A STUDY OF PHYCOPHYSIOLOGY IN CONTROLLED ENVIRONMENTS

TWELFTH SEMIANNUAL STATUS REPORT

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No. 1012

PREPARED BY DR. ROBERT W. KRAUSS

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COLLEGE PARK, MARYLAND

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A Study of Phycophysiology in Controlled Environments

Prepared by the Director

Dr. Robert W. Krauss

Head and Professor of Plant Physiology

Department of Botany

University of Maryland

College Park

April 1, 1966

Introduction

Progress reported in this review has been achieved under an extension of Research Grant NsG-70-60 made to the University for the period 1 April, 1964 to 31 March, 1966. The initial grant was for three years beginning 1 April, 1960. This report covers work performed between 1 October, 1965, and 31 March, 1966. It reports briefly the results of studies completed during the six-month period and includes manuscripts of work ready for publication. Publications from the reviewed literature are also forwarded with this report. Financial details are supplied directly from the comptroller's office.

Personnel

The personnel employed during the current report period are as follows:

Name	Title	Appointment
Professional Staff:		
Dr. R. W. Krauss	Director and Professor	4/1/60-present
Dr. C. Sorokin	Research Associate	4/1/60-present
Dr. R. A. Galloway	Associate Professor	4/1/60-present
Dr. G. W. Patterson	Assistant Professor	7/1/65-present
Technical Assistants:		
W. C. Schaefer, Jr.	Electronics Technician	4/1/64-present
A. Osretkar	Assistant	4/1/64-present
L. E. Hageman	Laboratory Mechanic	12/26/65-present
Victor Montviloff	Assistant	9/13/65-present

Part-time and Student Labor:

K. C. Chang	Draftsman
T. A. Smith	Laboratory Assistant
J. C. Houk	Laboratory Assistant
R. D. Godwin	Laboratory Assistant

During part of the report period, Dr. Galloway has been conducting his research on CO_2 fixation at the University of Freiburg in Germany while on sabbatical leave. A summary of his findings will be included in the next report. Mr. Leslie Hageman has been added to the technical staff. The work of graduate students, Mr. Gross, Miss Chimiklis, and Miss Reger, is in support of this study although salaries are not paid from this grant.

Research Summary

Increasing competence demonstrated in engineering necessary vehicles for long term space exploration has focused attention on the requirement for extended life support. Men in space require food, water, and oxygen, and there must be a method of carbon dioxide and waste disposal. In looking toward total or partial regenerative procedures to accomplish these ends, it appears that either a physical or a biological system must be engineered to provide such life support. The problem of determining the duration of space voyages that may be attempted as well as the kinds of systems which might be expected to provide life support is pressing. In general, for shorter flights of up to 30 - 60 days, physical systems involving the packaging of food and oxygen supplies are adequate. For longer periods

regeneration becomes increasingly logical--the longer the flight, the more logical. It has been the mission of the team supported under this grant to examine the physiology of organisms whose culture characteristics make it possible for them to serve in biological regeneration. There are two groups of organisms which give promise of such a role--the hydrogen bacteria and the unicellular algae. This report is concerned primarily with the latter although some of the culture techniques can be employed with either group.

It is worthwhile to point out that the criteria for the selection of any organism to play the oxygen and food producing role in a system where man is the consumer of these products will ultimately bear on the reliability of rapid production of a biomass which is complementary to man. In the last analysis the amount of oxygen produced will depend on the amount of carbon reduced and incorporated into the protein, carbohydrate, and fat of the organism. Therefore, the most useful measure of the suitability of an organism for space flight regeneration must be the magnitude of its <u>sustained</u> rate of growth coupled with the stoichiometry of its synthetic system and the nature of its cellular product.

There are always major differences in the performance of microorganisms in large scale culture compared to that in small scale laboratory apparatus. This is in part due to the engineering required for sustained growth and in part to the shifts in environment made by the organisms themselves in continuous culture. Predictions of efficiency made on small scale apparatus, though useful in a comparison of intrinsic cellular potential, cannot be trusted in the absence of data on performance. An organism that

may appear to be at a disadvantage with regard to efficiency, may turn out to be far from reliable and efficient in terms of sustained growth and the nature of the product of growth.

It should be emphasized that the magnitude of the task of selecting and employing an organism for life support in the sense of regeneration for space is a task of enormous magnitude. In comparison, the engineering of a new rocket motor is a small task indeed. The necessary energy and quality of talent needed to do this task will not be obtained with modest funding. However, in the absence of large scale engineering resources the most logical expenditure of effort is toward an understanding of as many of the basic principles which govern the growth of the primary candidates as possible. With such basic information in hand, the possibility of mistakes will be minimized. This is the rationale that has been employed in the study of algal physiology and biochemistry in this laboratory.

The aim of this team has been directed to the following questions. First, what are the characteristics of the growth of Chlorella in continuous, recycled sterile cultures? How do these characteristics change with changes in light, culture density, and nutrition? Second, what is the nature of the algal product of growth under different culture conditions--especially as it relates to certain components of the algal cell likely to be of significance in nutrition? Third, what can be learned about the effects of differing wavelengths on the characteristics of growth of the organism? In approaching these problems, it has been necessary to probe deeply into the physiology of the cell. The details of the comparatively well known human physiology make the difference between

survival and peak performance of man. The details of the physiology of whatever organisms is employed in bioregeneration will make the difference between final success or failure of the system.

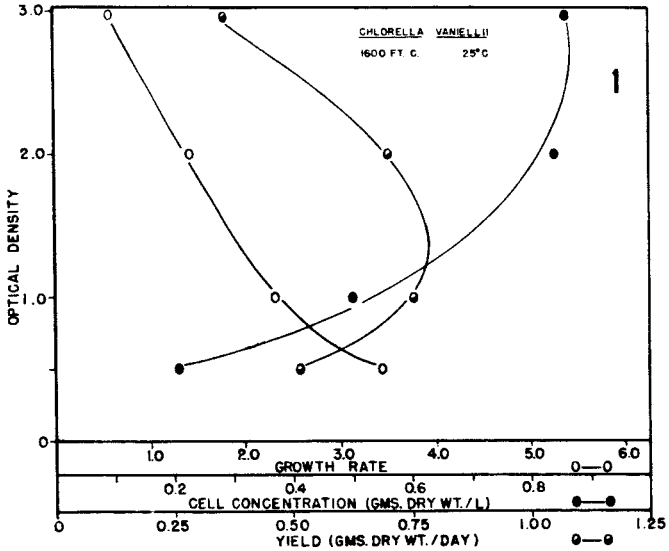
Recycled Cultures

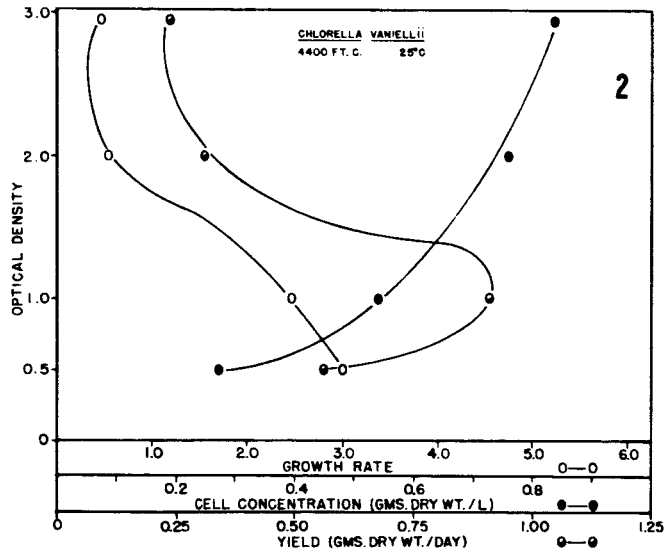
During studies designed to give information concerning the growth of algae at varying cell concentrations and light intensities, it became necessary to develop a device which would hold a cell population constant in a constant volume of solution by harvesting the increment automatically. Such a device was constructed and is now called the "Recyclostat". This apparatus has been engineered for continuous sterile culture of algae or other microorganism. It provides for a continuous record of growth, automatic harvesting to refrigerated disks, cell population control at any density, variable light intensity and quality, temperature control, and sampling and injection ports for cells and media. The device has been diagramed and described in detail in the last two progress reports and in NASA publication SP-70.

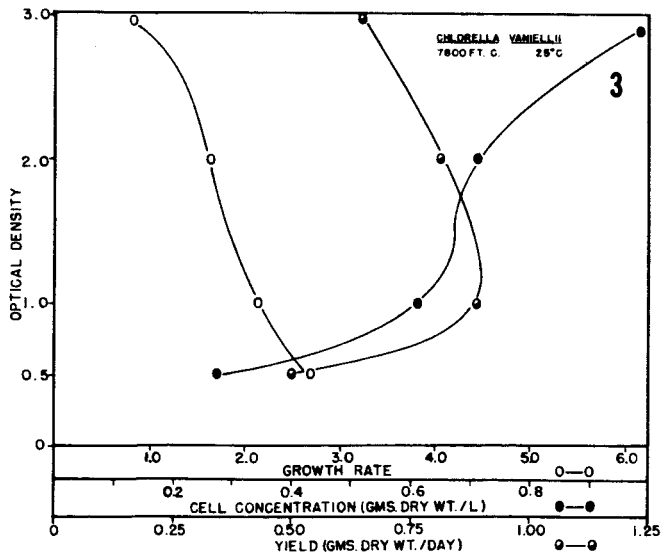
The questions that have been asked, and for which the Recyclostat was built to answer, is what is the optimal concentration of cells and at what light intensity and temperature should they be grown to give an optimal yield. The answer to this question has been sought in the framework of long term experiments of many days which do not produce misleading data.

Experiments have been completed during the current report period which show the yields expected from a typical species of Chlorella, Chlorella vannielii. Figures 1.1 - 1.3 show the responses of continuous cultures

Figures 1.1 - 1.3 A comparison of the yields, growth rates, and dry weights per liter of culture of Chlorella vannielii Shihira and Krauss grown sterilely at 1600 fc (1), 4400 fc (2), and 7800 fc (3) in the Recyclostat at 25° C.


CHLORELLA VANIELLII
1600 FT. C. 25°C
1
OPTICAL DENSITY
3.0
2.0
1.0
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (GMS. DRY WT./L)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (GMS. DRY WT./DAY)
●—●


CHLORELLA VANIELLII
4400 FT. C. 25°C
2
OPTICAL DENSITY
3.0
2.0
1.0
0.5
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (GMS. DRY WT./L)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (GMS. DRY WT./DAY)
●—●


CHLORELLA VANIELLII
7600 FT. C. 25°C
3
OPTICAL DENSITY
3.0
2.0
1.0
0.5
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (GMS. DRY WT./L)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (GMS. DRY WT./DAY)
●—●

grown under three different light intensities at four cell concentrations for C. vannielii. The response is measured in terms of growth rate and yield. The curves in Figure 1.1 illustrate the normal response of cells to changes in population density. As the density of the culture increases, the growth rate decreases; however, the yield increases to a point and then declines again. In managing a culture for optimal yield, it is necessary to determine at just what cell concentration that yield is achieved. The determination of this point is further complicated by the fact that cell densities do not change in a linear fashion with the changing absorbance of the culture--and it is absorbance (optical density) that is monitored by the photodiode in the Recyclostat. In this organism the cells become lighter per unit of absorbance as the population of the culture is increased. Reference to Figures 1.1 - 1.3 demonstrates that the shift in this parameter varies with the light intensity employed.

The optimum yield for Chlorella vannielii at the light intensities and cell populations examined is approximately 0.9 gram dry weight/liter/day. At that yield the cells are growing at 80% of their maximum possible laboratory growth rate--an efficiency that is quite respectable considering the duration of the experiments and further indicates the maximum yield that can be expected, assuming 100% of the maximum known growth rate.

The shape of the curves indicate that no significant gains in the yield can be achieved by increasing population densities inasmuch as the growth rate, and consequently the yield, drops dramatically. However, it should be remembered that any increases in the surface/volume ratio will improve this situation. The curves do indicate the limits of the improvement that engineering might accomplish. However, it is known that within

limits the cells do perform differently as the geometry of the vessel and its illumination change.

In order to develop a similar comparison with the high temperature Chlorella sorokiniana, a parallel set of experiments was run (Figures 2.1 - 2.3). As expected at the lower light intensities, and at the fairly dense population, both rate and yield were low. Even at 7,800 foot candles the rate and yield were less than for the low temperature strain. However, there is a curious shift in the yield curve at the high light intensities. Instead of declining with increasing cell density, the maximum yield is at the highest cell population tested (Fig. 2.3). This skewing of the curve indicates that the cells of this species may perform in an exceptional fashion at greater light intensities and population densities than any of our previous experiments would have led us to predict.

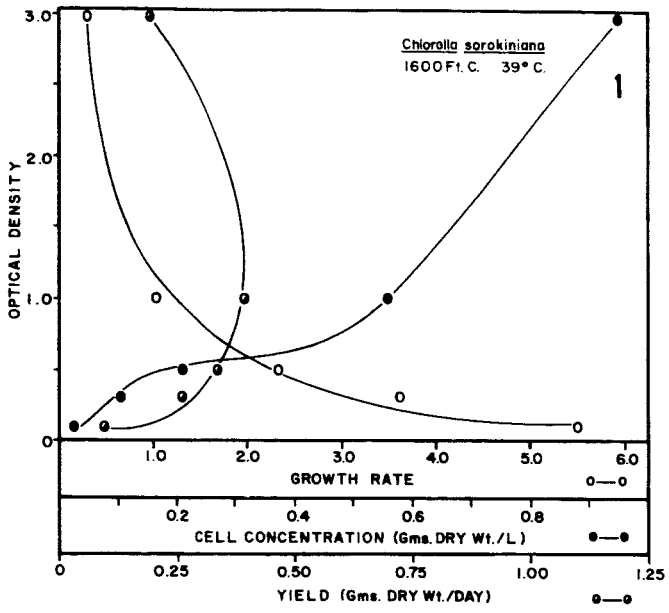
At present a new culture apparatus, designed to give definite data on performance at exceptionally high light intensities and cell population, is being built. With this apparatus the performance of cultures will be examined to determine if the yield curve continues to rise above cell densities of 1.0 gram/liter/dry weight.

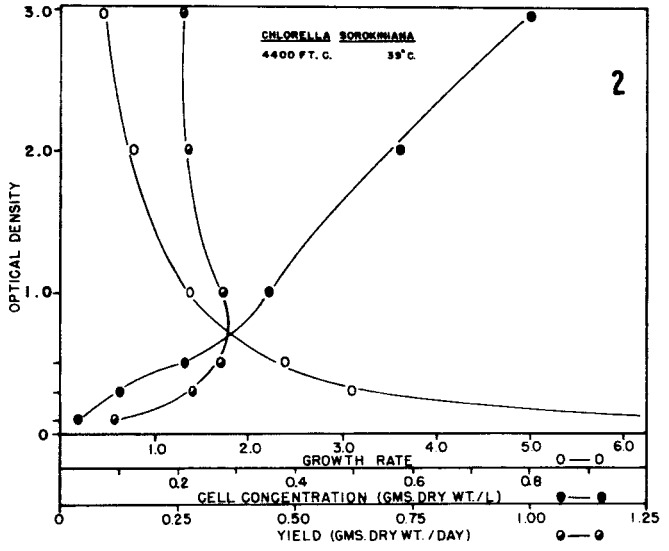
Experimental Gas Mixtures

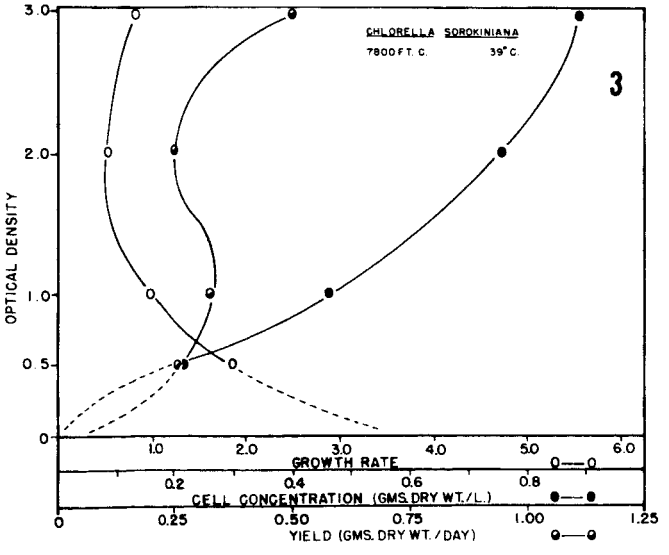
In view of the possible space use of mixtures of gases other than that normally encountered on earth, exotic mixtures which might be included in space vehicles were examined.

Investigations of the effects of substitution of helium for nitrogen and of helium for both nitrogen and oxygen were performed. Observations were made on:

Figures 2.1 - 2.3 A comparison of the yields, growth rates, and dry weights per liter of culture of Chlorella sorokiniana Shihira and Krauss grown sterilely at 1600 fc (1), 4400 fc (2), and 7800 fc (3) in the Recyclostat at 39° C.


Chlorella sorokiniana
1600 Ft. C. 39° C.
1
OPTICAL DENSITY
3.0
2.0
1.0
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (Gms. DRY Wt./L)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (Gms. DRY Wt./DAY)
●—●


CHLORELLA SOROKINIANA
4400 FT. C. 39°C.
2
OPTICAL DENSITY
3.0
2.0
1.0
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (GMS. DRY WT./L)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (GMS. DRY WT. / DAY)
●—●


CHLORELLA SOROKINIANA
7800 FT. C. 39°C.
3
OPTICAL DENSITY
3.0
2.0
1.0
0.5
0
1.0 2.0 3.0 4.0 5.0 6.0
GROWTH RATE
0—0
0.2 0.4 0.6 0.8
CELL CONCENTRATION (GMS. DRY WT./L.)
●—●
0 0.25 0.50 0.75 1.00 1.25
YIELD (GMS. DRY WT. / DAY)
●—●

1. The effect of the substitution of nitrogen for oxygen, that is, 95% nitrogen-5% carbon dioxide as compared with 95% atmospheric air-5% CO_2 gas mixture.
2. The inclusion of the high-temperature strain--1-9-30.
3. The extension of the range of temperature and light intensity.

The results indicate that a substitution of helium for nitrogen, that is a gas mixture consisting of 75% helium, 20% oxygen, and 5% carbon dioxide had no significant effect on algal growth when compared with standard conditions--95% atmospheric air and 5% CO_2. Of 21 experiments conducted in helium-oxygen-carbon dioxide atmospheres, positive differences were observed in 11 experiments and negative differences in 10 experiments. The average growth rate for all experiments conducted under various temperature and illuminance conditions for the helium-oxygen-CO_2 atmosphere constituted 99.5% of the average rate under the standard conditions (95% atmospheric air-5% CO_2).

A complete elimination of both nitrogen and oxygen by substitution of helium for both of them generally had a favorable effect on algal growth. The average growth rate in helium-CO_2 atmosphere calculated for 67 experiments conducted under different temperature and illuminance conditions constituted 110.1% of the rate in atmospheric air-5% carbon dioxide.

A substitution of nitrogen for oxygen, that is, a 95% nitrogen-5% CO_2 atmosphere, also proved to be beneficial. The average growth rate in the 95% nitrogen-5% CO_2 atmosphere calculated for 37 experiments conducted under different temperature and illuminance conditions constituted 115.1%

of the average rate in 95% atmospheric air and 5% carbon dioxide.

It has been thus established that substitution of helium for nitrogen generally had no effect on algal growth. Elimination of oxygen from the influent air by substituting nitrogen for oxygen, or by substituting helium for both nitrogen and oxygen, had a significant positive effect. Examination of experimental data indicates that the beneficial effect of the exclusion of oxygen from the influent air increases with the decrease in temperature and at a given temperature with the increase in light intensity. This is illustrated for the strain 7-11-05 studied at 20^{o}. At 310 foot candles there is a significant increase in the growth rates in helium (131.2% of control) and nitrogen (127.4% of control) (Fig. 3). As the illuminance is increased to 1600 foot candles, the growth rate at this temperature and in atmospheric air wanes and eventually the cells bleach (Fig. 4). Under the same conditions, growth rates in helium and nitrogen atmospheres (Fig. 4) are steady though lower than at 310 foot candles.

The favorable effect of elimination of oxygen from the influent air could be due to the suppression of photooxidation, a process particularly active under combination of low temperature and high light intensity. It has been reported that oxygen enhances photorespiration and inhibits photosynthesis. It has been also shown that low temperature and high light intensity inhibits cell division. Thus, the effect of the removal of oxygen on algal growth is complex and different processes such as photorespiration, photosynthesis, and cell division may be involved.

Figure 3 Growth of Chlorella sorokiniana at 20° and 310 foot candles.

Triangles -- in 95% nitrogen and 5% carbon dioxide

Circles -- in 95% helium and 5% carbon dioxide

Squares -- in 95% atmospheric air and 5% carbon dioxide

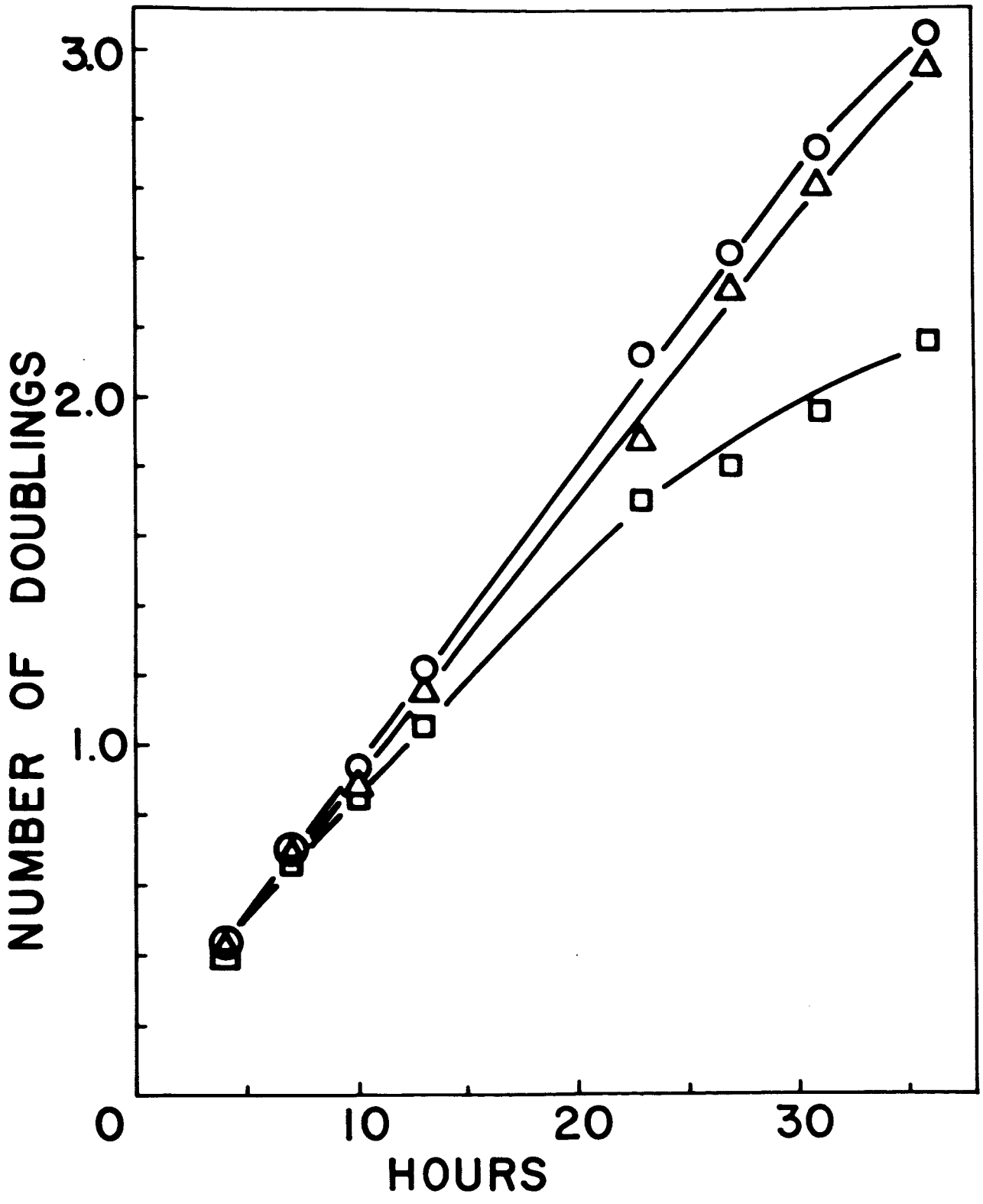
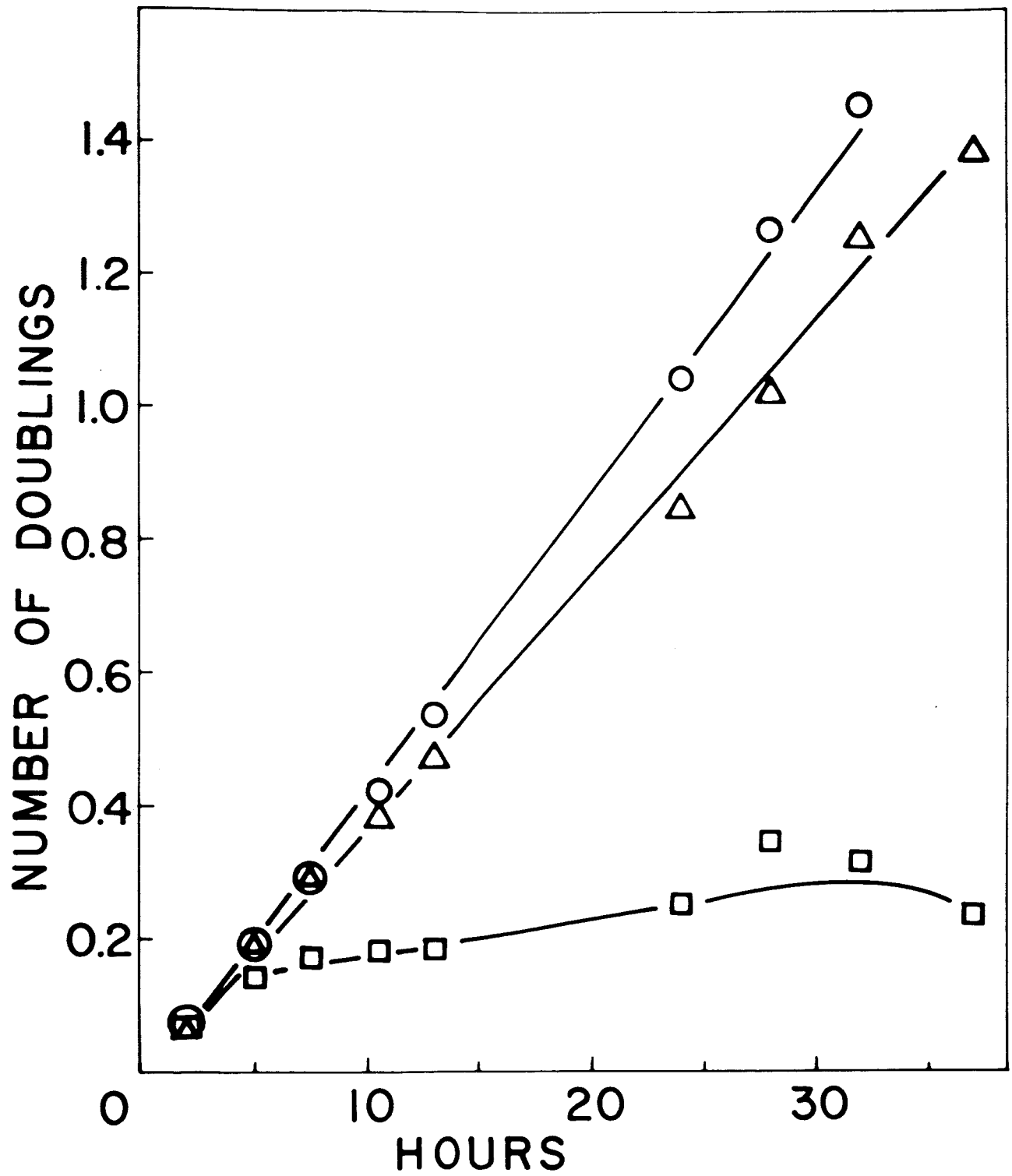

3.0
2.0
1.0
0
10
20
30
NUMBER OF DOUBLINGS
HOURS

Figure 4 Growth of Chlorella sorokiniana at 20° and 1600 foot candles.

Triangles -- in 95% nitrogen and 5% carbon dioxide

Circles -- in 95% helium and 5% carbon dioxide

Squares -- in 95% atmospheric air and 5% carbon dioxide


NUMBER OF DOUBLINGS
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
HOURS

Adaption and Mutation of Chlorella Species

In a continuing effort to determine the genetic stability of Chlorella, experiments have been performed on a species which is characterized by a marked sensitivity to mannose. With this physiological characteristic as a marker, we set out to determine whether the organism could lose this characteristic or if the toxicity of mannose could be lessened. Growth of a clone of Chlorella infusionum var. acetophila in the light was completely inhibited in 1.5 (10^{-2}) M D-(+)-mannose. Most cells of the culture were bleached and lysed over a period of 6 days; however a small number of cells retained a normal green color and after an additional period of several days a normal growth rate in the presence of mannose was observed. The selected strain did not utilize mannose as a substrate for heterotrophic growth. In contrast to the original clone, the surviving cells failed to utilize glucose as a substrate for growth in darkness. Incubation of this culture in 6.0 (10^{-3}) M glucose and darkness for 4 - 6 weeks eventually produced a culture that utilized glucose and was inhibited in the presence of mannose. Results similar to those with mannose were obtained with mannose derivatives in which the C-2 configuration was different, e.g., D-mannosamine, 2-deoxy-D-glucose, and glucosamine. Manometric studies showed glucose-induced O_2 uptake to be reduced in normal cells that had been exposed to mannose for 16 hours and then washed. Endogenous rates were unaffected. In order to account for the above differences between the normal culture and the selected strain, standard samples of each were exposed to glucose-U-^{14}C, mannose-U-^{14}C, and glucosamine-1-^{14}C in darkness. Normal cells exposed to glucose-U-^{14}C

took up 80% of the radioactivity in 4 hours, whereas the selected cell took up 3.2% in 16 hours. The accumulation of mannose-U-^{14}C by the normal cells was 20% and by the selected strain 3.1%--both in 16 hours. The uptake of glucosamine-1-^{14}C was equal for both types of cells.

These studies indicate that there can be changes in the physiology which can be attributed to mutation or adaption. Efforts are now concentrated on determining the nature of this physiological shift. Of especial interest is the determination of the enzymes that may have been lost or added as the result of the adaption. Such studies are basic to an estimate of the nature and degree of genetic shift in microorganisms employed to support man.

Organic Composition of Chlorella

Eight species of Chlorella were grown autotrophically and heterotrophically and their fatty acids, hydrocarbons, and sterols analyzed by gas-liquid chromatography. Of the eight Chlorella species studied, four contained $\Delta^{5,7}$-sterols, two had Δ^{7}-sterols, and two had Δ^{5}-sterols. The identity of sterols present did not change when the cells were changed from autotrophic conditions. One can distinguish between several of the Chlorella species on the basis of fatty acid content. The ratios of saturated/unsaturated fatty acids increased when the cells were grown in heterotrophic conditions. Fatty acid content changed only slightly as the cultures aged. Palmitic acid was present in large amounts in all species under all conditions studied. Very little difference was found in the hydrocarbon fractions of autotrophically grown Chlorella cells. However, significant changes occurred when the cells were grown heterotrophically. Significant changes also occurred in the hydrocarbon content of dense, aged cultures.

Publications

Karlander, E. P. and R. W. Krauss. 1966. Responses of heterotrophic cultures of Chlorella vulgaris Beyerinck to darkness and light. I. Pigment and pH changes. Plant Physiology 41(1): 1-6.

Karlander, E. P. and R. W. Krauss. 1966. Responses of heterotrophic cultures of Chlorella vulgaris Beyerinck to darkness and light. II. Action spectrum for and mechanism of the light requirement for heterotrophic growth. Plant Physiology. 41(1): 7-14.

3. Patterson, G. W., Sterols of Chlorella. II. The occurrence of an unusual sterol mixture in Chlorella vulgaris. Steroids (In press).

4. Patterson, G. W. and R. W. Krauss. 1965. Hydrocarbons and sterols from Chlorella. Plant Physiol. 40 suppl. XVII.

5. Sorokin, C., The secretory activity of algal cells in buffered media. Protoplasma. (In press).

6. Sorokin, C., Primary cellular aging. Proceedings of the VII International Congress of Gerontology. (In press).

PRIMARY CELLULAR AGING

By

Constantine Sorokin

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College Park, Md.

Cellular aging comprises deterioration processes occurring in the course of the development of an individual cell. In multicellular systems, these deterioration processes can be conceivably due to the effects of cell environment including mutual effects of cells on each other. Competition between cells for nutrients and space, accumulation of waste products of metabolism, and, in general, pathological condition of the organism as a whole are among well recognized factors which tend to deprive individual cells of optimal conditions essential for the full expression of potentialities with which a cell is endowed at the inception of its development.

Aging cellular processes, as caused by the deterioration in cell environment, are, however, secondary to the primary aging processes as due to the intrinsic determinant properties of the cell. Difficulties of demonstration of primary aging in cells of multicellular organisms led several authors to the denial of cellular aging as due to the intrinsic determinant properties of cells and to the assertion that cell deterioration in higher organisms is entirely due to the supracellular effects (Minot 1907, Pearl 1921). An ungrounded corollary from the above assertion is that cellular aging is absent in unicellular organisms (Strehler 1962).

Studies on primary cellular aging can be facilitated if factors known to cause secondary aging are eliminated or reduced to a possible minimum. This

is feasible in cultures of free-floating cells kept under optimal conditions and frequently transferred into a fresh nutrient medium or maintained in a so-called continuous dilution growth device. Observations on metabolic activity in the course of cell development can be done either on individual cells or on batches of synchronized cells (Zeuthen 1964). Due to difficulties of handling individual cells and to exceedingly high precision required to measure biochemical and physiological characteristics of a single cell, the single-cell technique usually lacks persuasiveness of observations made on batches of synchronized cells.

Metabolic activity has been shown to undergo rhythmic fluctuations concomitant with the developmental changes during the growth-division cell cycle (Sorokin 1964). In the majority of observations, a peak in metabolic activity coincided with the earlier phases of the cell cycle and the lowest activity with the end of the developmental cycle, that is, with the period shortly before and/or during cell division.

The decline in respiration rate in the course of cell development has been demonstrated for unicellular (Sorokin and Myers 1957) and colonial (Neeb 1952) algae, protozoa (James 1965, Zeuthen and Scherbaum 1954), microsporocytes and microspores of higher plants (Erickson 1947, Stern and Kirk 1948), animal eggs (Holter and Zeuthen 1957), and mammalian spermatozoa (Shettles 1939). Both the endogenous respiration and that in the presence of externally supplied substrates was often affected (Sorokin and Myers 1957).

In photoautotrophic organisms, the decline in the rate of photosynthesis in the course of cell development was shown for a number of strains and species of the unicellular alga Chlorella (Nihei, et al. 1954, Pirson and Lorenzen 1958, Sorokin 1957, 1960a) and Scenedesmus (Bongers 1958) and for the colonial alga Hydrodictyon (Neeb 1952). Older cells have lower quantum efficiency

(Sorokin and Krauss 1961) and lower saturating light intensity. They are more sensitive to the deleterious effects of strong light (Sorokin 1960b). Lower photosynthetic activity of older cells at light saturation implies lower activity of enzymatic portion of the photosynthetic mechanism (Sorokin 1963a). Higher fluorescence intensity demonstrated for older cells (Döhler 1963) may indicate lower efficiency of the pigment moiety of the photosynthetic machinery involved in the primary photochemical act.

Growth studies indicated that even under most optimal external conditions, the capacity for organic synthesis declines in older cells. This was shown for synchronized cultures of the green alga Chlorella (Pirson and Kowallik 1960, Sorokin 1963b), and Scenedesmus (Müller 1961), for the protozoan Tetrahymena (Hamburger and Zeuthen 1960) and for the single cells of Amoeba (Prescott 1955, Satir and Zeuthen 1961), as well as for bacterial cultures of Streptococcus faecalis (Mitchison 1961). Older cells are more dependent in their synthetic capacity on the external supply of nitrogen, while younger cells, in the absence of external nitrogen, possess a high ability to reuse nitrogenous intermediates from their metabolic pools (Sorokin 1963b).

A special place in the biochemical dissection of the growth-division cell cycle is occupied by studies of the time course of synthesis of nucleic acids and proteins. A consensus has been reached that in dividing cells of higher animals and plants, synthesis of deoxyribonucleic acid is a discontinuous process (Lark 1963). It largely occurs in the interphase cells during the so-called S period. During the preceding (G_1 period) and succeeding (G_2 period) portions of the interphase, as well as during mitosis (M period), there is no DNA synthesis. A great assemblage of data for bacteria (Barner and Cohen 1955, Burns 1959), algae (Iwamura, et al. 1955), protozoa (Prescott 1960)

and HeLa (Terasima and Tolmach 1963) cells generally conform to the above pattern. Few dissensions (Abbo and Pardee 1960, Cummings 1965) are probably due to the specificity of the employed experimental technique, particularly to not allowing sufficient time necessary to observe the decline in DNA synthesis in older cells.

Far less agreement has been reached in regard to the timing of the ribonucleic acid and protein synthesis during the cell cycle. A number of investigators demonstrated that, in several organisms and cell types, RNA and protein synthesis is a rhythmic process, with maxima usually distinct and generally following that of the peak of the DNA synthesis. Other authors prefer to consider RNA and protein synthesis as a continuous process, occurring at a steady rate over the whole growth-division cycle.

The controversy seems to be dissolving now with the advancement of understanding that, though protein synthesis as a whole in some organisms and under certain conditions may appear as a steady process, the synthesis of particular proteins--specifically of several enzymes--is rhythmic (Sussman 1965). If so, then the continuous nature of the total protein synthesis is only apparent and actually due to merging of the rates of a multitude of synthetic (as well as catabolic) reactions, each with specific maximum and minimum, during the cell cycle. The evidence is accumulating that, for several enzymes, the activity fluctuates in the course of cell development (Cole and Schmidt 1964, Gorman, et al. 1964, Masters, et al. 1964). The attempts are made to trace these fluctuations in the enzyme activity to the rhythmicity in the enzyme synthesis.

A number of other cellular characteristics have been shown to fluctuate in the course of cell development. Of particular interest are changes in sensitivity to adverse conditions during the life cycle of cells. This has

been shown for a number of organisms and for mammalian cells in regard to the deleterious effects of high (Scherbaum 1963) and low (Koch 1959) temperatures, and visible (Sorokin 1963b), UV (Sasa 1961), and X-ray (Tolmach 1963) radiation. A higher sensitivity usually coincides with later stages of cell development.

An overwhelming evidence indicates that microbial cells, as well as several types of dividing cells of higher animals and plants, undergo regular cyclic changes of higher and lower metabolic activity, each cycle of activity corresponding to one growth-division cycle. The amplitude of fluctuations and timing during the cell cycle may differ for particular cellular characteristics. Both the amplitude and timing depend on the hereditary constitution of the organism and environmental conditions during and prior to observations.

The lowest activity, in most cases, usually coincides with the end of the growth-division cycle. In a line of indefinitely dividing cells, the activity must come during the subsequent portion of the life cycle to the original level to make the next and all successive cycles possible. In some kinds of cells, the complete restoration of cell capabilities in the process of cell division is lacking. The imperfections of restoration mechanisms make cells of succeeding generations to be endowed with lesser and lesser potentialities, with the result that eventually the cell line comes to an end.

The declining portion of the metabolic activity during each growth-division cycle is identified as cellular aging. If cell division in a normally dividing cell is delayed or interrupted, the aging processes characteristic of the normal growth-division cycle continue and intensify until the cell eventually divides or dies. In cells of higher organisms, which differentiate to become specialized cells, the division activity is interrupted.

The nature of control mechanisms which direct one of the two cells originating from the same stem cell to stop dividing and to begin differentiating is obscure (Holtzer 1963). Even more obscure is the nature of aging processes in differentiating and differentiated cells. Do these processes continue in differentiating cells or stop, or are they replaced by other aging processes specific to specialized cells? At present, more is known about the course of aging processes in nondifferentiating cells than about those in specialized cells.

Speculations concerning the nature of rhythmicity in biochemical and metabolic events during the cell division cycle include assumptions as to the accumulation of poisons and inhibitors, feedback mechanisms, immobilization due to molecular cross-linkages, and dependence of timing of enzyme activity on the timing of the duplication of specific genes responsible for the synthesis of specific enzymes. The relevance of these hypotheses to cellular aging is uncertain and may be clarified only in intensive studies of aging as distinctly due to the intrinsic determinant properties of cells or to supracellular effects.

REFERENCES

Abbo, F. E. and A. B. Pardee (1960) Biochim. Biophys. Acta 39: 478.

Barner, H. D. and S. S. Cohen (1955) Fed. Proc. 14: 177.

Bongers, L. H. J. (1958) Meded. Landbouwhogeschool Wageningen 58: 1

Burns, V. W. (1959) Science 129: 566.

Cole, F. E. and R. R. Schmidt (1964) Biochim. Biophys. Acta 90: 616.

Cummings, D. J. (1965) Biochim. Biophys. Acta 95: 341.

Dohler, G. (1963) Planta 60: 158.

Erickson, R. O. (1947) Nature 159: 275.

Gorman, J., P. Taruo, M. LaBerge, and H. O. Halvorson (1964) Biochem. Biophys. Res. Commun. 15: 43.

Hamburger, K. and E. Zeuthen (1960). C. R. trav. Lab. Carlsberg 32: 1.

Holter, H. and E. Zeuthen (1957) Pubbl. Staz. Zool. Napoli 29: 285.

Holtzer, H. (1963) in General Physiology of Cell Specialization (D. Mazia and A. Tyler, eds.) McGraw-Hill Book Company, New York, p. 80-90.

Iwamura, T., E. Hase, Y. Morimura, and H. Tamiya (1955) A. I. Virtanen Homage Volume, Biochemistry of Nitrogen.

James, T. W. (1965) Exptl. Cell Res. 38: 439.

Koch, A. L. (1959) J. Bacteriol. 77: 623.

Lark, K. G. (1963) in Molecular Genetics (J. H. Taylor, ed.) Academic Press, New York, p. 153-200.

Masters, M., P. L. Kuempel, and A. B. Pardee (1964) Biochem. Biophys. Res. Commun. 15: 38.

Minot, C. S. (1907) Popular Sci. Monthly 70: 481; 71: 97, 509.

Mitchison, J. M. (1961) Exptl. Cell Res. 22: 208.

Müller, H.-M. (1961) Planta 56: 555.

Neeb, O. (1952) Flora 139: 39.

Nihei, T., T. Sasa, S. Miyachi, K. Suzuki, and H. Tamiya (1954) Arch. Mikrobiol. 21: 155.

Pearl, R. (1921) Sci. Monthly 12: 193.

Pirson, A. and W. Kowallik (1960) Naturwissenschaften 47: 476.

Pirson, A. und H. Lorenzen (1958) Naturwissenschaften 45: 497.

Prescott, D. M. (1955) Exptl. Cell Res. 9: 328.

Prescott, D. M. (1960) Exptl. Cell Res. 19: 228.

Sasa, T. (1961) Plant Cell Physiol. 2: 253.

Satir, P. and E. Zeuthen (1961) C. R. trav. Lab. Carlsberg 32: 241.

Scherbaum, O. H. (1963) in The Cell in Mitosis (L. Levine, ed.) Academic Press, New York, p. 125-151.

Shettles, L. B. (1939) Anat. Rec. 75: 145.

Sorokin, C. (1957) Physiol. Plant. 10: 659.

Sorokin, C. (1960a) Physiol. Plant. 13: 20.

Sorokin, C. (1960b) Arch. Mikrobiol. 37: 151.

Sorokin, C. (1963a) Nat. Acad. Sciences U. S., Publ. 1145: 742.

Sorokin, C. (1963b) Arch. Mikrobiol. 46: 29.

Sorokin, C. (1964) Experientia 20: 353.

Sorokin, C. and R. W. Krauss (1961) Biochim. Biophys. Acta 48: 314.

Sorokin, C. and J. Myers (1957) J. Gen. Physiol. 40: 579.

Stern, H. and P. L. Kirk (1948) J. Gen. Physiol. 31: 243.

Strehler, B. L. (1962) Time, Cells and Aging, Academic Press, New York.

Sussman, M. (1965) Ann. Rev. Microbiol. 19: 59.

Terasima, T. and L. J. Tolmach (1963) Science 140: 490.

Tolmach, L. J. (1963) J. Cellular Comparative Physiol. 62 (Suppl. I, Part II): 141.

Zeuthen, E. (ed.) (1964) Synchrony in Cell Division and Growth, Interscience Publishers, New York.

Zeuthen, E. and O. Scherbaum (1954) in Recent Developments in Cell Physiology (J. A. Kitching, ed.) Academic Press, New York.

STEROLS OF CHLORELLA. II. THE OCCURRENCE OF AN UNUSUAL STEROL MIXTURE IN CHLORELLA VULGARIS

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The first identification of a sterol in Chlorella was by Klosty and Bergmann (1) in 1952 when they isolated ergosterol from Chlorella pyrenoidosa. More recently, Otsuka (2) identified ergosterol and a Δ^5 sterol in Chlorella ellipsoidea. Ergosterol was the principal component of the mixture. Recent work in this laboratory has been centered about the identification of sterols from other species of Chlorella. A mixture of the Δ^5 sterols, poriferasterol, clionasterol, and 22-dihydrobrassicasterol was found (3) in C. ellipsoidea and C. saccharophila, but no ergosterol was detected. Chlorella vulgaris was shown to contain chondrillasterol as its principal sterol component, and although two other sterols were present in significant quantities, they were not isolated. This paper describes the isolation and identification of each of the sterols occurring in C. vulgaris.

Cells of Chlorella vulgaris Beyer., Emerson's strain, were grown heterotrophically on basal inorganic medium containing 0.5% glucose in fifteen-liter carboys equipped with bubbling tubes for

air (3). The cells were harvested in a Sharples Super Centrifuge and freeze-dried before extraction. Average yield was 3 grams dry weight per liter. Lipid material was extracted from the cells with acetone in a soxhlet apparatus, saponified under nitrogen, and the non-saponifiable matter extracted with ether in a liquid-liquid extraction apparatus. The non-saponifiable lipid was fractionated as described by Heftmann (4) et al. on Woelm Grade III neutral alumina. The fraction containing sterols was acetylated and rechromatographed under the same conditions described above. The fraction containing the mixture of sterol acetates was subjected to column chromatography on Anasil B which was added to the 3 cm x 40 cm column in a slurry of n-hexane. The sterol acetates were added to the column in a minimum amount of n-hexane and eluted with 2% ether in n-hexane. Gas chromatography was used to assay the 15-ml fractions which were collected. The three sterols isolated were named sterol I, sterol II, and sterol III in the order of their elution from the Anasil column.

Nearly 100 mg of the sterol mixture was obtained from 100 g of C. vulgaris. The approximate composition of the sterol mixture was sterol I, 10%; sterol II, 25%; and sterol III, 65%. Sterol III has previously been identified as 24β-ethyl-$\Delta^{7,22}$ cholestadienol or chondrillasterol, a sterol which also occurs in another unicellular alga, Scenedesmus obliquus (5). Sterols I and II also gave rapid Liebermann-Burchard reactions indicative of a Δ^{7} double bond and failed to show the typical ultraviolet absorption spectrum

of a $\Delta^{5,7}$ sterol. Thus these sterols, like chondrillasterol, appear to be Δ^{7} sterols. The infrared spectrum of sterols I and II were essentially identical. They were also identical to the spectrum of chondrillasterol except for the absence of the strong band at 10.3μ. The presence of this absorption band indicates the presence of a trans double bond at C-22 (6).

Gas chromatographic retention times (Table I) indicate that sterol I is a Δ^{7} C-29 sterol and sterol II is a Δ^{7} C-28 sterol since the GLC retention times of sterol I coincide with those of 24α-ethyl-Δ^{7} cholestenol (Δ^{7} stigmastenol) and the retention times of sterol II coincide with those of 24β-methyl Δ^{7} cholestenol (Δ^{7} ergostenol).

Table I. GLC relative retention times of Chlorella sterols and certain known sterols

Compound	Relative Retention Time[a] SE-30[b]	QF-1[c]	NGS[d]
Chondrillasterol	3.06	4.00	11.8
Δ^{7} Stigmastenol	3.47	4.48	14.1
Sterol I	3.47	4.46	14.2
Δ^{7} Ergostenol	2.82	3.80	11.0
Sterol II	2.82	3.80	11.1

a/ Relative to cholestane.

b/ Column 6 ft x 3.4 mm ID, 2% SE-30 on 100-140 mesh Gas-chrom Q, 20 psi, 236°C, cholestane time 7 minutes.

c/ Column 6 ft x 3.4 mm ID, 1% QF-1 on 100-140 mesh Gas-chrom P, 20 psi, 236°C, cholestane time 3 minutes.

d/ Column 6 ft x 3.4 mm ID, 1% NGS on 100-140 mesh Gas-chrom P, 20 psi, 216°C, cholestane time 4 minutes.

In Table II, melting point and optical rotation data of Chlorella sterols are compared with values obtained from the literature for Δ^7 ergostenol (7) (also called fungisterol) and Δ^7 stigmastenol (8). The values obtained for sterol II are essentially identical to those found in the literature for Δ^7 ergostenol. Agreement is not as good in the comparison of melting points of sterol I and Δ^7 stigmastenol.

Table II. Melting point and optical rotation data for Chlorella sterols and certain other sterols

Sterol	M.P. Sterol	M.P. Acetate	Optical Rotation Sterol
Chondrillasterol	169-70	174-5	± 0
Δ^7 Stigmastenol	145	157	+ 8
Sterol I	140-2	161-163	---
Δ^7 Ergostenol	148	160	0
Sterol II	148-9	159-161	- 2

Although the melting points for Δ^7 stigmastenol and sterol I are not conclusively different, the following evidence suggests that these compounds are not identical but are isomeric at C-24. Chondrillasterol and Δ^7 ergostenol have β-ethyl and β-methyl groups, respectively, as the asymmetric carbon #24 of the sterol side chain. Sterol I also has an ethyl group at C-24 which would be expected to originate in the same manner as the ethyl group in chondrillasterol. In addition, it is of interest to note that all other sterols isolated in pure form from algae have also been 24β sterols.

except fucosterol and cholesterol which are not asymmetric at C-24. Therefore, the unknown sterol appears to be the 24β isomer of Δ^7 stigmastenol, 24β-ethyl Δ^7 cholestenol (Δ^7 poriferastenol). Gas chromatography cannot distinguish between isomers of this type (9).

This is the first reported isolation of Δ^7 poriferastenol from natural sources. This is also the first reported isolation of Δ^7 ergostenol from a green plant.

The sterols of Chlorella vulgaris are identical to those previously isolated from Chlorella ellipsoidea except that C. vulgaris contains Δ^7 sterols while those of C. ellipsoidea are Δ^5 sterols. Chlorella vulgaris differs from C. ellipsoidea in its apparent inability to convert the steroid Δ^7 double bond to the Δ^5 double bond as most plants do.

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REFERENCES

1. Klosty, M. and Bergmann, W., J. AM. CHEM. SOC. 74, 1601 (1952).

2. Otsuka, H., PLANT AND CELL PHYSIOLOGY 4, 293-297 (1963).

3. Patterson, G. W., and Krauss, R. W., PLANT AND CELL PHYSIOLOGY 6, 211-220 (1965).

4. Heftmann, E., Wright, B., and Liddel, G., ARCH. BIOCHEM. BIOPHYS. 91, 266-270 (1960).

5. Bergmann, W., and Feeney, R. J., J. ORG. CHEM. 15, 812-814 (1950).

6. Jones, R. N., J. AM. CHEM. SOC. 72, 5322 (1950).

7. Bergmann, W., COMPARATIVE BIOCHEMISTRY, VOL. III, Editors, M. Florkin and H. Mason, Academic Press, New York (1960).

8. Idler, D. R., Kandutsch, A. A., and Baumann, C. A., J. AM. CHEM. SOC. 75, 4325 (1953).

9. Thompson, Malcolm J., Robbins, William E., and Baker, Graeme L., STEROIDS 2, 505-512 (1963).