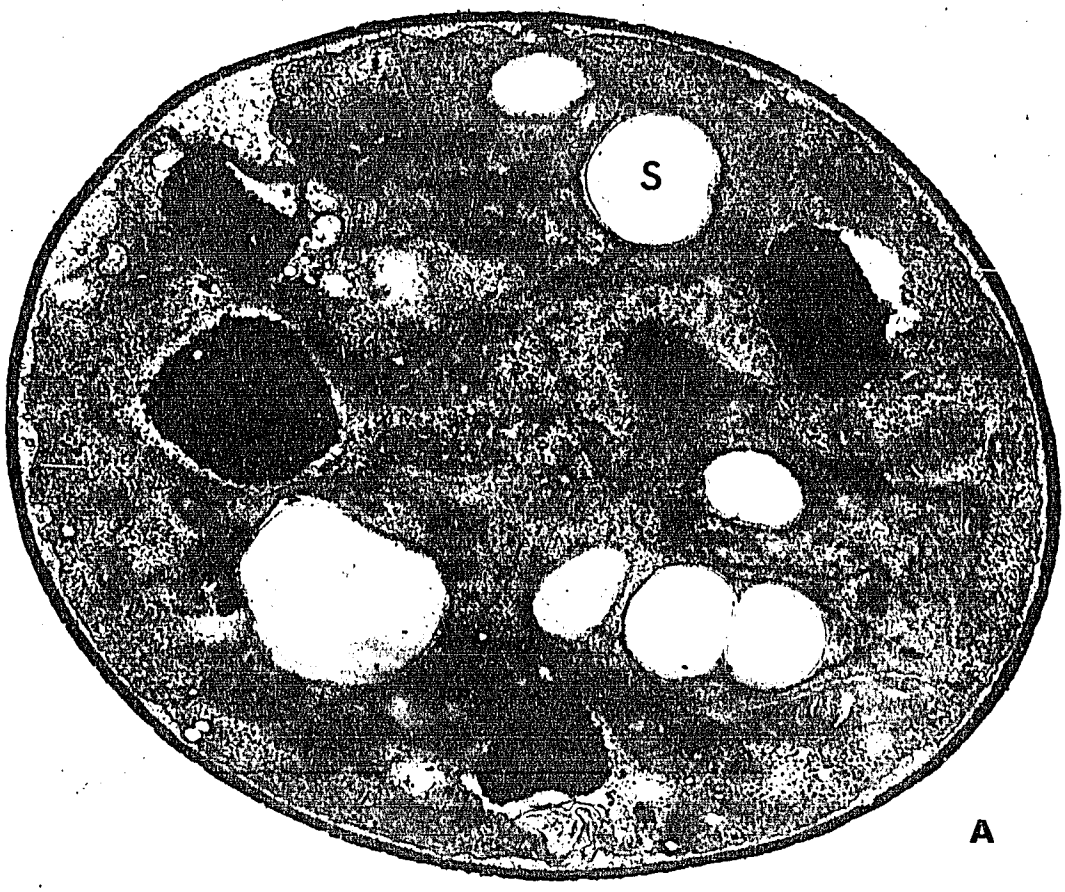

B

Figure 11

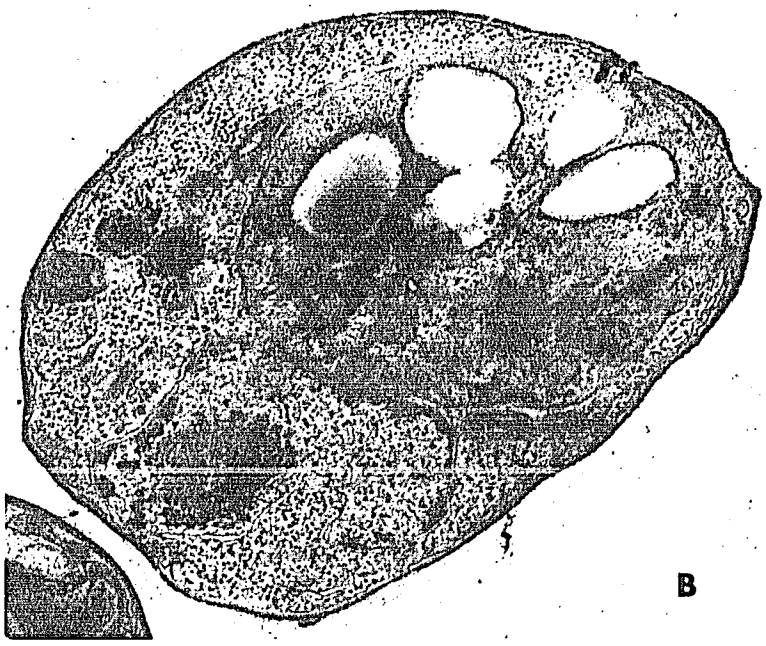
Typical (+) exposed to 100% oxygen for several days, showing distinctive features.

A) An intact cell with increased numbers of mitochondria, starch, and polyphosphate deposits. about 27,500 X

B) A small (young?) (+) cell, appearing relatively normal on 100% oxygen. about 27,500 X



A



B

Figure 12

Wild type exposed to 100% oxygen, showing general cellular disorganization. Six dictyosomes are visible within the nuclear region. about 34,000 X

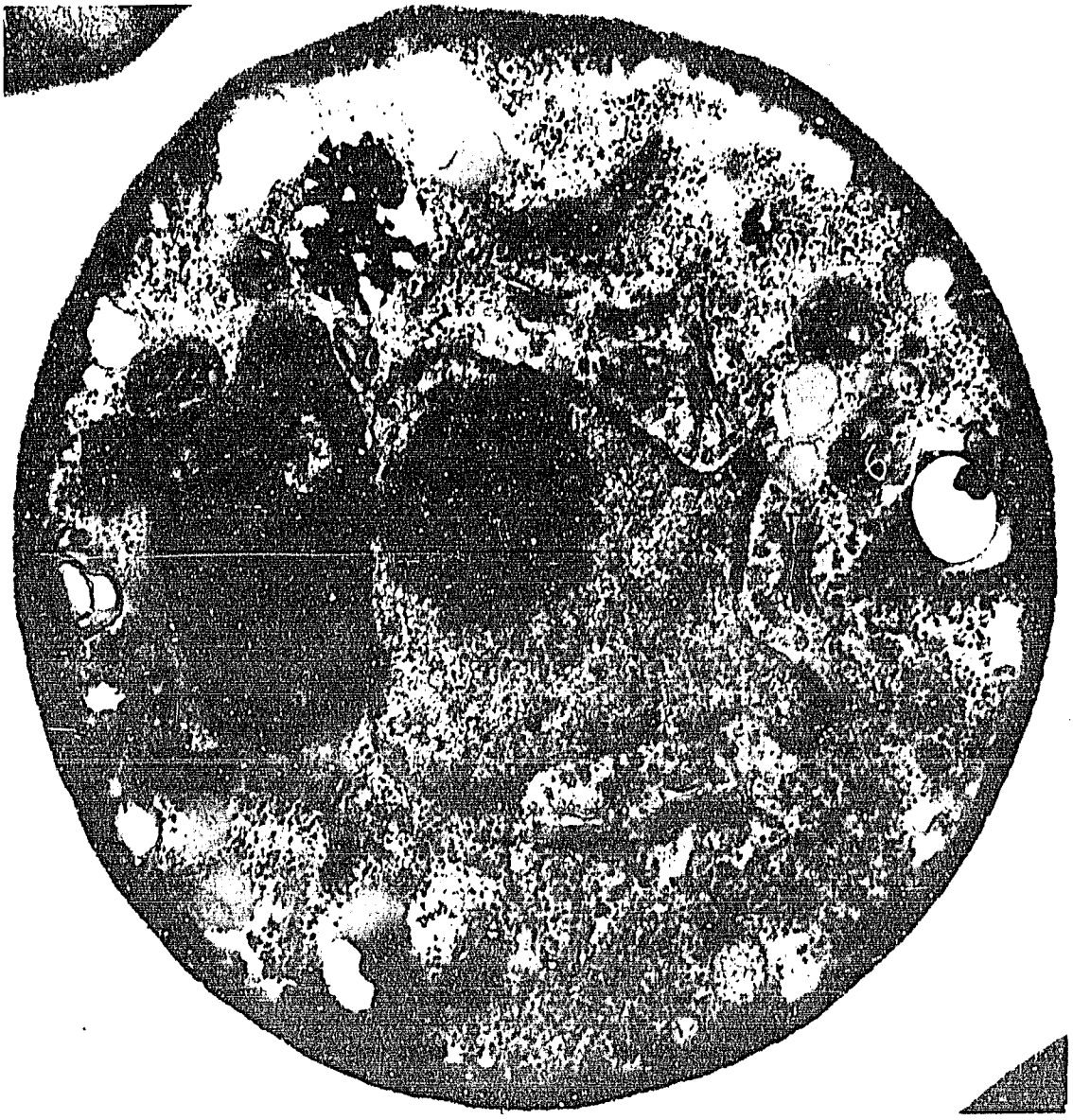
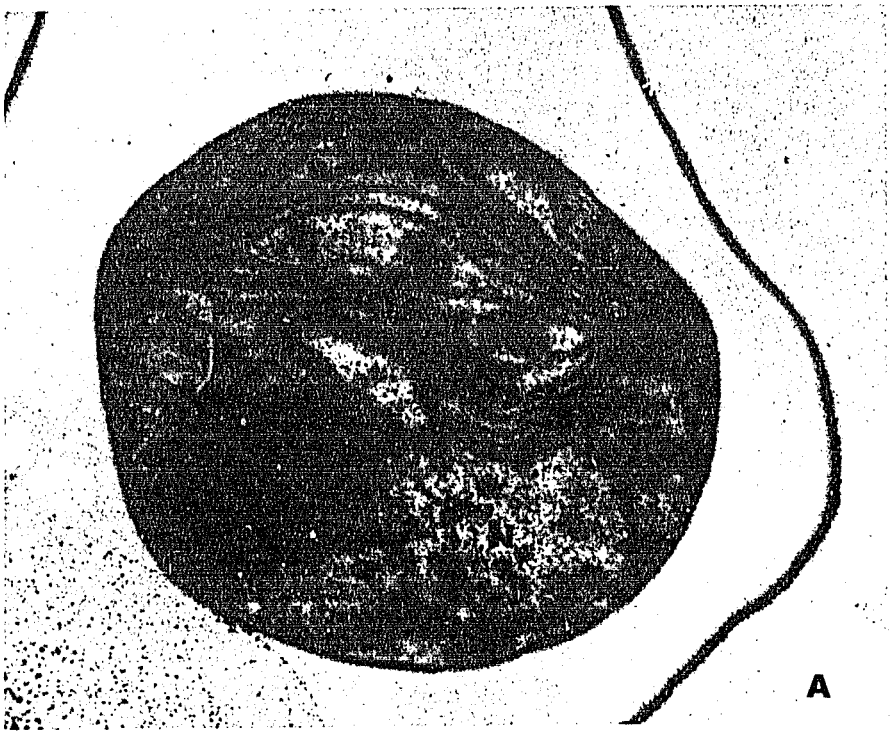


Figure 13

Typical ORS grown on 100% oxygen, showing general cell morphology.

A) The chloroplast and nucleus occupy much of the intracellular volume, while cytoplasmic vesicles containing medium density material are conspicuous. about 27,500 X

B) Cytoplasmic vesicles are abundant, as is starch in the chloroplast. Endoplasmic reticulum becomes quite prominent (arrows), especially around the periphery of the cell. Mitochondria appear poorly-developed. about 27,500 X


A

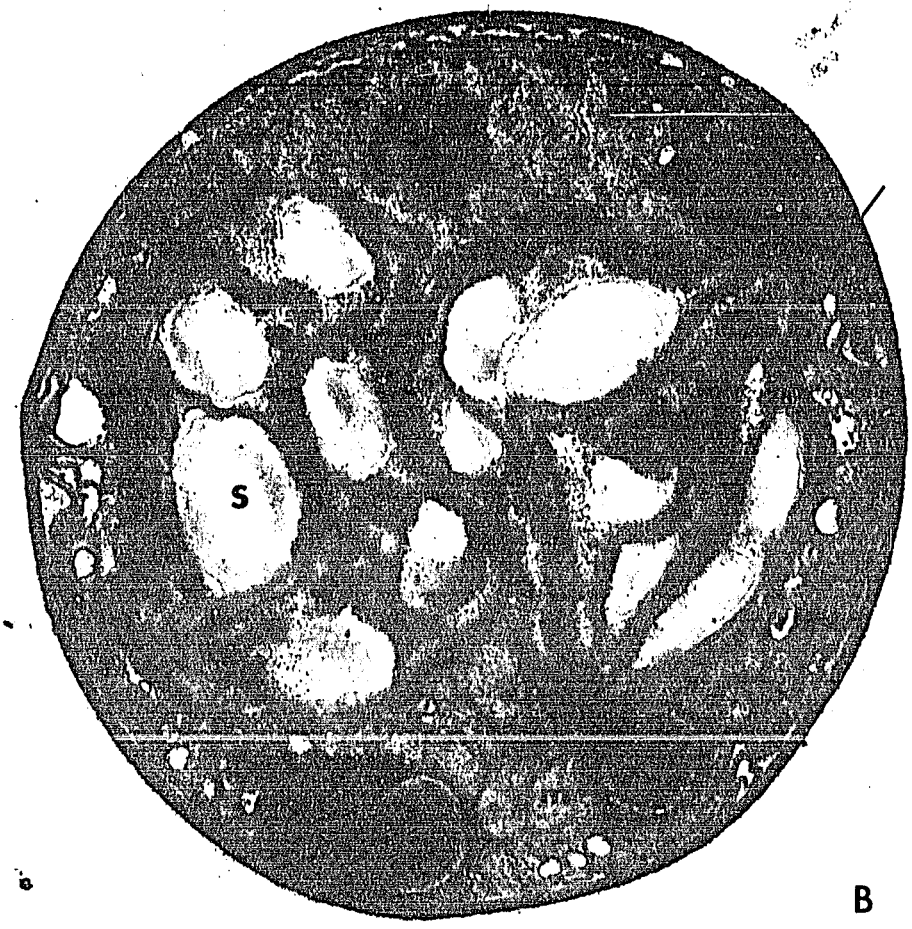
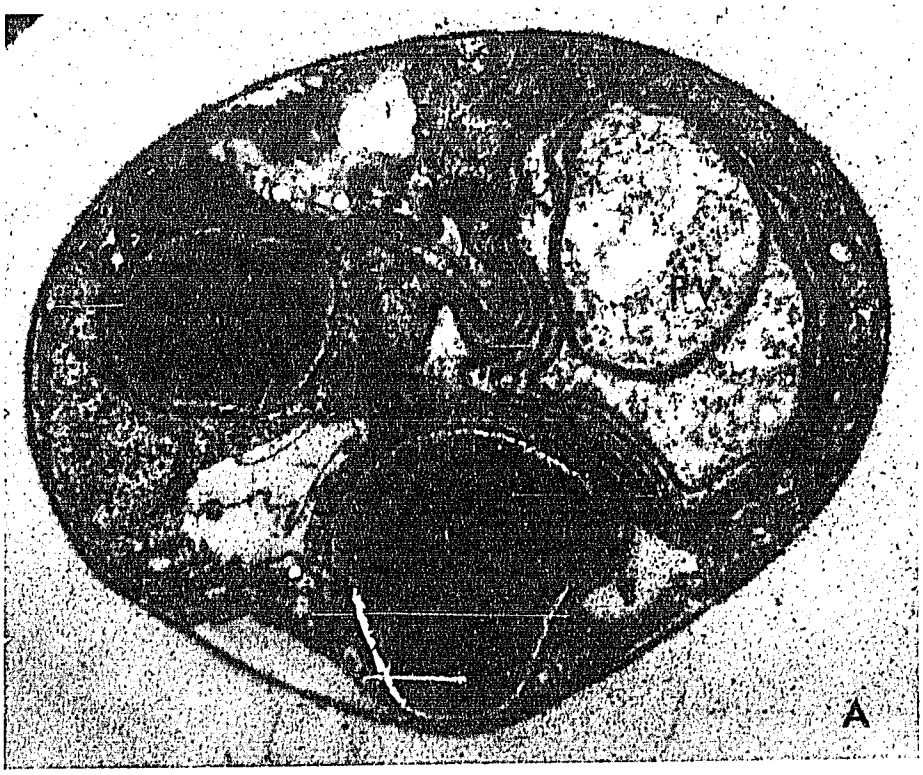

S
B

Figure 14

ORS grown on 100% oxygen, showing the state of general cellular dedifferentiation under this condition.
A) The chloroplast is often vacuolated and cytoplasmic vesicles are widely distributed. about 27,500 X
B) Atypical gramular endoplasmic reticulum can be seen in cross section (arrow) around cell periphery. A section through the prolamellar body is apparent.
about 27,500 X


PV
A

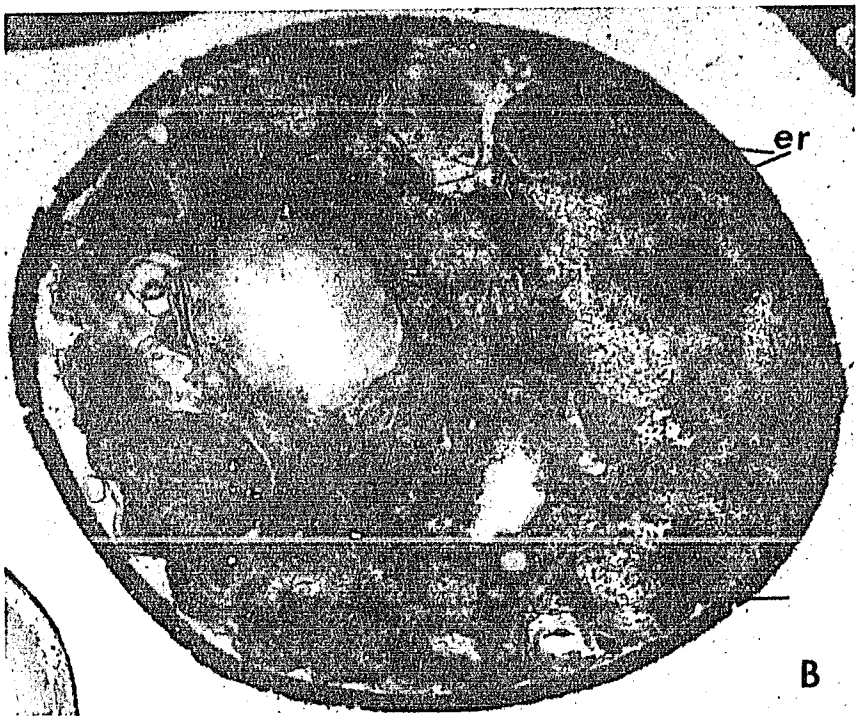
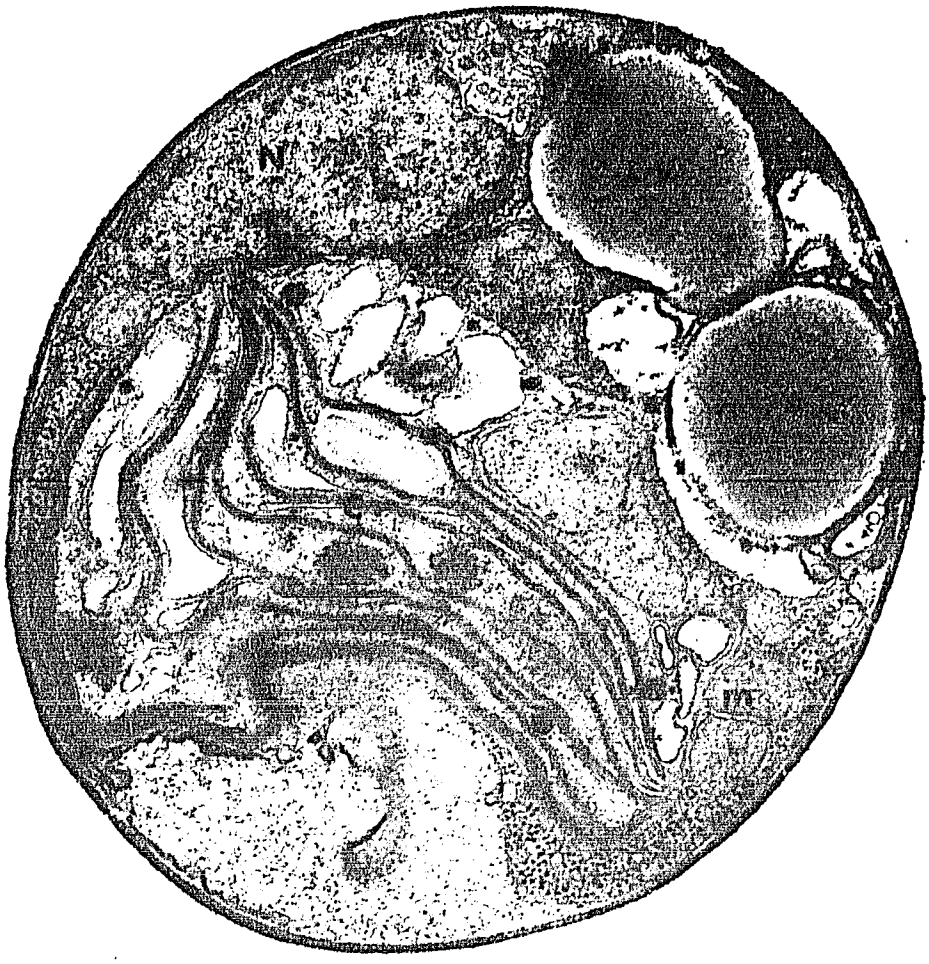

er
B

Figure 15

ORS grown on 100% oxygen, showing general cell morphology. Nucleus, chloroplast with starch, cytoplasmic vesicles, and several non-descript mitochondria are pictured. about 34,000 X


N
CM