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ALGAL CULTURE

FROM LABORATORY TO PILOT PLANT

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FOREWORD

The Carnegie Institution's project on the mass culture of algae is a collateral development or offshoot of its program of research on photosynthesis. One part of this program was concerned with the nature of the products which the plant makes in the photosynthetic process [200]. Several widely different species of plants were used in these investigations, but particular attention was paid to the unicellular alga <u>Chlorella pyrenoidosa</u> [201], because of the broad range of environmental conditions under which it can grow, and because this organism has been extensively used as a subject of study of many other aspects of the photosynthetic problem.

The experience gained in the culture of Chloreila was applied to a wartime investigation, namely, the search for an antibacterial substance that might be isolated from the culture solution in which Chlorella was grown [202]. It turned out that the antibiotic activity resulted from a complex photooxidation of the unsaturated fatty acids in the Chlorella itself, and that a similar reaction could be induced in fatty acids from a variety of sources [203, 204, 152].

After the war the investigation of the influence of environmental factors on the chemical composition of Chlorella was resumed. The conditions for varying the protein and fat content over wide ranges were established [151]. The nature of the lipides in the Chlorella was also investigated [91],

By the winter of 1947-1948 the possibility of growing Chlorella on a large scale for food was being considered seriously. H. A. Spoehr and Harold W. Milner prepared a preliminary analysis of the problem for publication in Carnegie Institution of Washington Year Book No. 47 [206]. The sort of development that they suggested, involving applied research and engineering, lay outside the scope of the Institution's usual activities. The Institution therefore turned over this aspect of the problem to Research Corporation of New York, continuing its own fundamental scientific, studies on photosynthesis. Research Corporation contracted with Stanford Research Institute in September 1948 for an engineering study of the factors involved in the design of a pilot plant.

The small culture unit that was constructed and operated at Stanford showed that continuous culture of Chlorella was possible; but the results were not conclusive enough for Research Corporation to feel justified in financing further experimentation. The work at Stanford Research Institute was therefore terminated in the spring of 1950.

During the latter part of 1950 the American Research and Development Corporation in Boston^ Massachusetts, became interested in the subject of algal culture and made a careful review of the economic aspects of the subject on the basis of the experiments at Stanford Research Institute. This review showed clearly that there was still much uncertainty as to whether large-scale culture was economically feasible.

It was generally agreed at that time that further work of a fundamental sort was needed, and that it was especially desirable to discover whether any new problems would be introduced by a large increase in the size of a culture unit. It was essential also to obtain larger quantities of the alga than had so far been available, so that its potential usefulness could be evaluated. In order to accomplish these purposes, the Carnegie Corporation of New York joined with the Institution in making funds available for Arthur D. Little, Inc., a consulting research and development organization in Cambridge, Massachusetts, to set up a pilot plant for the culture of Chlorella.

The staff of the Department of Plant Biology turned its attention to the problem of the factors that affect the rate of growth of Chlorella as one aspect of its program of fundamental research on photosynthesis. The Institution also sponsored laboratory investigations of algal culture at the University of Texas by Dr. Jack Myers, who spent the summer of 1951 as a visiting investigator at the Department of Plant Biology. The Institution also, in July 1951, granted a fellowship to Dr. Robert W. Krauss, of the University of Maryland, extending him support for his fundamental research on algal nutrition. In addition to this research, Dr. Krauss is collaborating with the Institution in culturing a variety of different algae.

By no means all the problems involved in algal culture have been solved; but partial answers to the principal questions have been obtained. The alga Chlorella has been grown and harvested continuously on a large scale; it has nutritive value in animal feeding; possibly it or one of the other algae contains useful chemical raw materials; and estimates have been made as to possible yields of Chlorella in commercial quantities, based on the best data at present available.

At this stage of our program it is desirable to publish a fairly complete account of the work to date, so that our accumulated experience may be conveniently available. Such an account should be an aid to all the workers in the field and especially should help any organizations that may be interested in setting up pilot plants for the culture of algae.

Most of the present monograph is made up of reports of investigations under the auspices of the Carnegie Institution of Washington. We are privileged, however, to include also accounts of some parallel investigations in other countries. It is hoped that, as a progress report of a rapidly developing field of science and engineering, this book will serve to stimulate even more interest in the subject, and will result in increased progress toward the large-scale culture of algae.

The manuscripts of the chapters originally planned for this monograph were received during the first quarter of 1952. The first chapter, which summarizes them, was also written at that time. Later that year the Carnegie Institution had the privilege of having Professor H. Tamiya as a guest worker at its Department of Plant Biology, through a fellowship supported by the Office of Naval Research of the United States Government. Professor Tamiya has prepared an account of studies of the growth of algae in mass culture that he and his colleagues had performed at the Tokugawa

Institute for Biological Research, Tokyo. A delay in the publication of the monograph made it possible to include this new material, which is presented as chapters 16 and 18. It was not possible, however, to incorporate a discussion of it in the summary. Furthermore, it was not feasible to ask the other authors to revise their respective chapters so as to include more recent results.

Active participation by other groups is essential for the ultimate success of the development of algal culture. The Carnegie Institution has felt that it had an obligation to lead the way in exploring the new field, for a discovery of great potential importance to humanity had been made through the fundamental research in one of its laboratories, and this discovery might have remained unused for many years unless further action was taken. Indeed, exactly this had happened much earlier in the case of hybrid corn. The basic knowledge concerning hybrid corn was published by George H. Shull, of the Institution's Department of Experimental Evolution, in 1909, but almost ten years went by before any effort was made to apply that knowledge for the benefit of man.

Rather than let the development of algal culture proceed by chance, the Institution has been actively carrying it on to the point where enough information is at hand to assess it fairly. At this stage we are not able to recommend any particular type of installation or to state positively the economic possibilities of this method of producing foodstuffs. The ramifications of the subject are so wide that we cannot yet be sure what form the future development may take.

The need of the world for additional sources of high-protein food is so great, especially in overpopulated areas, that serious effort in tracking down every promising lead is certainly warranted. Such great advances in technology have already come from the coupling of engineering with biology that it seems inevitable that the production of food, at least in certain areas, will eventually be carried out by "process" industries. The large-scale culture of algae may well become the first of them. In regions of the world where population is especially dense, and fertile land is limited, it is entirely possible that process-industry methods of producing food may furnish a respite from the threat of famine and so contribute toward more salutary conditions for civilized living. If algal culture can serve such a purpose, it is well worth development for that reason alone.

It is hoped that such a development may take the form ultimately of a multitude of individually owned, relatively small establishments, combining the culture of algae perhaps with utilization of the product for animal feeding on the spot. The new industry that would result would thus enter into our economic life in such a manner as not to produce disruption, but rather to strengthen individual enterprise.

Whatever form an algal culture industry takes eventually, the programs for its commercial development may need patent protection in order that venture capital may be secured for necessary risks. The Institution recognizes that it has an obligation to protect the public interest in a matter

of this sort. Accordingly, it has obtained assignments of a number of
patent applications dealing with the culture of algae.   If the development
reaches the stage where one of these patented processes is needed, it will
be made available as broadly as is consistent with the optimum public
benefit and on a reasonable basis.   Any income received by the Institution
from this source will be used either in support of further investigation for
the improvement of the process, or in connection with its regular program
of basic research.

V. Bush

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PART I

INTRODUCTION AND SUMMARY

Chapter 1

CURRENT STATUS OF THE LARGE-SCALE CULTURE OF ALGAE 1

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A

Introduction

Microbiological processes have been used by man for centuries in the preparation of certain foods and beverages; but it is only during the past half century that they have become of increasing technical importance. Not only has man learned how to prevent the spread of disease and to eliminate harmful effects from microorganisms; he has also learned how to put microorganisms to work for him on a large scale.

Beginning in 1919 with the introduction of the fermentation process for producing citric acid from sugar by means of the mold Aspergiilus niger, microorganisms have become a tool of the chemical engineer. For example, he now uses a mold to produce riboflavin (vitamin 83), & yeast to produce either ethanol or glycerol from sugars, and bacteria to produce acetone and butanol simultaneously from starch. The present monograph recounts the progress made in a current attempt to exploit still another group of microorganisms, namely, the unicellular algae.

Interest in algae as a possible source of food was stimulated by the results of an investigation at the Carnegie Institution of Washington's Department of Plant Biology dealing with the nature of the products of photosynthesis by algae. The Institution believed it desirable to find out whether the large-scale culture of algae is feasible, because of the important implications for human betterment that such a process would have.

The first attempt in this country to translate the biological requirements of algal culture into engineering specifications for a large-scale culture plant was made at Stanford Research Institute in 1948-1950 [279]. The problem was tackled at about the same time in Germany (see chapter 11). During 1951 a further advance was made through the construction and operation of a Chlorella pilot plant for the Carnegie Institution by Arthur D. Little, Inc.

The Chlorella pilot plant showed that the large-scale culture of algae is technically feasible. Nearly 100 pounds (dry weight) of Chlorella was grown and made available for investigations of its nutritive properties and its possible applications as an industrial raw material. An extrapolation

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[1] Chapters 16 and 18, on important investigations in Japan, were received after this summary was prepared. See Foreword, page iv.

of pilot-plant results suggests that an annual yield of 11^1_2 tons per acre is not an unreasonable expectation with the present technological knowledge. In view of the high protein content of dried algal cells (about 50 per cent) and their high vitamin potency, this yield seems promising enough to warrant further investigation of the problems that were not solved by the initial operation of this pilot plant.

Besides some additional small-scale experiments on factors that affect the growth rate of algae, what is needed next is a demonstration plant having an area, of about an acre. Such a plant would be large enough to give operating experience on which the design of a commercial unit could safely be based; and it would provide enough algae for experiments on processing them as food.

The present monograph is a comprehensive treatment of the subject of the large-scale culture of algae. In addition to the pilot plant, many other investigations both in this country and abroad are reported. The aim of this first chapter is to summarize their results and to present some background information, so that the reader not previously acquainted with this field of biological research may understand the detailed reports that follow.

B
Why Algae Are Interesting

General Characteristics

The algae, like other plants containing chlorophyll, are able to convert inorganic compounds into organic matter by means of light energy through the process of photosynthesis. A majority of the algae are aquatic plants. They range in size from the largest oceanic species, some of which reach a length of over a hundred feet, down to microscopic unicellular forms. It is with these unicellular plants that this monograph is chiefly concerned.

Even the larger algae with seemingly complex structure are, botanically speaking, much less specialized than the land plants. Algae lack the elaborate transport system that is common to nearly all land plants. That is, material manufactured in one part of an alga does not readily move to another part. Hence, functionally distinct structures analogous to the leaves, stems, and roots of higher plants are not found in algae. The reproductive process also is less complicated in algae than *in* land plants. In unicellular algae all the functions of a plant are carried out in a single microscopic cell.

Animals and also plants without chlorophyll lack the ability to form organic matter by photosynthesis, and have to live at the expense of the green plants that make their own food. In the large-scale culture of algae, attention must be paid to the parasitic organisms which are likely to be contaminants. These are the microscopic animals called protozoa, and microscopic aquatic plants such as molds, yeasts, and bacteria. A few of the bacteria are photosynthetic, the rest require organic carbon.

Algae have been studied intensively for over sixty years, during which time some progress has been made in gathering and co-ordinating information about the 17,000 species that have been described. (See chapter 3 for a summary of their general biology.) During the past thirty years a few species have been used as research tools by plant physiologists in the study of the mechanism of photosynthesis. One such investigation by Spoehr and Milner [151] about five years ago showed that the composition of an alga such as Chlorella pyrenoidosa could be controlled. This finding gave added interest to the idea that unicellular algae might be grown on a large scale as a source of food.

Comparison with Higher Plants as a Food Source

In ord^r to understand the interest in algae as a possible source of food, we must recall the general features of the structure of higher plants. Except for the leafy vegetables that are eaten because of their flavor or vitamin content, man's vegetable foods are derived froiti a portion of the plant, such as its fruit, seeds, or roots. These parts contain the largest concentrations of protein, which is essential for the reproduction of the plant, and of fats and carbohydrates, which are forms of food stored for the use of the next generation. The sum total of these nutritive parts of the plant, however, is usually half or less of the total dry weight. Most of the plant structure serves mechanical purposes; roots to anchor it and to draw food and water from the soil, leaves to expose large areas of cells to sunlight, and stems to support the leaves and fruits in the light and air.

The primitive character of their cellular organization gives microscopic algae a number of advantages over higher plants as a source of food. In the first place, essentially the entire plant is nutritious, for little of it is devoted to indigestible structures.

A second advantage concerns the kind of food produ-ced. Dried algal cells grown under favorable conditions contain over 50 per cent protein, or more than is found in the edible parts of any of the higher plants. Furthermore, this protein should be suitable for human nutrition, for it contains the ten amino acids now considered essential (see chapter 20) and has a low molecular weight, which means that it can be digested readily.

The possibility of growing a high-protein plant food in large quantities is of paramount importance in connection with long-range planning for the feeding of an ever expanding world population. In certain quarters of the world today large numbers of people do not have access to sufficient protein, with the result that they exist in a state of malnutrition, even if they have adequate quantities of carbohydrates. There is little hope for relief by the traditional method of raising animals for food, because of the large areas of land required to grow grass and other crops to support them. Furthermore, the local production of fish is not always feasible. In this sort of situation, algal culture may fill a very real need.

There is still a third way in which algae have an advantage over higher plants, namely, in the utilization of the sunlight that falls on a particular area of land.   We shall leave to the next section a discussion of the absolute amount of solar energy that algae can utilize in making organic material, and consider here only the relative performance of algae and of higher plants.

A plant utilizes solar energy at its maximum rate only if other environmental conditions are optimum.   The air temperature must be within a fairly narrow range and there must be an abundance of water and mineral nutrients.   Man now regularly provides the last-named requirement by the use of fertilizers, and he provides water in some places by irrigation. His crops are still at the mercy of the weather, however, and there seems to be no way to provide temperature control.   Furthermore, even though greenhouse experiments [287] have shown that the rate of production of organic matter can be increased several-fold by moderate increases in the carbon dioxide content of the air, such increases have not yet been applied to a farm crop.

In the large-scale culture of algae all these conditions are easily maintained at their optimum values over long periods of time.   Also the concentration of algal cells is kept high enough so that all the sunlight shining on a particular area is absorbed.   When field crops are grown for food, there is wide variability in this regard, the full absorption ordinarily taking place for only a relatively short period of time before harvest.   In algal culture, on the other hand, every sunny day gives the same result: the algae are always at the height of the growing season.

Efficiency of Utilization of Light Energy

In some discussions algag have been mentioned as being much more efficient than higher plants in their inherent capacity to utilize the energy of visible light.   The latest evidence, presented in chapter 5, is that algae and higher plants appear to be about equal in this respect.

When growing under light of very low intensity, both types of plant have utilized as much as 20 per cent of the incident energy in the visible part of the spectrum.   When they are growing in full sunlight, the conversion in both cases is reduced to 2 to 3 per cent of the visible part of the spectrum (see chapter 5).   The favorable aspect of algal culture is that there is prospect that this big reduction, which is caused by the phenomenon of "light saturation,"[JJ] can be partially eliminated, as is brought out in a later section of this chapter.

The maximum efficiency of light utilization in photosynthesis is a controversial subject. Different workers report values from about 30 [34, 35] to 80 [17, 198] per cent, each supposedly under optimum conditions, using oxygen evolution as an index of the rate of photosynthesis in experiments lasting a few hours or less. These results are not really in disagreement with the 20 per cent efficiencies found in the growth experiments: the high efficiencies apply only to the first step in the

complex photosynthetic process, whereas growth requires a sequence of reactions within the cell, for each of which there is some loss of energy.

The maximum average daily yield can be computed from the result of the Chlorella growth experiment described in section B of chapter 5, for which the highest efficiency of energy utilization was observed.[2] It would amount to 70 g/m^. The fact that this maximum yield is over five times the best yield obtained in the 1951 Chlorella pilot plant gives rise to the hope that a very great increase in yield during large-scale culture of algae may be realized, once we have a better understanding of the optimum conditions for growth on a large scale.

Relation to Natural Resources

Looking into the future we may forecast some of the long-term effects of the culture of algae. Assuming that half the per capita requirement of 65 grams of protein a day was to be obtained from algae, the total area required for algal culture would be less than a million acres for the present world population. This is such a relatively small area--just a little bigger than the state of Rhode Island—that we may conclude that the introduction of algal culture need not displace existing agricultural crops. Rather, we may consider it a means of utilizing land not suited for agriculture. The use of increasing areas of such land for algal culture would be a means of keeping the food supply in balance with a steadily growing world population.

Land area is not the only requirement for the large-scale culture of algae. For every pound of dry algae harvested, a minimum of two pounds of carbon dioxide and one-twelfth pound of combined nitrogen are required. The latter can be obtained from the atmosphere by fixation processes that have been in commercial use for many years. Carbon dioxide is also abundantly present in the atmosphere; but its concentration must be increased several-fold before it can be used in the forced feeding of algae. Hew to do this cheaply is an unsolved problem. Fortunately there are more concentrated sources of carbon dioxide that can be used for a long time to come.

Besides adding to the food supply, algae may "some day become a source of energy. During the growth of algae (and other plants) solar energy is transformed into latent chemical energy, which, can be released later by burning the plant material.

The growing of algae on a large scale uses up energy for pumping, centrlfuging, and drying. The over-all process, however, has a positive energy balance. That is, the harvested algal cells contain more stored solar energy (expressed as heat of combustion) than the nonsolar energy that is used up in growing and harvesting them. Therefore, an algal

[2] The efficiency was 23.5 per cent. The heat of combustion of the Chlorella was 5.77 kcal/g. The daily insolation in the wave-length range from 4000-to 7000 Å has been taken as 170 cai/cnv*, which is the average value during a year at San Jose, California [279],

culture unit when combined with a steam generating plant in which the
algae were burned would become an energy converter, capable of convert-
ing solar energy into high-temperature heat which in turn could be convert-
ed into electric power. In this case the source of carbon dioxide would not
be a problem, since the combined plant would be a closed system for car-
bon, except for some losses in operation.

Such a solar energy converter would not be economically feasible today;
but it may become of practical importance at some future date.   The way
that all forms of energy that man uses are derived ultimately from the sun
has been described by a number of writers in recent years (for example,
see [185] and [251]). Our coal and oil supplies of course represent a
storage of solar energy from photosynthesis during an earlier stage of the
earth's history. An algal energy converter might be used to take their
place after they have been exhausted.

Sufficient data are not yet available to make an estimate of the power
output of such a plant in terms of growth area and capital investment.
Therefore, it is not possible to give a definite answer to the question of
how large an area would be required to grow enough algae to meet all the
food and energy needs of the expected world population of 7 billions in the
year 2050, when the estimated per capita energy requirement will be 150
million calories a day. 3

A preliminary estimate of the area required can be made if we assume
an arbitrary value for the efficiency of conversion of energy from algae
into electricity and other forms. Taking 30 per cent for this efficiency
and a daily yield of dry algae of 22 g/m2, the area required would be
slightly over 1 acre per capita or 11 million square miles for a world
population of 7 billions. This area, about 19 per cent of the land surface
of the earth, is small enough so that one may think seriously of algal
culture as a means of utilizing solar energy on a day-by-day basis when
all the coal, oil, and fissionable materials will have been used up.

<div align="center">C</div>

Biological Aspects of Large-Scale Culture

Before the above-described advantages of large-scale algal culture
can be realized, a number of practical problems have to be solved. Most
of the chapters of this monograph deal with these problems, which for
convenience in discussion here may be classed as either biological or
engineering. Before summarizing the progress that has been made in
their solution, however, it is desirable to consider what is meant by the
term "growth of a culture, " for it is through their effect on growth that
changes in environmental conditions are evaluated.

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[3]Thi4ʐ question was posed by Palmer Putnam in conversation with the author. The values for
populatiim and energy requirements in 2050 are those given in his forthcoming book on "Energy
in thi· Future*/*

Microbiological Growth

The term "growth" when applied to algae has the same statistical significance that is attached to it in microbiology in general. It refers to the increase of a population, in a manner analogous to its use in the phrase "growth of a city." Because we seldom observe an individual algal cell for an extended period of time, we cannot tell much about the growth of a cell during its life history. The important thing to remember is that, barring accident, an algal cell does not die; it divides into more algal cells.

All of us are accustomed to dealing with plants and animals that are large enough so that we can notice individual differences between specimens of the same species—a visual process that makes possible a dog show or flower show. With the algae, as with other microorganisms, such differentiation is not possible, even with a microscope. In fact, even a skilled taxonomist has difficulty in distinguishing between some related species. Therefore, we have to acquire a new concept when we begin to think about algae: the concept of defining a species in terms of certain group characteristics of large populations of that species.

It may be well to pause a moment to see how large such populations are. There are about 20 billion cells in one quart of a bright-green moderately thin suspension of Chlorella pyrenoidosa (concentration, 0.2 g/1 dry weight), which is ten times the number of people in the world. The daily increase of such an algal suspension is also enormous. While it is growing vigorously during a sunny day in summer, the number of cells may easily double.

The time required for a culture to grow to twice the number of cells is sometimes used as a measure of the rate of growth; this is called the "generation time." A more frequently used measure is the logarithmic growth constant, which is the natural logarithm of the ratio between the number of cells at the end and that at the beginning of a fixed period of growth, usually one day.

Environmental Conditions

The growth constant for a given species has a maximum value when measured under optimum conditions of growth. It is decreased markedly by departures from the optimum conditions with respect to temperature, light, carbon dioxide concentration, nitrogen content of the medium, and concentration of other nutrients, as is discussed in chapter 4. When the Chlorella pilot plant described in chapter 17 was first set up, a considerable background of experience was available concerning these environmental conditions. This was useful for the initial planning; but as soon as experience was gained in actual operation of the plant, additional information was needed.

One of the important accomplishments of the 1951 pilot-plant operations was the delineation of this aspect of the problem of large-scale culture.

Some of the environmental factors were investigated during the operation of the pilot plant (chapter 17), and others were investigated at the Department of Plant Biology (chapter 9). Further information is still needed concerning some variables. For instance, operating a culture continuously makes *it* difficult to maintain the proper concentration of micronutrients, a subject discussed in chapters 8, 9 (section C), and 17.

The acquiring of information about the effect of environmental conditions on the growth rate of algae is complicated by the fact that some of these conditions are interrelated. This is especially true with respect to the inorganic nutrients, as is brought out in section C of chapter 8.

Shift from a Steady State

An even more serious complication is the effect on the growth rate of a shift from a steady state. The best measurements of growth rate have been made with a culture growing under constant environmental conditions, including the density of the culture itself. By the use of artificial illumination, growth can be continued without interruption for the duration of the experiment.

Under these standardized conditions, as explained in chapter 4, the same value is obtained for *the* growth-rate constant by measuring either (a) the number of cells, (b) the volume occupied by the cells in the bottom of a centrifuge tube (referred to as the "wet volume" or "packed cell volume[5]"), (c) the weight of the cells after they have been centrifuged and dried to constant weight, or (d) the amount of light absorbed or scattered by the cells in suspension (referred to as the "ootical density").

The four methods of evaluating the growth rate are not equivalent for cultures growing under variable illumination. Four independent investigations reported in the present monograph (see chapter 7; chapter 9, sections B and C; and chapter 17) reveal for the first time the strong influence of diurnal alternation of light and dark periods. The initial light-sensitive reaction of photosynthesis, of course, stops when the light goes off and resumes when it comes on. Cell division, on the other hand, may continue during the night at the same rate or even at an increased rate, deoending on the temperature at that time and the preceding history of the culture.

Another investigation (chapter 9, section C) has revealed that the yield of a culture (that is, the dry weight of cells produced in unit time) is increased by the use of low night temperatures. Furthermore, a higher daytime temperature can be tolerated when a lower night temperature is used. Additional experiments of this sort, using a continuous culture apparatus instead of the batch method, are highly desirable. Specifications can thus be evolved for the application of this phenomenon, to increase the yield in large-scale culture units.

Variation of illumination and variation of temperature are not the only disturbances to steady-state conditions. A change in any other environmental factor will also affect the growth rate. Therefore, the testing

of variant operating conditions is complicated, because when one condition of the environment is changed purposely for the sake of the test, another one is often changed inadvertently. Greater sophistication is necessary in the design of tests. Above all, tests of operating variables should be performed with daylight illumination. The effect of variability in illumination can be eliminated by using a parallel test unit as a control. Only in this way will the results have any validity when applied to large-scale culture in sunlight.

Effect of Old Culture Medium

A puzzling question is the effect of the re-use of a culture medium in which algae have been grown. Recycling of the medium is essential in large-scale continuous culture. Some experiments by Pratt [131] on the growth of Chlorella vulgaris had been interpreted as indicating that the algae produced a growth-inhibiting substance, to which he gave the name "chlorellin." Myers, in reviewing Pratt's work in section B of chapter 4, suggests that the effect reported may be limited to C. vulgaris.

Recent experiments with C. pyrenoidosa, reported by Davis in section C of chapter 9, bring up the question again. He found that cell division of a very dense culture was inhibited in an old medium, although the accumulation of organic matter within the cells (as measured by increase in dry weight) was not affected. During the operation of the pilot plant, some of the medium in which a large quantity of cells had been grown was used in a check experiment in connection with recycling of the medium. As is reported in chapter 17, cells growing in this old medium continued to divide. The cell density was much greater in Davis' experiment.

This subject certainly needs re-examination. One possible explanation of these seemingly contradictory results may be the effect of bacterial contamination. In a wartime study of antibacterials from plants [152], it was found that the medium in which a pure culture of Chlorella pyrenoidosa was grown did contain a small quantity of an antibacterial substance. Since bacteria were constantly present in the pilot-plant culture medium, it may be that they eliminated the "chlorellin" from the medium by reacting with it. One bit of evidence in favor of this hypothesis is that the number of bacteria was fairly constant. It may be that an ecological balance between bacteria and algae determines the concentration of "chlorellin" and hence the upper limit of density at which vigorous growth can be maintained in a continuous culture in which the medium is recycled.

Contamination

Although bacterial contamination was not a serious problem in the operation of the 1951 pilot plant, contamination by protozoa was. Similar experiences are reported by other workers using large outdoor culture units (see chapters 5, 9, 10, and 11). Selective centrifugation to eliminate

contaminants, as proposed in chapter 17, needs to be tried under routine operating conditions to make sure that it is effective; for the susceptibility of a culture to contamination is greater when it is not growing vigorously.

The hazard of contamination is so great that provision for control needs to be incorporated in the design of a large-scale plant. An important element of such control will be avoidance of mixing the culture medium from all the many small culture units of which the large plant will be made up. In this way contamination of one part will not necessarily affect the whole plant.

Algal Species

Most of the experiments on the large-scale culture of algae have been performed with some species of Chlorella. This choice was made largely because the species of this genus grow rapidly and tolerate a variety of cultural conditions—Chlorella has been termed an algal "weed. "

Already some attention is being paid to the possibility of growing some other algae as a source of sterol (see chapter 22). Another interesting possibility is the large-scale culture of those species of blue-green algae that have the power of nitrogen fixation [40].

With so many species of algae to choose from, Chlorella is not necessarily the best genus for large-scale culture in the long run; but it is certainly a convenient one for the initial development. Once a large-scale process is operating smoothly with Chlorella, it will be relatively easy to modify it to grow some other species which small-scale experiments in the meantime will have shown to be preferable.

D

Engineering Approach

Contrast with Natural Conditions

Algal cells growing in a pond or other natural habitat lead a simple existence. They float about in the water, utilizing the small amount of carbon dioxide that is dissolved from the air or that comes from the decay of debris on the bottom. The light may not be very bright, but they are adapted for photosynthesis at a light intensity as low as 10 f.c. Most of the time the available nitrogen and other inorganic nutrients are less than is required for rapid growth.

Then one day conditions improve, perhaps because rain has washed fertilizer from a near-by field into the pond. The algae respond quickly to the increased supply of nutrients, and in a few days an algal "bloom" covers the pond—the number of algae becomes so great that the water is colored* This condition does not last long. Larger organisms feast on the algae, and since the nutrient supply soon diminishes, the algal population fast declines to its former level.

Large-scale culture, by contrast, consists in forced feeding in a protected habitat. The algae are given as much carbon dioxide, nitrogen, and other nutrients as they can utilize. Fortunately they have a large capacity for them. The one serious limitation is that the individual cells cannot utilize light of high intensity, as will be discussed in section E.

The main engineering problems of large-scale culture are, first, to provide the means for the algae to grow at the maximum rate continuously, and, second, to harvest some of them without disturbing the growth of the remainder.

Yield per Unit Area

An elementary analysis of the economics of large-scale culture indicates that the total cost of the algae produced will be approximately proportional to the growth area. Hence, as a first approximation, the unit cost (per pound, let us say) is inversely proportional to the yield per unit area. By "yield" is meant the weight of algae grown in unit time, which may be a day or a year. The yield per unit area and the cost per unit area are the two fundamental parameters in planning for large-scale culture.

The yield per unit area is a measure of the efficiency with which light energy is utilized by the culture. The volume and depth of culture and the concentration of algae do not have the fundamental significance that attaches to the area illuminated. The same yield per unit area can be obtained with almost an infinite number of combinations of volume, depth, and concentration. One requirement is that the product of the depth and concentration be great enough for optical extinction to occur, so that none of the light is wasted by absorption on the bottom.

In growth experiments performed in flasks, bottles, and tubes, it is often difficult to determine the exact area illuminated. Hence many investigators have expressed yield in terms of concentration, for example, "grams of algae per liter of culture. " For comparative experiments, to see what effect a particular variable has on the yield, this form of expression is satisfactory. A result so expressed, however, cannot be translated into a cost factor, unless some supplementary comparison is available.

Design of Plant

The elements of a plant for growing algae on a large scale are relatively simple: (1) a container with a transparent upper surface; (2) a means of circulating the culture medium within the container so that the algae do not

settle; (3) a means of controlling the temperature; (4) a means of introduc-
ing carbon dioxide and other nutrients continuously; (5) a means of harvest-
ing the algae almost continuously; and (6) a means of processing the harvest
so that it is preserved until used.   The way these requirements were met
in the pilot plant is described in chapter 17.   The container and the methods
of harvesting and drying will be discussed briefly here.

Choice of a Container

The choice of a container is at once the most important and the most
troublesome.   The container is more specialized than the other equipment
and in part determines how the other equipment shall be used.   The plant
proposed in the report from Stanford Research Institute [22, 279] was to
have long steel tanks 11 inches deep covered with glass.   The cost was
estimated at $1.30 a square foot in 1950; and the cost of similar concrete
or wooden structures was estimated to be even higher.   One of the pur-
poses of the Chlorella pilot plant was to try a radically different material
of construction in an effort to reduce this cost.

The container used in the first unit of the pilot plant consisted of a long
tube of transparent polyethylene plastic only 0. 004 inch thick.   When partly
filled with liquid it flattens out to an elliptical cross section, with a width
of nearly 4 feet and a height of 8 to 12 inches, depending on the proportions
of liquid and gas.

Such a tube has a number of advantages besides low initial cost.   It
facilitates the introduction of carbon dioxide as a gas phase in contact with
the entire layer of liquid; and it provides a convenient barrier against
predators.   Although some installation techniques still need to be worked
out, the experience with this plastic tubing in the pilot plant was suffi-
ciently encouraging to justify further trial.

Open Culture

It might be thought that the simplest container for large-scale culture
of algae would be an open pond.   Certainly the initial cost is low; but dis-
advantages are met with during operation.   Without a cover it is difficult
to keep the concentration of carbon dioxide high enough to permit maximum
growth.   Also a pond is a poor form because of the impossibility of ade-
quately stirring the suspension without a large investment in equipment,
and because of the difficulty of harvesting.

A possible exception to these objections is a sewage oxidation pond.
Here there is a plentiful supply of nitrogen, and considerable carbon di-
oxide is provided by bacterial decomposition of the sewage.   Since the
algae are a by-product of an operation conducted for another purpose,
almost the sole cost would be that of harvesting.   A recent report [88]

of a project by the Sanitary Engineering Laboratories of the University of California suggests that "the algae cells may be readily separated from the culture medium by simple alum flocculation followed by settling." The use of the algae from such ponds for cattle feed was suggested in the same report.

A modified form of open-pond algal culture involves having fish eat the algae and then harvesting the fish. Fertilizing a pond to increase the growth of algae does give an increased yield of fish, as was shown by the investigation of Smith and Swingle [274]. The average annual production of fish from the well fertilized ponds was about 0.25 ton per acre, as compared with about a fifth this amount from unfertilized ponds. This method of increasing fish production is followed currently at some of the goldfish ponds in northern Maryland. Although the result is worth while in a specialized case of this sort, it is important to remember that the concentration of algae and the yield of protein are smaller by an order of magnitude than those envisioned in the large-scale culture of algae. In the experiments of Smith and Swingle [274] the most advantageous algal concentration (measured simply as "organic matter") was 15 to 30 parts per million, which corresponds to a density of 0.015 to 0.030 g/1, or only one-tenth the lowest concentration to which the Chlorella pilot plant was harvested. Furthermore, when the algae were grown in very large amounts, some of the algae decayed and the fish died from suffocation.

An alternate form of container, costing more than a pond but less than a closed container, is an open trench, in which flow can be used to keep the culture stirred and to facilitate continuous harvesting. It also has the disadvantages of lack of confinement of the carbon dioxide and of not affording protection against dirt and invading organisms. The results obtained in Germany with this form of container, reported in chapter 11, are sufficiently encouraging so that it should not be dismissed without further consideration.

Harvesting and Drying

The suspension of algae in a large-scale culture unit will be quite dilute, containing less than 1 per cent of algal cells dry weight. The algae can be removed readily by means of a supercentrifuge. The only drawback to this means of harvesting is the cost of the equipment.

The cost of a very high-speed centrifuge increases rapidly with the increased size that is necessary to handle large volumes of liquid. Growing the culture at as high a density as possible will decrease the cost of centrifuging. Even for a dense suspension, it may be economical to use two centrifuges in series. The first one, large enough to handle the whole flow, could operate at a moderate speed to give a partial separation. The concentrated material from this centrifuge could then be separated completely by a smaller machine operated at a higher speed.

Another means of accomplishing this same result might be the use of gravity sedimentation to give a partial concentration prior to centrifuging. Laboratory experiments reported in section D of chapter 9 indicate the feasibility of this method. It is estimated from those results that the set-tling basin would need to have an area about one-tenth that of the growth area, for a moderately dense culture (about 2. 5 g/1 dry weight). One possible disadvantage of so large a settling basin is that the invasion of protozoa might be more difficult to control. A detailed design of a set-tling basin has not been drawn up, and no estimate has been made of its cost as compared with that of a centrifuge.

The algal cells when removed from a centrifuge form a thick paste, which contains about 75 per cent water. In this condition the material spoils quickly—in less than an hour in a hot room. For convenience during the operation of the pilot plant, the product was frozen as soon as it was removed from the centrifuge; but this procedure might not be satis-factory on a large scale.

After the algal cells have been frozen or dried thoroughly they can be kept without spoilage for a long time. Drying has been performed experi-mentally In three ways: by freeze drying, by spraying, and by defatting. The freeze-dried (or lyophilized) product is least likely to have suffered a change in vitamin content, and therefore material from the pilot plant intended for nutritional studies was dried in this way. This is a very ex-pensive process that could not be applied on a large scale without exten-sive development.

Spray drying appears to be the method most easily applicable on a large scale. It should be possible to connect the output from a centrifuge directly to a spray drier, so that the product would not remain long in the condition In which spoilage occurs.

The process used by the VioBin Corporation for simultaneous defatting and dehydration [245] has been found to be suitable for drying Chlorella. A separate liquid fatty fraction is obtained in addition to a dry, bland powder that contains the protein. This separation of components might increase the opportunities for utilizing the product; and therefore the Vio-Bin process should be investigated further, as soon as sufficiently large quantities of algae become available.

E

The Challenge of Light Saturation

Nature of the Problem

The cells of green algae (with which experiments relating to large-scale culture have*been performed) can utilize in photosynthesis only a limited amount of light energy at a time. This phenomenon of light satu-ration is probably a corollary of the Internal mechanism whereby they can photosynthesize with a very small amount of light. The upper limit of

intensity at which sunlight is utilized with full efficiency by Chlorella pyre-
noidosa has been estimated by Myers in section B of chapter 4 as 400 f.c.,
on the basis of his measurements of the saturating intensity of artificial
light.   The occurrence of this saturation effect imposes a serious limita-
tion on the efficiency with which solar energy can be utilized by algae.

In a culture that is deep and dense enough to absorb practically all the
light, mutual shading of the cells gives a gradation of light below the sur-
face that offsets this limitation to some degree.   It has been shown experi-
mentally [279] that in a Chlorella culture the light intensity decreases
with depth and concentration according to the Beer-Lambert law of light
absorption.   There will be a depth at which the light intensity just equals
the saturation intensity Ig.   All the light that penetrates to greater depths
will be utilized with maximum efficiency.   At lesser depths only the frac-
tion Ig/Ij of the light of intensity Ij will be utilized.   It has been shown by
V. Bush (memo to the files, December 27, 1949) that in this situation the
fraction f of the energy of bright light (of appropriate wave lengths and
having an incident intensity I|) that is utilized in photosynthesis by an
algal culture is given by equation (1):

$$f = \frac{I_g}{J_f} \left(\ln \frac{I}{i} \Big| + I \right),$$ 　　　　　　　　(1)

During summer in temperate latitudes the incident intensity of sunlight
in the middle hours of the day is at least 8000 f.c.   Taking that value for
Ij and 400 f.c. for Ig, the value of f according to equation (1) is 0. 20,
which means that only 20 per cent of the energy in the visible spectrum of
the sunlight having the high intensity of 8000 f.c. is utilized photosyntheti-
cally, the remainder being wasted as heat.   This utilization is four times
as great as it would be if the light intensity were just at the saturation
value of 400 f.c.; and it is common experience that an algal culture grows
faster on a sunny day than on a cloudy one.   The light-saturation effect
accounts for the fact that a twentyfold increase in the incident energy re-
sults in only a fourfold increase in the amount utilized by the algae.

We are faced, then, with the problem of arranging matters so that
algal cells can utilize high-intensity sunlight just a§ efficiently as they
can weak light.

<u>Intermittent-Light Effect</u>

There is another property of the photosynthetic apparatus of the algal
cell that may solve this problem.   It has been known for a long time-that
algae can utilize light in very short flashes.   A recent investigation by
Kok, reported in chapter 6, has resulted in numerical values (of the order
of a few milliseconds) for the critical flash time as a function of the inci-
dent intensity, and has shown that for Chlorella pyrenoidosa the dark time
must be at least ten times as long as the flash time for fully efficient util-
ization of the incident light in photosynthesis. Burk? Cornfield^ and
Schwartz [197], using a different approach that involves the time constant

of a "back reaction" in the photosynthetic cycle, have deduced a considerably longer critical dark time.

Despite this unresolved disagreement concerning the duration of the critical dark time, all workers in this field agree on the significance of this phenomenon for large-scale culture. It means that if an algal cell is exposed to light of high intensity for a short time, it can absorb all that light in the first stage of photosynthesis and then utilize it in succeeding stages in the dark. Much recent research effort has been devoted to finding a means for shifting the group of cells exposed to the light in such a manner that each cell will receive just its quota of light and then will immediately be replaced by another one, so that none of the light will be wasted.

Turbulent Flow

One means by which it is hoped to accomplish this legerdemain is turbulent flow of the culture. What is sought is a flow pattern of a very dense culture such that the cells will rapidly move in and out of a thin layer at the surface of the culture. Only while they are in this thin layer will they receive light, the absorption being complete below this layer. In this way the entire surface of the culture will always be occupied by cells that are utilizing ?11 the incident light through the intermittent-light effect, the actual cells in this layer being different at every instant of time.

The expected increase in yield can be computed by means of equation (1) for a given incident intensity, being $1/f$ for a culture in which turbulent flow permits all the algal cells to utilize the visible light energy with full efficiency. For a day in midsummer with full sunshine the increase might be as high as sevenfold. On cloudy days and in other seasons it would be less. Perhaps threefold is about as high as might be expected for an annual average in a temperate latitude.

An increase in yield by this means was anticipated even before Kok's measurements had been made; and its realization was one of the objectives of the 1951 pilot plant. At the start there was no quantitative guide to the densities and flow rates necessary. After Kok's measurements had been made, it was realized that both the densities and the flow rate at which the culture unit had been operated were too low. A trial was then made with the special unit referred to as no. 3 in chapter 17. The highest density that could be achieved before cold weather called a halt to the experiment was not great enough to give a definitive answer.

In the meantime French and Davis made some small-scale experiments with a special growth chamber in which high turbulence could be created. As is reported in section C of chapter 9, an increase in yield of 67 per cent was obtained with a very high-density culture illuminated with high-intensity artificial light. This experiment has demonstrated the reality of the expected effect of turbulence, but the result cannot be applied directly to large-scale culture.

Dilution of Sunlight

An entirely different means of overcoming the effect of light saturation is to be tried in the proposed pilot plant in Israel that is described in chapter 15. By means of an optical system the high-intensity sunlight incident on the surface of a deep culture is to be diluted throughout the depth of that culture in such a way that the illumination received by any algal cell will not be above the saturation intensity.

Another means of arriving at this same result was proposed by W. A. Wood (memorandum, April 18, 1951). It made use of inclined surfaces so designed that a thin layer of culture flowing over them was exposed to oblique illumination of the correct intensity.

Improved Strains of Algae

A very hopeful approach to the problem of light saturation is the search for a strain of alga that will be able to use solar energy more efficiently. Some progress in this direction has already been made through the discovery of a high-temperature strain of Chlorella, reported in section B of chapter 4. Not only is this strain capable of growing vigorously at a much higher temperature than the usual ones, but also it can utilize light of slightly higher intensity. With the knowledge that some variation in this respect is possible, a search should be made, perhaps among artificially produced mutants, for an alga that can utilize light of very much higher intensity.

A somewhat related approach to the problem is that suggested by Burk, Cornfield, and Schwartz [197] for studying the dark reaction that limits the rate of photosynthesis at high light intensities. They point out that if this reaction were better understood, its rate might be increased.

Summary

The practical importance of circumventing light saturation during the growth of algae may be emphasized by recalling the daily yield of dry Chlorella that might be grown if bright sunlight could be utilized with the same efficiency as weak light. The yield of 70 g/m^ mentioned in section B corresponds to an annual yield of dry matter of 114 tons per acre, which is several times that of any agricultural crop. This comparison is the basis for the hope that eventually algal culture may surpass agriculture in productivity, provided the deterrent of light saturation can be overcome.

F

Program for the Future

Economic Appraisal

So far we do not have a sound basis for estimating the eventual cost of production of algae by large-scale culture. Further investigation of the

possibilities of increasing the yield per unit area is needed before a
reliable estimate can be made. The desirability of continuing the devel-
opment of the large-scale culture of algae, however, does not depend
entirely on the hope for a cheap product.

There are some contingent factors related to governmental policy that
need to be considered in order to obtain a well rounded economic appraisal
of the large-scale culture of algae. In some countries today the need for
more protein is so acute that such a process might be worth a government
subsidy, since it would provide a means of increasing the protein supply
without requiring foreign currency. This is the basis of the present inter-
est in the subject in Israel, as reported in chapter 15.

In other areas of the world there are millions of people living on the
verge of starvation, and in particular without adequate supplies of protein.
If the large-scale culture of algae can be successfully instituted in such a
place, the conventional dollar analysis of the cost will not apply. The pro-
tein produced in return for labor will be something that could not have been
obtained in any other way.

The large-scale culture of algae could have survival value. It is con-
ceivable that if normal food imports were cut off from some isolated part
of the world, large-scale production of algae could play a decisive role
in the survival of the inhabitants. HeFe again conventional cost analysis
would not apply.

Still another potential application of algal culture might be in a space
station. Ley [246] has described recently the sort of structure now being
planned as an artificial satellite. A great deal of machinery will be needed
in the space station to maintain a pseudo-terrestrial environment suitable
for man. The addition of an algal culture unit for production of food for the
inhabitants would not seem to increase in very large proportion the com-
plexity of the structure and it would eliminate many costly trips of a space
ship bringing food from earth besides providing a supply of oxygen.

Use of Microscopic Algae as Food

Throughout this review the point of vierw has been that the large-scale
culture of microscopic algae has as its main objective the production of
food. This is a well founded assumption, although, so far, it has not been
possible to do much toward utilizing algae for this purpose.

Florence Meier Chase's interesting essay on "Useful Algae" [208]
gives a full account of the use of a number of species of macroscopic ma-
rine algae as food. Along the coast of Europe some forms are regularly
collected and dried for use as cattle fodder. In Japan certain seaweeds
have been considered a delicacy for centuries. In our own country Chon-
drus crispus or Irish moss is harvested commercially along the New Eng-
land coast for use in puddings and other food products.

Since there is a great deal of similarity in the general chemical nature
of all algae, as is brought out in chapter 19, it might be safe to assume
from the foregoing evidence of the use of macroscopic algae as food that

microscopic algae also are suitable. Fortunately we have one piece of direct evidence in favor of this conclusion, namely, the report in chapter 14 of the successful results that have attended the feeding of "plankton soup"--consisting largely of algae--to human patients in a Venezuelan leper colony.

Before the Chlorella pilot plant was set up in 1951 there was not a large enough supply of this alga available in this country for adequate feeding trials. Some preliminary rat-feeding trials in England, which are reported in chapter 13, indicate a protein value equivalent to that of yeast, which is one of the most nutritious protein foods. Some of the product from the pilot plant has been used in chick-feeding tests, with very encouraging results, as reported in chapter 20.

Dried Chlorella has a vegetable-like flavor, resembling that of raw lima beans or raw pumpkin. An effort should be made to learn how to cook it and otherwise process it as human food as soon as an additional supply of the alga has been grown.

Algae as an Industrial Raw Material

A considerable portion of the product from the Chlorella pilot plant was used in investigations of its possible usefulness as an industrial raw material, as reported in chapter 21. Chlorella was not found to contain any components of interest to the pharmaceutical industry, and the percentage of glutamic acid is too low to make it a satisfactory source of this amino acid. The chlorophyll content is much higher than that of the plant sources now being used for this material, and Chlorella presumably could be used as a raw material for this compound; but this does not seem to be a sufficiently large use to justify production for this purpose alone.

The proteins were reported to be difficult to extract from Chlorella and were found to be of such low molecular weight that they were not suitable for use as a substitute for casein in making either adhesives or artificial wool. In a different process for making adhesives, however, low molecular weight may be an advantage. The fat fraction contains a large percentage of unsaturated acids, and its suitability as a drying oil for paints and varnishes [92] is under investigation.

An especially interesting possibility is the use of algae as a source of sterol to be used as a starting material in the synthesis of cortisone. Although Chlorella pyrenoidosa does not contain a suitable sterol, Scenedesmus obtiquus does contain a small amount. An active research program is under way at the University of Maryland, as reported in chapter 22, to try to find an alga that will contain enough of a sterol to be a large-scale source of supply. In order to utilize fully the new resources represented by these different algae that are being grown in quantity for the first time, samples of them are being assayed for the presence of a number of biochemical components besides sterols.

Although these preliminary investigations have not revealed any startling applications of microscopic algae as an industrial raw material,

further search is warranted. For one thing, as more experience is gained in the separation of algae into their principal components of protein, lipid, carbohydrate, and pigment, some possibilities previously overlooked may be revealed. The situation is similar to that which existed ten years ago with respect to the problem of the utilization of vegetable wastes. The research carried out since then by the U. S. Department of Agriculture at its Eastern Regional Research Laboratory [238] has yielded basic knowledge about the handling of such materials, much of which may be applicable to the study of algae. Furthermore, some industrially useful components of algae might be removed without destroying the nutritional value of the material,

Demonstration Plant

The twenty-fold increase in growth area from a large laboratory-scale culture unit to the 1951 pilot plant made possible a tremendous advance in our understanding of the problems associated with the large-scale culture of algae. Not all the problems have been solved, but they are now in clearer focus.

Many of these problems can be solved by further experiments with small-scale units. Laboratory culture chambers will be best suited for a search for more prolific strains of algae. Questions concerning the nutrient medium and other operating conditions will best be studied in moderate-sized multiple growth units with natural illumination. Units of this size are being used in the current survey of different species of algae. A further attempt to achieve the intermittent-light effect through turbulent flow will require a unit about the size of the 1951 pilot plant. Operation of a unit at least this large is also desirable for the purpose of providing sufficient quantities of algae for more extensive nutritional studies and initial experiments on processing as human food.

It is important to emphasize that basic laboratory research should be continued in parallel with this engineering development. There is still much to be learned about the process of photosynthesis in algae* For instance, the properties of the pigments in algae and the transfer of energy from one pigment to another are not well known. The separation of the photosynthetic cycle into its component reactions is another broad problem. Delineation of the factors that control cell division among the algae is also of prime importance.

Application of the knowledge gained from these different kinds of experiment will eventually require another large increase in the size of the experimental culture unit. In fact, it is not too early to begin planning what might be termed a demonstration plant.

This demonstration plant would be an installation in the open country with a total growth area approaching one acre. It would be situated in a sunny location not subject to great extremes of temperature. (This specification by no means implies that algal culture may not eventually be adapted to a cold.climate.) The growth area might include several different designs so that they could be compared directly under similar conditions.

Here data would be obtained concerning the labor required and the cost of operation and maintenance under conditions closely approximating those of a commercial plant. At the same time the demonstration plant would produce a sufficiently large amount of product--perhaps as much as 200 pounds a day—to permit extensive feeding trials and other experiments in its utilization. Collateral studies should be made of modifications necessary to meet special requirements imposed by location, availability of raw materials in certain specified forms, and specialized product.

Just as the jump in size to the pilot plant of 1951 brought progress toward the large-scale culture of algae, so too will the next jump to the demonstration plant bring further progress. Two years of operation of such a plant should furnish sufficient information for designing full-sized units for any particular application or location.

Chapter 2

THE NEED FOR A NEW SOURCE OF FOOD[1]

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The problem of food supply is as old as mankind itself. With the apparent shrinking of the world, particularly since the last war, and with our growing awareness of the ever-increasing world population demanding a rising standard of living, the grimness and formidable proportions of this problem are causing concern to even this, the most favored of nations. Of the tremendous amount which has been written and spoken on this problem it would seem that there are three principles on which a fair degree of agreement can be reached:

1) The food problem is above all a practical one, practical in the sense that contributions towards its solution must ultimately demonstrate actual usefulness within the medium of present-day society. By contrast, the oft-repeated schemes of the artificial synthesis of food stuffs, independent of the plant or animal, must for the immediate purpose be regarded as fanciful and unrealistic.

2) The production of food, as practised throughout the world today, is an extraordinarily complex process, presenting almost innumerable economic, politico-social, practical, and scientific problems. Hence it is the meeting ground for many disciplines, speaking different languages with all too little common understanding. Of the many facets to the problem no single one can be permanently regarded as the dominant one.

3) In consequence of this great complexity, involving climatic, edaphic, economic, and social variables, it would be highly hazardous to rely upon some single revolutionary step or procedure as the key to the solution. There has been an abundance of visionary schemes. Hope of success seems rather to lie in the direction of concerted evolutionary effort along a wide front, through the critical cooperation of many disciplines.

This is no time to indulge in the spirals of quixotic dreams. True, vision is necessary, but the vision must be a disciplined and practicable one.

It is always very hazardous to over-simplify the problem of food production. Yet from one point of view food production is essentially dependent upon the photosynthetic capacity of an area of land. In the past increased

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food production has been attained primarily through the extension of the land areas which have been put under cultivation. To a considerable extent this has been possible through the use of machinery and scientific application of fertilizers, the development of adapted genetic strains and through the control of diseases and pests. Fortunately we have been given hope of developments in all of these directions for increasing world food production. It is, however, important to bear in mind that in all of these possible extensions the fundamental process, that of trapping the energy of the sun through photosynthesis remains the principal synthetic force.

Let us look at the matter for the moment from the point of view of the utilization of solar energy by the plant in the simplest terms. As a matter of practical experience, the plant under field conditions converts only a very small percentage of the solar energy falling on it into organic material. Of the* 7,300,000,000 kilocalories of the sun's radiation falling on an acre of land per year only about 0.1 to 0.5 per cent is fixed as organic material in the temperate zone. And when we speak of organic material synthesized by the plant it is well to bear in mind that only a portion of this, usually not more than one-half, is in the form of what is ordinarily considered a crop of food stuff.

The factors which make for this astonishingly small return are varied and some of them are rather complex. But we may examine for the present purpose some of the more obvious ones. In the temperate zone the period during which plants can grow is only about four months. During much of this time the plants are small and can utilize but a small percentage of the area of land, thus making use of only a fraction of the energy falling thereon. Actually, it has been found that during a period of four months, representing the growing period of a corn crop, a total conversion of about 1.5 per cent was attained counting the shelled corn, cobs, leaves, stalks, and roots. Of this total dry-weight about one-half is in the form of grain. Obviously, the tropical regions have a distinct advantage because of the long growing season, which.accounts for the relatively high conversion factors in undertakings like the Hawaiian cane sugar plantations.

There is another important circumstance which makes for the low efficiency in the utilization of solar energy by the plant. About one-half of the total solar radiation is composed of heat rays and infrared radiation. These wave-lengths are not effective in photosynthesis. Consequently, the plant is capable of utilizing for the synthetic process only one-half of the radiation incident on it.

Given a well-functioning machine, in this case the elaborately constructed leaf with its complex system of pigments for absorbing light energy, its series of enzymes for promoting the chemical reactions and disposing of the end products, it is essential that this machine should be provided with an adequate supply of raw materials, in order to obtain maximum production. The raw materials for photosynthesis are primarily carbon dioxide and water. Reduced to its very simplest terms, the water serves the process of photosynthesis largely as a source of hydrogen, which in turn is used to hydrogenate, or reduce, the carbon dioxide, the oxygen of the water being

eliminated as a byproduct. The carbon dioxide, on the other hand? serves the process of photosynthesis as the raw material from which are formed the organic substances through this reduction process. The water and carbon dioxide economy of the plant are consequently of the greatest significance. Because of its more obvious nature much attention has been given to the water. But clearly one of the most significant factors in the production of organic matter is the concentration of carbon dioxide in the air, for it is from this source that all the material produced by the plant is derived. There is only 0.03 per cent carbon dioxide in the atmosphere and on this thin thread hangs our very existence. If the percentage of carbon dioxide is doubled, the rate of photosynthesis is also doubled, and it has long been known that the production of crops can be very materially increased through enrichment of the air with carbon dioxide. This, then is a positive means of increasing the effective use of the quantity of sunlight falling on a given area of land.

To some of us who have been looking at the problem of world food supply from a point of view and scientific background somewhat different from that of the food economist, of the agronomist, or of the soil expert, the question has repeatedly occurred whether it might not be worth while to make some preliminary examination of other approaches to the problem. One naturally wonders how an engineer would regard the problem. If he had charge of engines which converted only about one per cent of the fuel consumed by his prime mover, and it was demanded of him to produce more power, what would be the course of his approach to the problem? Would he not at least look into the question as to whether he could increase the efficiency of his generator system? Or would he simply construct more generators of the same low efficiency?

In probably no important endeavor is man so strongly bound to the past as he is in the matter of the production of his food. In provision for shelter, means of transportation and communication, protection against weather, and methods of waging war he has made truly fundamental advances. In comparison with these activities, agriculture is to an astonishing degree still bound to the past. Most of our crop plants were brought into cultivation by primitive man. He took the plants which were at hand in the wild. Through a long and exceedingly complex process these plants have been brought to give higher yields and food of better quality. The application of modern concepts of genetics to plant breeding has, it is estimated, on the whole increased yields by some 40 per cent. It seems doubtful whether improvements in this direction can be expected to improve yields so that they even approximate the estimated increases in demand for food. The question quite naturally arises whether we have sufficiently explored the synthesizing capacity of plants for the fundamental materials constituting our food stuffs, carbohydrates, proteins, and fats, throughout the tremendous diversity of form and function which characterizes the plant kingdom. The portions of the plants on which we now rely for our food sources— 3eeds, tubers, and other storage organs—are, as a matter of fact, derived from an astonishingly small portion of the plant kingdom. They are for the

most part higher land plants. Of these only a relatively small portion is suitable for human consumption, much of the organic material which is synthesized by the plant is cellulose and as such has little nutritive value.

A striking feature of the plants which man has used as a source of food is not only that a small portion of the plant is usable as food, but the very structure of the plant seems ill-adapted to his purposes. The primary manufactory of the plant is in the green leaves. It is here that the synthesizing processes occur which produce the food stuffs. From the leaves the organic materials migrate to other parts of the plant and are laid down in seeds and tubers as storage materials. The distance between the manufactory and the storage organ is often great. Much of the material which the leaves manufacture is used in the construction of the transportation system and another portion is used in the maintenance of this system. In brief, in the plants commonly used for food production a very considerable proportion of the material produced by photosynthesis is not directly available to man.

We are here concerned with the most effective utilization of the solar energy incident upon an area of land by the usual crop plants. The very nature of the development of these plants makes for relatively low utilization. The seeds are planted in the soil and a long time of growth is required before sufficient leaf surface has developed to form anything like a complete coverage of the land area to absorb the maximum quantity of light. For example, with wheit, oats, and barley during the first six weeks of growth only about one-quarter of the maximal leaf surface is developed. During the next two weeks an additional one-quarter is formed. In the ninth week alone the final half of the maximal leaf surface is developed. Thereafter, for only two or three weeks is the plant manufacturing organic material at its maximal capacity. [1]

Essentially, therefore, this usual system of utilizing solar energy constitutes what is industrially termed a batch process. The start is at zero efficiency and is slowly built up until maximal efficiency of the system is attained. This is used for a very short time, after which the process ceases, and must again be started anew. Although this course is largely determined by climatic factors, it raises the question $.s to whether some of the principles of a continuous process, which have been so eminently successful in many industrial operations, could not be applied to manufacture of organic material by plants.

The shortcomings of higher plants as photosynthetic machines for the production of consumable food are, therefore, fairly obvious. In contrast to these organisms, lower forms of plant life may present some advantages. For example, unicellular green algae are relatively highly efficient photosynthesizers. Each cell contains a large amount of the pigments essential to photosynthesis; the organic material formed in the process is stored within each celL The growth of the plant takes place primarily through the division of each cell into 4, 8, or 16 new cells, each of which is capable

1 Walter, H. Dei* Assimilathaashalt unserer Kulttirpflanzen unter feldmSssigen Bedingungen, Biol. Zentralb. 67: 89-94, 1948.

of photosyntnesis and of further division about every twelve hours unaer favorable conditions. Thus the photosynthetic machine consists of a huge number of minute cells, each about 3 to 8 microns in diameter, the population freely suspended in water. For the growth of the population or culture there are required only simple inorganic salts dissolved in water, carbon dioxide as the only source of carbon, light to carry forward the photosynthetic process and a temperature about 25° C. The organic material synthesized consists mainly of proteins, carbohydrates, and lipids.

Lower organisms of this nature, of which the unicellular alga, Chlorella, has been given the most attention have, moreover, another remarkable feature which may prove to be of significance for a source of food. They are remarkably flexible, in the sense that their composition can be altered at will. The composition of higher plants in regard to the relative amounts of carbohydrates, proteins and fats is fairly constant, because their very structure demands that a relatively large amount of the plant is composed of cellulose. Not so in the case of a plant such as Chlorella. For example, by careful control of the composition of the mineral nutrient solution, of the light intensity and the age of the culture, a wide variation in the relative amounts of the chemical constituents can be attained.

PART n

CONDITIONS FOR GROWTH OF ALGAE

Chapter 3

THE BIOLOGY OF THE ALGAE: A BRIEF SUMMARY

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It is the purpose of this chapter to provide a framework of reference on the biology of the algae. Information of importance to the problem of mass culture has accrued from work along three different lines initiated from the different points of view of botany, microbiology, and general physiology. As a result the literature is badly scattered and nowhere summarized for the benefit of those concerned with mass culture. The present attempt contains all the approximations and errors of abridgment and oversimplification inherent in any brief summary of a broad field.

A
Botany (or Phycology)

The algae include a "heterogeneous assemblage of simple plants" [275] of great diversity in size, pigmentation, reproductive mechanism, composition, and habitat. Classically, the algae have been assigned to the lowest division of the plant kingdom, the Thallophyta, distinguished from the higher plant groups by the lack of differentiation into stems and leaves. This is not a completely satisfactory distinction [275]; it correctly emphasizes, however, the comparatively low level of cell specialization characteristic of the group.

Botanical study of the algae has been concerned primarily with the descriptive problems of morphology, reproductive mechanism, systematic classification, and ecology. An excellent treatment is available in a Manual of Phycology [276]. From this source the more recent system of classification, which is based on seven major divisions, has been summarized and is presented in table 1. So brief a summary has little merit except as an attempt to point out the diversity of algae. The particular species treated in the present monograph constitute a small sample of a large and heterogeneous group.

Algae are typically aquatic organisms, although a number of forms are normal inhabitants of soil and certain species have become adapted to bizarre habitats. They are found abundantly in fresh waters and in the sea, but a given species is limited to one or the other environment. They

<u>Table 1</u>

Summary of the major divisions of algae

Division	Pigmentation[a]	Other characteristics
Chlorophyta	Grass green	Unicellular, multicellular, a few macroscopic; cell wall of cellulose and pectins
Euglenophyta	Grass green	Unicellular, motile; lacking a cell wall
Chrysophyta	Yellow green to golden brown; xanthophylls and carotenes may mask the chlorophylls	Microscopic, mostly unicellular; includes the large group of diatoms, which have a cell wall containing silica
Pyrrophyta	Yellow green to dark brown; xanthophylls predominant	Unicellular, motile; cellulose cell wall
Phaeophyta	Olive green to dark brown; fuco- xanthin and other xanthophylls predominant	Principally macroscopic and marine; cellulose and pectin cell wall
Cyanophyta	Blue-green; phycocyanin	Multicellular but usually micro- scopic; some forms become unicellular in turbulent media; usually with a gelatinous sheath
Rhodophyta	Red; phycocyanin, phycoerythrin	Usually macroscopic; marine; cellulose and pectin cell wall

[a] Chlorophyll, carotenes, and xanthophylls are present in all divisions.

are widely dispersed; few species are endemic to a localized geographic region. Among the aquatic forms it has proved useful to distinguish between the plankton or free-floating algae and those attached to the shore or bottom*

Like those of other green plants, the algal cell typically contains a nucleus, one or more chloroplasts, and other less regularly defined formed-structures suspended in a fluid or semisolid cytoplasm. The chlorophyll and carotenoid pigments are localized in the chloroplasts. (An exception to this arrangement occurs in the blue-greens [Cyanophyta], in which there is no distinct nucleus or chloroplast organization.) The outer layer of the cytoplasm is bounded by a plasma membrane with distinctive limiting characteristics of permeability which vary widely among species. The cell is usually surrounded by a sheath or cell wall and often embedded in a gelatinous layer of pectinlike materials of variable thickness. In some forms motility is provided by the whipping action of one or two hairlike flagella.

Many algae are unicellular. In others the cells remain more or less permanently attached in a colonial organization. A colony may still be

microscopic in size.   In other cases the colony may take the form of a macroscopic mass or an extended threadlike filament or a flat sheet.

Sexual reproduction, with attendant cyclic changes or life histories which mark the transition from one generation to the next, is found in many algae. Others, for example the blue-green, Chroococcus, are limited to asexual types of reproduction such as binary fission, the division of one cell into two approximately equal cells. Among the variations of asexual reproduction is the production of autospores as in Chlorella or Scenedesmus; here a large cell becomes walled off into numerous smaller cells which are liberated by the bursting of the old cell wall. The rate at which this process takes place is discussed in the next chapter.

Chlorella and Scenedesmus, which are the principal algal subjects of this volume, belong to the group of green algae, the Chlorophyta. They are common inhabitants of fresh waters and soils. Each is a genus, a group of distinctly different but closely related species. Cells of a typical species of each genus are sketched in figures 1 and 2. The simple morphology and life history of these two genera provide little of interest to the classic phycologist; the few pages allotted to them in any of the standard textbooks of phycology present little information of value for the experimental problems of mass culture.

B

Microbiology

The smaller algae, particularly the unicellular forms, have submitted to the same techniques that are applied to the bacteria, protozoa, yeasts, and molds. Standard microbiological procedure is to obtain a pure culture, a collection of cells restricted to the progeny of a single parent cell.   All the individuals of a pure culture are, therefore, of the same species; and in the absence of sexual reproduction all individuals are provided with an identical genetic make-up. Mutations do occur, but unless environmental conditions are unusual, severe changes due to mutations are not likely to be perpetuated in the culture. Individual variations arising from minor genetic changes, diversity of environment within the culture, or cellular "age" are statistically ironed out, since a sample which submits to macroscopic study usually contains billions or trillions of individuals.

Because of the air- or dust-borne dispersal of many microorganisms, a pure culture is maintained only by special precautions for the exclusion of contaminants. By diverse and often laborious procedures pure cultures of a number of algae have been isolated and are maintained by repeated transfer under aseptic precautions [16, 137], In some cases it is necessary or desirable to use a culture containing a single algal species without excluding other microorganisms; this is referred to as a unialgal culture. The most extensive collection of algal cultures is that assembled by Professor E. G. Pringsheim as the "Culture Collection of Algae and Protozoa/[1] Botany School, Downing Street, Cambridge, England.
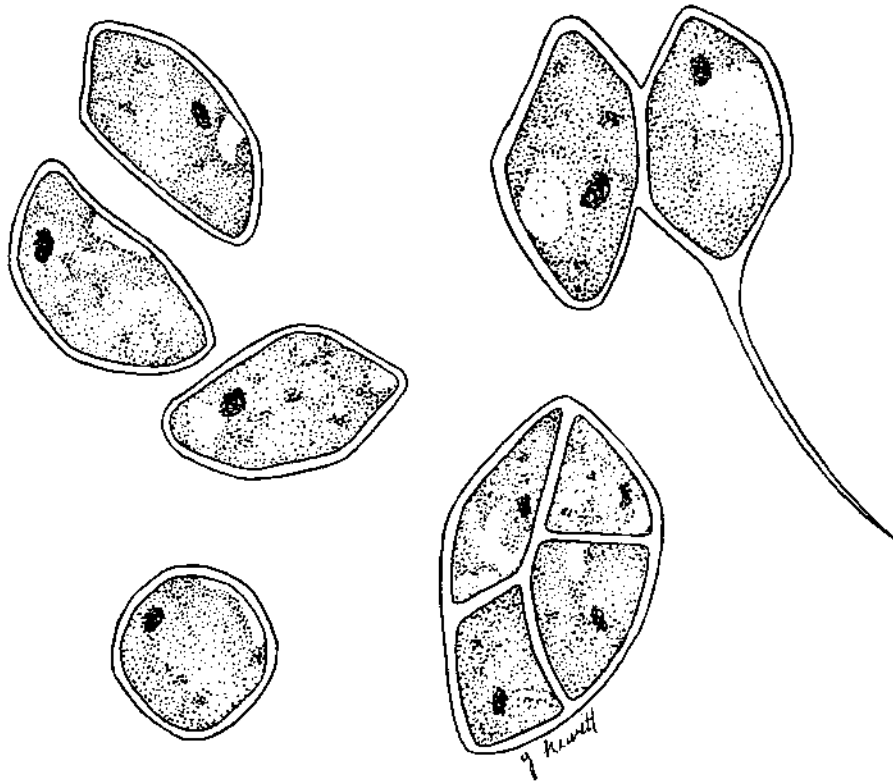
Fig. 1. <u>Scenedesmus obliquus</u>, strain D3 of H. Gaffron. Cells as observed in a rapidly growing liquid culture. (Magnified about 3000 times.)
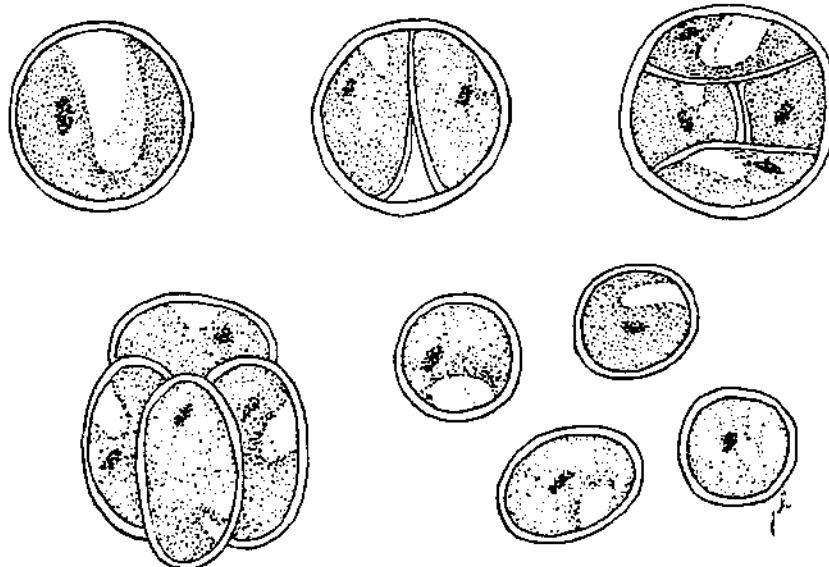

Fig. 2. <u>Chlorella pyrenoidosa</u> (Emerson strain). Cells as observed in a rapidly growing liquid culture. (Magnified about 3000 times.)

Microbiologists have been less concerned with classification and less rigorous In taxonomic usage than botanists or zoologists. Classification based on structure becomes more and more difficult as the number of observable structural characteristics decreases; it breaks down entirely when it can be shown that two organisms quite different in their cellular activities are microscopically indistinguishable. Among the bacteria, structural characteristics are used to define the large groupings[3] and physiological characteristics are frequently Introduced in order to distinguish between species. Unfortunately, a physiological classification has not yet been applied to the algae. Confusion of terminology is illustrated by the case of Chlorella. Over a dozen cultures of different origins and characteristics have been used in physiological work reported for this genus. Four of these which have found rather widespread use in England and the United States may be identified as follows: Chlorella pyrenoidosa (Emerson strain), C. vulgaris (Emerson strain), C. vulgaris (Trelease or Columbia strain), C. vulgaris (Wann or Cornell strain). All these have been maintained as laboratory cultures for the past twenty or thirty years. They are referred to as different strains of Chlorella, although this usage merely reflects their inadequate taxonomy. By microbiological criteria they are distinct species. Chlorella pyrenoidosa (Emerson strain) is the organism which has been used most widely in mass-culture studies in the United States. It was the one grown on a larse scale in the pilot plant described in chapter 17,

C

Physiology

Because of their lack of cellular specialization, algae have been used for the study of a number of physiological processes of general importance. Of particular usefulness here are the developments which have arisen from the study of photosynthesis. Since Its introduction by Otto Warburg in 1919, Chlorella has become a standard organism for studies on the mechanism of photosynthesis. Much of the information on the growth of Chlorella and other algae Is found as incidental material in publications primarily concerned with the process of photosynthesis. Warburg's technique of growing the algae in illuminated cultures in inorganic media provided with 5 per cent carbon dioxide in air has been varied widely In detail but not in its essential features. Unfortunately, this technique has not been used generally by microbiologists. Many studies which might have yielded valuable quantitative information on algal growth are useless In this respect since carbon dioxide was supplied only by diffusion from air through cotton stoppers.

The same factors which contributed to the usefulness of Chlorella as an experimental organism for photosynthesis have led to its selection for exploratory work on the problem of algal mass culture. It is a hardy and rapidly growing form, an algal weed. Its chloroplast takes up a large

fraction of the cell, and its very high rate of photosynthesis exceeds its rate of respiration by a factor of 10 to 100 times. It excretes relatively little organic matter, so that even without maintenance of pure culture conditions *he bulk contamination by bacteria is relatively small.

Information on the growth and physiology of the algae is fragmentary at best; the first general treatments have appeared only within the past few years [99, 194]. Fortunately the more numerous data on the factors affecting photosynthesis can often be used to make at least approximate predictions with respect to growth. Not all conditions which affect growth do so because of effects on photosynthesis; but conditions which seriously affect photosynthesis must have corresponding effects on growth. A useful reference work on photosynthesis is the monograph by Rabinowitch [265].

Chapter 4

GROWTH CHARACTERISTICS OF ALGAE IN RELATION
TO THE PROBLEMS OF MASS CULTURE[1]

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Biological information of significance for the mass culture of algae can best be treated by considering, first, the characteristics of growth under controlled and measurable conditions; then the extrapolation of these characteristics to the case of high-density cultures; and, finally, the limitations which it is expected will be introduced by the use of sunlight illumination. The discussion will center around Chlorella pyrenoidosa (Emerson strain), since extensive data are available for this species.

A

Growth of Chlorella under Measurable and Controlled Conditions

Growth Constant k

Growth of Chlorella and other unicellular algae can be expressed in terms of the generalization that the rate of population increase is a function of the number of cells:

$$f^\wedge f(N). \qquad\qquad (1)$$

(Equation (1) is not a completely restricting description, since, as will be noted below, rate of increase is also a function of other conditions.) When every cell in the population is maintained under constant environmental conditions, the relation becomes

$$^\wedge = k\,N\,, \qquad\qquad (2)$$
$$\text{at}$$

or alternately, in its integrated form and converted to log^Q?

$$\log_{10} \frac{N}{W_0} = k_* \bullet \qquad\qquad \textbf{(2a)}$$

Equation (2) affords a means of evaluating k, the specific growth rate, which is an intrinsic characteristic of an organism under a given set of environmental conditions. Alternately, k for a given organism may be measured as a function of the variation in any environmental condition and used as a quantitative measure of effects of that condition on growth. Attention is now directed to the experimental requirements for the measurement and interpretation of k.

Equation (2) describes a steady-state system. In Chlorella, growth is a repeating process in which the business of cellular machinery is to build more cellular machinery; the smallest practically useful unit is the cell. In a given steady state the cells of successive generations are identical in average composition, and the average generation time A~*-B and B->C is constant. The gross composition of Chlorella varies within wide limits as a function of a number of environmental conditions [95, 96, 151] and it is to be expected that a steady-state system will be maintained experimentally only under careful control. A particular problem is light intensity, since mutual shading of the cells increases during growth and decreases the effective light intensity even when the incident intensity is held constant.

<u>Measurement of k</u>

Equation (2) is formulated in terms of the number of cells, N. The experimental method for determining cell number is a laborious procedure of inherently low precision. It involves counting cells under a microscope in a chamber of known dimensions, such as that provided by a hemocytometer. About 1000 cells must be counted to obtain a standard deviation of ±3 per cent. It is therefore more convenient, and often desirable, to use some other index of cellular quantity. Cell volume may be determined with a precision of about ±1 per cent by centrifuging an aliquot of suspension in a calibrated capillary tube until a minimum volume is reached. Cell weight may be determined with a precision of about ±1 per cent by heating or otherwise desiccating to a constant weight the centrifuged and washed cells from an aliquot of suspension.

The optical density may also be used, since it is linear with any of the above indices for a given suspension within certain limits. Optical density, however, is entirely a relative measure and not reproducible as between different laboratories, since the value for a given suspension varies widely with the optical characteristics of the instrument used. An instrument with a large light-sensitive receiver and without focusing of the optical system responds principally to light absorption by the pigments (example: the Evelyn photoelectric colorimeter); one with a small receiver and a long-focus optical system responds principally to-light scattering and is more sensitive to number and size (example; most densitometers, see e.g. [249]).

For a given steady-state system, cell number, cell volume, cell weight, optical density (with a given instrument), and all other measures of cell quantity are related by conversion constants and can be used interchangeably. Equation (2) has been verified experimentally for a number of different algae under approximately steady-state conditions by evaluating N in terms of various choices of the above measures of cell quantity; references are cited in table 1. From one steady state to another, or if steady-state conditions are not maintained at all, the conversion factors between different measures of cell quantity are not constant and may vary within wide limits.

B

Factors That Affect the Growth Rate

k as a Function of Light Intensity

Data are available on the effect of light intensity on k at 25° C as obtained by a continuous dilution method for very thin suspensions [95, 96], The data, schematically reproduced in figure 1, give a saturation curve in
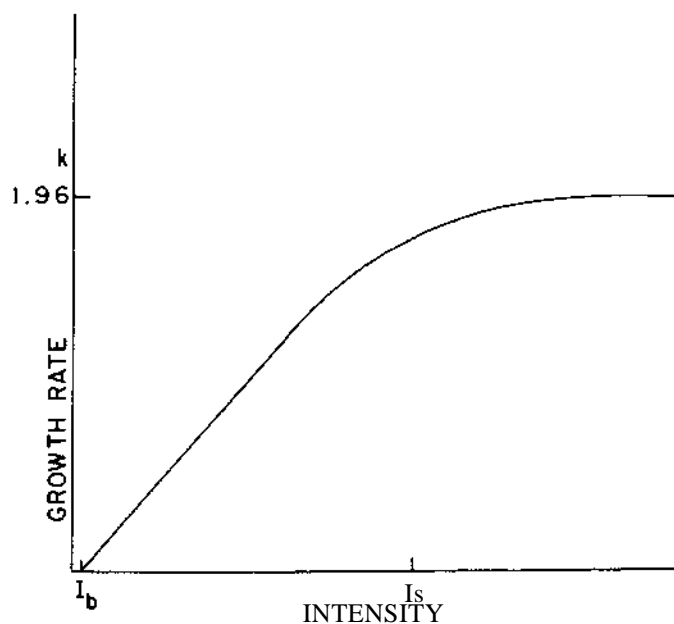
Fig. 1. Growth rate of Chlprella pyrenoidosa as a function of light intensity. The specific growth rate k is expressed in units of $\log_e$ per day. Units of intensity are purposely omitted for reasons discussed in the text; the data on which the figure is based assign a value of about 100 f.c. to I_s, the transition point between light limitation and light saturation.

which a maximum value for k of 1.96 per day is achieved at about 100 foot-candles. Extrapolation of these data to problems of mass culture must be done with recognition of several limitations and uncertainties.

In the first place, lower values on the curve must extrapolate, not to zero intensity, but to some finite value, 1^, required to maintain the endogenous respiration or basal metabolism, which is the cellular overhead without any increase. (The curve of figure 1 has been drawn to show this theoretical requirement, although the original data were not good enough to demonstrate it.)

In the second place, the intensity system of luminosity (i.e., foot-candles) is difficult to standardize and use for comparison between different light sources. The data of figure 1 were obtained with tungsten illumination. In absolute accuracy the important minimum value of 100 f.c. for light-saturated growth may easily be in error by 100 per cent when applied to sunlight illumination.

In the third place, the data of figure 1 were obtained under conditions in which each cell received approximately equal radiation from all sides. If the fractional light absorption by an individual cell is high, then unilateral illumination will require a higher intensity for attainment of saturation. Direct data on absorption by an individual Chlorella cell are not available. Data compiled by Strain [283] for a 5-micron layer of a 2 per cent chlorophyll solution indicate an 80 per cent absorption of red and blue light. Calculations from measurements on the transmission of a Chlorella suspension lead to an estimate of 30 to 40 per cent absorption of incident red and blue light by a single cell (see section F). The minimum intensity required for maximum rate of growth of Chlorella is in the neighborhood of 400 f.c. under unilateral illumination, according to recent determinations in this laboratory by a less elegant method.

Finally, the data of figure 1 describe response to continuous illumination. At high cellular concentrations a turbulent suspension exposes the cells to intermittent periods of light and darkness. The extent to which a growing cell integrates intensity x time under the various possible conditions of flashing light is the subject of chapter 6 and also is given further consideration in section D of the present chapter.

k as a Function of Temperature

Effects of temperature are not worked out in detail. It is common experience that for Chlorella pyrenoidosa at light saturation the growth rate is higher at 25° than at either 20 or 30° C. Recent experimental data obtained by Milner, which are presented in chapter 9, section C, show that in sunlight maximum growth occurs at a higher temperature if the cells are held at a low temperature (15-20° C) during the night. No data are available on effects of temperature on k at light intensities below light saturation; such data are needed, since dense cultures are growing at effectively low light intensities even when the incident intensity is full sunlight.

k as a Function of Carbon Dioxide Concentration

It is assumed that carbon dioxide affects growth only by virtue of its effect on photosynthesis. Chlorella absorbs carbon dioxide principally in the undissociated form (CO_2 or H_2CO_3) and little if any as HCO_3^- or $CO_3^=$ Two studies on effect of carbon dioxide concentration on photosynthesis indicate that carbon dioxide saturation is achieved at or below 0.1 per cent [33, 232]. Above about 5 per cent, toxic effects become operative, although the upper limit is not definitely known. It is expected, therefore, that growth rate will be independent of carbon dioxide concentration between 0.1 and 5 per cent. 2 These values are expressed in terms of the composition of the gas phase in equilibrium with the suspension. If the rate of carbon dioxide uptake is high, a considerable diffusion gradient between the suspension and the gas phase may be required; hence the common use of 5 per cent CO_2.

In contradiction to the above conclusion based on photosynthesis measurements, Spoehr and Milner [151] have reported a difference in growth yield at carbon dioxide concentrations between 3 and 5 per cent in nitrogen, roughly in the ratio of 3 to 5. This experiment was not critically designed to attack the question of carbon dioxide concentration required for maximum rate of growth, nor did the authors offer any conclusion on this point. The question is now of sufficient importance to be answered by specifically designed experiments.

k as a Function of Components of the Medium

Media for growth of algae have been compounded by rule of thumb based on years of ancestor worship, with occasional modification to fit the needs of batch cultures. Our present information ^ on effects of composition of the medium comes from experiments in which there was determined the minimum quantity (initial concentration) of a component needed to give maximum amount of growth. No data are available on the relation between rate of growth and phosphate concentration (for example); such information could be obtained by the use of a continuous-dilution culture unit such as is described at the beginning of section C in chapter 9.

Algae are known to be efficient accumulators of inorganic ions. For the specific case of <u>Chlorella pyrenoidosa,</u> the following data may be cited from our experience. Cells grown in a Knop's solution containing 3,75 g/1 of total inorganic salts have a typical ash content of 5 per cent of their dry weight or 1.25 per cent of their wet weight. Thus 1 liter of packed cells contains 12.5 g of ash, as compared with a liter of medium containing 3.75 g of total salt. The differential is really greater than 12.5/3.75, since ashing results in loss of all the nitrogen and some of the sulfur and phosphorus. It has been demonstrated that the magnesium sulfate, potassium phosphate,

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[2] New data that support this conclusion over the range 0.56 to 4.43 per cent are presented by Davis and co-workers in chapter 9, section C.—Ed.
[3] The available data are reviewed in chapter 8.--Ed,

and potassium nitrate components of the Knop's medium may be varied in the range of 0.02 to 0.001 M without effect on the growth rate of thin cultures of C. pyrenoidosa [97]. Independence of growth rate and salt concentrations over wide limits is to be expected, since the calculated energy for ion accumulation is small as compared with the energy required for synthesis of organic cellular materials.

The relation of k to the concentrations of the various microelements is a problem which requires special consideration. The concentration range between requirement and toxicity is narrow and at such low values that the usual analytical procedures are not applicable. Unfortunately, several of the heavy metals (iron, manganese, molybdenum) slowly form large ionic structures which are reversible with difficulty and apparently unavailable to the cell; continued maintenance of desirable ionic concentration levels may be difficult, particularly at pH values above 7. The chelating agent ethylenediamine tetraacetic acid is useful for this purpose [104].

Effects of Autoinhibitors on k

In other microorganisms a decrease in rate of growth with age of a culture has often been attributed to inhibiting effects of accumulating metabolic products of the cells. In the algae, because of their high synthetic capacity and low level of excretion, it may be expected that effects of this type will be less important.

There is, however, one clear-cut case in which an autoinhibitor has been demonstrated in an alga. Working with Chlorella vulgaris (Trelease strain), Pratt ([131]; see also previous work cited in this reference) demonstrated that growth of a culture is inhibited by very low concentrations of a diffusible material produced by the cells and excreted into the medium. The inhibitor was called "chlorellin," although its chemical identity was not established. It also inhibits the growth of other algae and certain bacteria. The original, suggestion of the occurrence of chlorellin came from extensive analysis of growth curves of cultures in Erlenmeyer flasks. Unfortunately, the growth curves also reflecMight-intensity limitations, so that effects of the inhibitor on the growth rate cannot be evaluated independently.

In subsequent work Spoehr et al. [152] demonstrated that inhibitors of bacterial growth, probably photooxidized fatty" acids, are produced by (L fiZrenoidosa (Emerson strain). The phenomenon of growth inhibition due to a chlorellin-like material has not been demonstrated, however, in this or any other aleja except that used by Pratt. In this laboratory we have carried cultures of the Emerson strain up to more than 50 g/1 dry weight without evidence of any growth limitation save those due to light limitation and depletion of certain nutrients [104]. For the mass culture of C. pyre-!12MS§2i limitations due to autoinhibitors are judged not to be any more important than other second-order effects; from Pratt's observation it is expected that the same conclusion will not hold for all algae.

k as a Characteristic of the Organism

When temperature, light intensity, and all other conditions are adjusted to optimum values, Chlorella pyrenoidosa achieves a limiting growth rate, k_{max}, which is an inherent characteristic of its internal biochemical machinery. At 25° C (close to the optimum temperature) this value is 1.96 per day. The temperature coefficient of k, though known only roughly, is much too high to permit explanation of $k_{m}ax$ as being established by a diffusion process.

According to the following argument, k_{max} is not established by the capacity for photosynthesis. The k value of 1.96 per day corresponds to an increase of 8.2 per cent per hour. Cne milligram dry weight of ceils, containing about 0.50 mg of carbon, would thus increase to 1.082 mg dry weight and 0.54 mg of carbon after steady-state growth for an hour at maximum rate. But if such cells growing at bare light saturation (i.e., 100 f.c. as in fig. 1) are studied in a short-time manometric experiment at the still higher intensity (e.g., 500 f.c.) needed to saturate photosynthesis, then it is found that they can assimilate an amount of carbon dioxide equivalent to 0.08 mg carbon per hour. If another aliquot of the same cells is allowed to establish a new steady state of growth under 500 f.c, it is found that the growth rate is still 1.96, corresponding to 0.04 mg carbon per hour.

The growth experiment places a limit of 0.04 mg carbon per hour as the maximum assimilation allowed by the growth rate under steady-state conditions; but in the short-time photosynthesis experiment, 0.08 mg is actually observed. Since excretory products are negligible, the discrepancy must lie in the departure from steady-state conditions during the photosynthesis measurement. The following explanation has been proposed. Cells grown at 500 f.c. average over twice as large as cells grown at 100 f.c, but do not have any greater chlorophyll content per cell [95, 96]. They are restricted in rate of division, but not in size, and adjust to the more than adequate carbon assimilation by producing extra quantities of reserve materials in the transition to the new steady state. Whether or not this explanation is correct in detail, the argument requires that k_{max} is not established by limitations of photosynthesis.

The same kind of conclusion may be reached from entirely different data. The specific growth rate of Scenedesmus costulatus at 24.5° C was measured by Bristol-Roach [196] in the light plus carbon dioxide, in the dark plus glucose, and in the light plus glucose plus carbon dioxide. High and low light intensities were used. At low light intensity the rate k was 0.35; in the dark plus glucose, 0.48; at low light intensity plus glucose, 0.87. Thus the contributions to growth by glucose assimilation and light-limited photosynthesis are additive. At high light intensity, however, the growth rate of 1.08 was not affected by addition of glucose, a phenomenon which has been demonstrated also for Chlorella pyrenoidosa (J. N. Phillips, unpublished data). Again the conclusion follows that at light saturation, rate of growth is limited not by rate of carbon assimilation, but by some other process.

The rate-limiting process which establishes k_{max} must be some cellular process other than photosynthesis. Tentatively this is regarded as the rate of synthesis of some cellular component. Unfortunately, the severe permeability restrictions of Chlorella speak against the likelihood of success in supplying either the catalyst or the product of the hypothetical rate-limiting reaction.

One other comment should be made on the interpretation of the maximum growth rate of Chlorella. Here, as for other microorganisms, k_{max} is a definite and real value, experimentally observable by different methods. In no case, however, is it known that k_{max} is a perfectly constant characteristic of every individual cell. For example, the generation time

Table 1

Growth rates for algae and other microorganisms [a]

Organism	k [b]	Temp. (°C)	Conditions	Reference
Bacteria				
Escherichia coli	60.0	c	Lactose broth	[247]
Azotobacter chroococcum...........	f14.0	c	Urea, glucose	[247]
	[3.0	c	Sugar, mineral salts	[247]
Yeast				
Willia anomala.......................	14.0	30	Glucose, yeast extract	[225]
Protozoon				
Tetrahymena geleii...................	4.1	25	Yeast autolysate	[259]
Algae				
Anabaena cylindrica.................	0.74	23	Light, CO_2, NO3-	[221]
Chlorella pyrenoidosa (Emerson strain)	'1.96	25	Light, CO_2, NO3-	[95, 96]
	0.92	25	Dark, glucose, NC3~	e
	0.48	25	Dark, acetate, NC3"	e
	2.0	25	Light, CO_2, glucose, NO3-	e
Chlorella wlgaris (Wann strain)..	1.1	23	Light, glucose, NH4NO3	[117]
	0.67	23	Dark, glucose, NH4NO3	[114]
Chlorella (Tx 115)....................	5.8 [d]	39	Light, CO_2, NO3-	
Euglena gracilis (Vischer strain).	1.4	25	Light, CO_2, NH4+, Bi, B_{12}	f
	0.58	25	Dark, butyrate, NH_4, Bj, B_{12}	f
Euglena gracilis var. bacillaris ...	0.97	25	Light, CO_2, NH4+, Bi, B_{12}	f
	0.97	25	Dark, butyrate, NH4+, Bi, Bi_2	f
Prorocentrum micans....	0.46	18	Light, sea water, soil extract	[186]
Scenedesmus quadricauda...........	2.02	25	Light, CO_2, NO3-	[109]
Scenedesmus costulatus	1.08	24.5	Light, CO_2, **NO3"**	[196]
	0.48	24.5	Dark, glucose, NO3-	[196]
	1.08	24.5	Light, CO_2, glucose, NO3-	[196]

& Additional useful data on other algae are available (see, e.g., [172]) but not adequate for accurate estimate of k.

bin terms of **$\log_e$** units per day; a value of 0.69 corresponds to a generation time or doubling of once per day.

[c] At the optimum temperature of the organism.

[d] Preliminary value only, from unpublished data by C. Sorokin.

e J. N. Phillips, unpublished data.

f M. Cramer, unpublished data.

of the bacterium Escherichia coli, which divides by the simple process of binary fission, is 20 minutes. It is not known whether every cell divides in precisely 20 minutes or whether this value is merely the median value on a distribution curve. Intuitively one prefers the latter explanation; but in this case the same distribution curve must be perpetuated; otherwise the generation time would continually decrease because of the selective advantage of the more rapidly growing cells.

In Chlorella, cell division is accomplished by the splitting of a large cell into a number of smaller cells which break away and increase in size, repeating the performance. Exactly the same argument applies here, however, as with E. coll. A population of Chlorella cells growing in a steady state must have for the growth rates of individual cells a distribution curve just as stable as that for the velocities of individual gas molecules in a fixed volume at constant temperature and pressure.

Characteristics of Growth of Other Algae

Values of the specific growth-rate constant, k, are presented in table 1 together with comparative data for other microorganisms. It will be noted that the growth of algae is typically slower than that of other microorganisms. Among the algae studied to date, Chlorella pyrenoidosa has one of the highest growth rates at 25° C.

Temperature Tolerance

Since some algae are found growing abundantly in nature at temperatures above 30° C, it was expected that it would be possible to find species with a higher temperature tolerance and a higher value of kmax than are found in C. pyrenoidosa. From accumulation cultures at 32° C inoculated by collections taken from shallow ponds and streams near Austin, Texas during the summer months of 1951, C. Sorokin (unpublished data) isolated a number of strains which grew logarithmically at 32° and subsequently were adapted to logarithmic growth at 39° C in the laboratory at the University of Texas. Preliminary data on one of these, included in table 1, indicate a remarkably high growth rate. All the strains appear to be species of Chlorella. A number of additional strains have been isolated from accumulation cultures at 39° C but have not yet been studied. These strains are under further investigation as to temperature limits and metabolic characteristics. Their high temperature tolerances and growth rates appear to offer distinct advantages for mass-culture applications.

C
Growth in High-Density Cultures [4]

Economy in harvesting procedure requires cultures of high density for mass-culture operations. Limitations of nutrient provision and the produc-

[4] A paper by Tamiya {285} on the kinetics of algal growth was received after preparation of the first draft of this chapter. This revised section now incorporates certain of **Tamiya's** ideas, although in an entirely different development, which is probably less rigorous and certainly more simple.
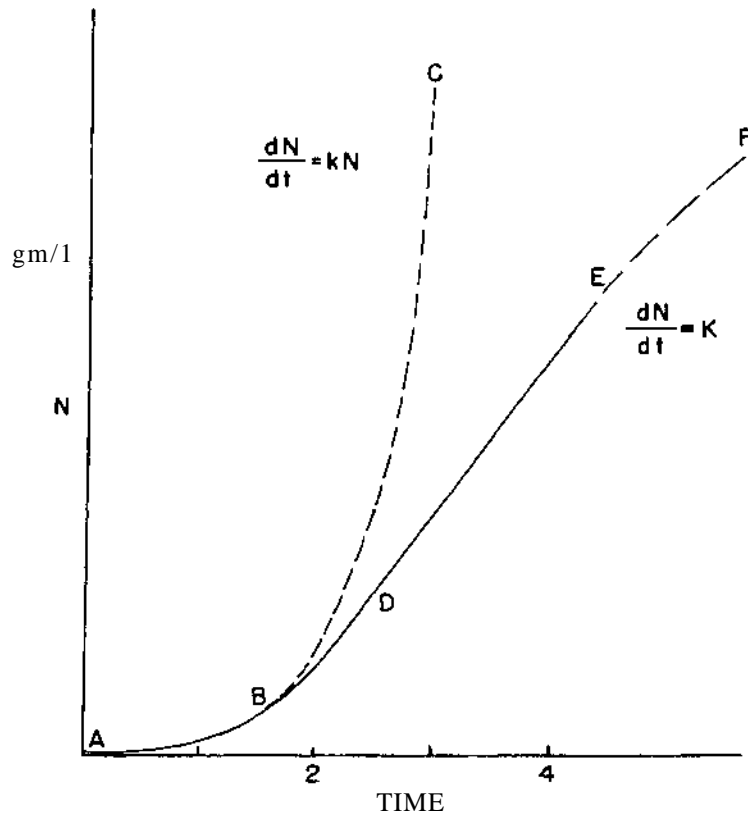
Fig. 2. Growth curves for Chlorella

tion of autotoxins become more difficult problems as the culture density
is increased. When such technical difficulties are surmounted, light alone
remains as the final limiting factor. It is instructive, therefore, to see
how well growth in dense cultures may be understood in terms of light
limitations alone.

Typical Growth Curve

For a culture of Chlorella growing without limitation by carbon dioxide
or nutrient and at constant temperature and constant high light intensity, a
growth curve similar to the solid line ABDEF of figure 2 is observed
experimentally [104, 172]. At low cellular densities, growth at first pro-
ceeds exponentially along the curve AB, then along a linear segment DE,
and finally along a region of decreasing slope EF. The nature of the three
important regions of the curve may be predicted from light-intensity con-
siderations, according to the following argument.
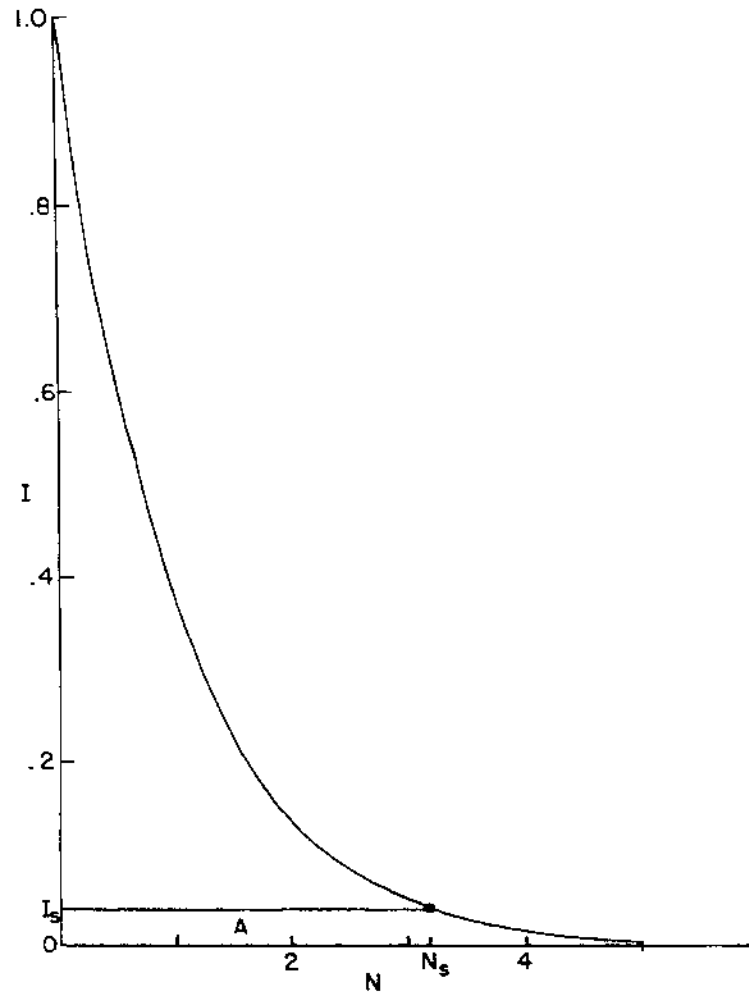
Fig. 3. The light intensity transmitted by a Chlorella suspension as calculated from Beer's law, $I \sim I_Q e^{-aNx}$. Unit values have been assigned to I_Q, oc, and x. I_s has been assigned a value of 0.04 corresponding to 400 f.c. when I_o is the lu,000 f.c. of full sunlight.

The curve ABDEF of figure 2 may be described with reference to figures 1 and 3. Consider a culture of unit volume illuminated at high intensity on one surface so that the light path through the suspension is of unit length. The only variables are cell quantity, N, and time. The intensity of light emerging from the back surface is described as a function of N by the curve of figure 3. An important value of intensity, I_s, represents the light intensity above which growth is light-saturated as indicated in figure 1.

Exponential Growth

At the beginning of the growth curve, when N is small, the intensity of light emerging from the back surface of the culture is above I_s, and all cells in the culture are light-saturated. Growth proceeds exponentially along AB according to equation (2),

$$<\S = kN, \tag{2}$$

since k remains constant at its maximum value. This condition continues to a population N_s at which the emerging light has intensity I_s. As the population passes N_s, some of the cells at the back surface become less than light-saturated, and for an increasing fraction of the population k decreases/ Growth at populations above N_s continually increases the fraction of cells which are illuminated at intensities less than I_s, so that k of equation (2) will decrease as some function of N.

Linear Growth

Growth in a dense culture proceeds through a second important phase, DE of figure 2, described by

$$\mathbf{f\text{-}K,} \tag{3}$$

in which K is the constant over-all rate of increase for the culture as a whole. Combining equations (2) and (3) gives

$$ff^{N} = K = k_c N, \tag{4}$$

in which k_c means the average growth rate per cell.

Explanation of equation (4) in terms of light limitations requires some measure of the effective light intensity in the culture which can be used to estimate k_c from figure 1. It is convenient to define the effective intensity per cell, I_c, as that portion of the incident intensity per cell which is metabolically active. I_c may have values ranging from zero to Is . For any intensity greater than I_s, I_c remains equal to Is, since only this portion of the intensity is metabolically active. The average growth rate per cell is directly related to I_c by

$$k_c = m(I_c - I_b), \tag{5}$$

where m is the slope of the curve of figure 1 determined by the fraction of incident light absorbed and by the quantum efficiency. Ib is the small value of light intensity required to maintain the cellular overhead. So long as Ic is large as compared with Ib, kc approaches a simple linear function of I_c,

$$k_c = ml_c . \tag{6}$$

In figure 3 the effective light intensity per cell, Ic, may be evaluated as the shaded area A divided by N. For the reasonable value of Is chosen in the figure, A has already reached 76 per cent of its maximum area when $N = N_s$, and reaches 99 per cent of its maximum area when N = 2N$. Further increase in N during growth will cause only negligible increase in the area A, with the result that

$$I_c \simeq \frac{x}{-} , \tag{7}$$

where A_{max} is a constant, the maximum value of the area A. From the approximate equations (6) and (7),

$$k_c N = m A_{max}, \tag{8}$$

that is, $k_c N$ is a constant as required by equation (4). From these considerations it is clear that the phenomenon of linear growth may be established by the light factor alone if the incident intensity is large as compared with Is and practically all the incident light is being absorbed.

Limiting Density

The third region EF of the growth curve of figure 2 is reached when I_c is reduced to a value no longer large as compared with Ib- Equation (5) must now be used in place of the approximation of equation (6), so that

$$\frac{dN}{-ft} = k_c N = m (Amax - Nib). \tag{9}$$

The over-all rate of increase dN/dt now decreases with increasing N, approaching zero as Ic approaches Ib-

Optimum Density

Since maximum yield per day is an important objective of mass culture, it is clearly desirable to maintain a culture in the region DE of figure 2 under conditions such that I_c is on the early linear segment of the curve of figure 1, but large with respect to Ib - If for economy of harvesting procedure it is desirable to obtain very high cell densities, this should be accompanied by a decrease in the thickness of the layer exposed to illumination, so that I_c never becomes too low. However, losses due to the contribution of % and gains due to intermittence effects (see section D of chapter 6) may require some compromise in the choice of the most desirable density and thickness of the suspension,

D

Growth at High Densities under Sunlight Illumination

The use of sunlight illumination for the mass culture of algae introduces two complications: illumination is at a very high and variable but

not controllable intensity, and illumination is available only about one-half
of the total time. The biological problem is to adjust conditions so as to
use the available energy most efficiently.

Efficiency of Utilization of Energy

Unfortunately the plant chloroplast is a photochemical system which
works efficiently only at low light intensity. The data on which figure 1
is based indicate that 100 f.c. is the maximum intensity at which maximum
efficiency is achieved by Chlorella. Because of the uncertainties previous-
ly noted, however, a value of 400 f.c. is adopted for the present discussion.

The difficulties of the system are made apparent by the curve of fig-
ure 3. Of the total energy absorbed, i.e. the total area under the curve,
only the small shaded fraction is utilized for photosynthesis and growth by
an unstirred algal suspension. The difficulty is that the algal cell is capa-
ble of working only at a low energy level, whereas the incident energy is at
a high level. There are three phenomena which should be explored in at-
tempts to increase the effectiveness of light utilization.

Flashing Light

Studies on photosynthesis have demonstrated that the rate of assimila-
tion at light saturation is determined by thermal or enzymatic reactions
and not by the photochemical process itself. It has been shown that short
flashes of high intensity can be used with high efficiency if separated by
sufficiently long dark periods [215, 268, 286, 296]. By high turbulence of
culture which will expose cells to alternate periods of high light at the
front surface and darkness at the back surface, it may be possible to take
advantage of such flashing-light effects. It may be possible to make the
cells integrate light intensity x tim'e so that the effective light intensity
per cell will become identical with the average intensity per cell existing
in the culture.

Studies are now in progress at the University of Texas on the growth
of Chlorella in thin suspensions under flashing light of high intensity. The
objective is to determine the range of light and dark periods under which
flashes of high intensity (up to 10,000 f.c.) can be used most efficiently for
growth. A parallel investigation by Kok is presented in chapter 6. In con-
nection with such studies it would be a very great aid to have calculations
made of the range of duration of light and dark periods which might be
experienced by cells of a suspension in a vessel of some given thickness,
at some given measure of turbulence, and at a density such that practi-
cally all the light is absorbed at about one-tenth of the thickness.

It may be noted, incidentally, that an important possible advantage of
algal culture, as compared with the farming of higher plants, depends up-
on the flashing-light effect. One may propose the plausible hypothesis that,
except for minor species differences, every plant chloroplast containing
unit amount of chlorophyll has about the same light-intensity curve (e.g.,

fig. 1). In the nearly opaque leaf of a higher plant the considerations set forth for figure 3 must hold rather completely, since the chloroplasts are relatively fixed in position; there must be rather great losses in efficiency of utilization of full sunlight intensities. In an algal suspension it appears possible to interchange mechanically the positions of the chloroplasts with time so as to average out the light-intensity distribution and attain much greater efficiency of utilization.

Improved Strains of Algae

A second possible way to increase the effectiveness of sunlight utilization is to isolate or develop strains of algae with higher values of $k_m$ax and I_s. From the small but selected sample of algae studied for rate of growth it appears unlikely that an alga will be found with a higher growth rate at 25° C (cf. table 1). The high-temperature strains of Chlorella isolated by Sorokin (see above) have significantly higher growth rates. Strain Tx 115, now under study, also has a much higher value of Is, somewhere near 1000 f.c. at 39° C, so that the area A of figure 3 should be about twice as great as that for Chlorella pyrenoidosa at 25° C. If the efficiency of utilization of the absorbed light for growth is the same for both strains, then strain Tx 115 should show about twice as great a yield per unit area of culture.

Diurnal Intermittence of Illumination

There is a third phenomenon which may operate under certain conditions to increase the effectively used light above the base area in figure 3. This effect operates by virtue of the higher capacity for photosynthesis than for cell division, and the great possible variation in size and composition, of the Chlorella cell. Average cell size, as observed under steady-state conditions in thin suspensions, may vary by a factor of four times in the range of light intensities between 10 and 360 f.c. [95, 96]. Under daylight illumination growth does not maintain a steady-state condition. It may be that at the end of the light period, large cells with a high level of storage materials continue to divide in the dark, giving rise to minimum-sized cells for the beginning of the next illumination period.

This "huff-and-puff" phenomenon is entirely a theoretical prediction. [5] The theory might be tested by comparing the effects of continuous illumination with those of 12-hour light and dark periods on thin suspensions under high light intensity. It predicts that for equal light and dark periods the growth rate k at 25° C will be greater than the $0.5 \times 1.96 = 0.98$ expected if the process did not operate at all. This effect will be significant only when the effective light intensity per cell is above the growth-saturating value, and probably not at all under the conditions anticipated for mass-

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5 Chapter 7, the manuscript of which was received after this statement was written, describes an experimental verification of this prediction.—Ed.

culture work, where I_c will probably be kept below a growth-saturating
level.

Finally, it should be noted that daylight illumination with equal light
and dark periods will approximately double the value of Ifc, the effective
intensity needed to maintain the endogenous respiration or cell overhead,
since I_c is present for only 12 hours whereas the overhead operates for
all 24 hours.

E

Summary

The biological aspect of the problem of mass culture concerns quanti-
tative characterization of conditions affecting growth in terms which allow
extrapolation to engineering development. It has been the purpose of this
chapter to present the extent and limitations of our available information
and to call attention to specific problems on which better or more exten-
sive experimental data are needed.

F

ApE^ndix: Calculations on Light Absorption by Chlorella

In considering the practical effects of turbulence and flashing light in
dense suspensions, it is desirable to have data on light absorption such as
may be calculated from results obtained by Emerson and Lewis [217]
incidental to their quantum yield work. They measured the absorption
curve for a suspension of Chlorella pyrenoidosa (as grown at low light
intensity for their measurements) using a monochromator and a large
barrier-layer cell immediately behind the suspension to pick up most
(but not all) of the scattered light. Their data are values of log IQAX
for a layer 1.4 cm thick containing 0.96 mm^ cells per milliliter. Table 2
presents calculations of a Beer's law absorption coefficient for three sali-
ent points on the wave-length curve and for use with concentration in grains
per liter dry weight and length in centimeters.

From the data of table 2, Beer's law curves for percentage of absorp-
tion at a concentration of 1.0 g/1 dry weight may be drawn as in figure 4.
It will be noted that at this concentration over 95 per cent of the red and
blue and 60 per cent of the green is absorbed in the first centimeter. At
a concentration of 10 g/1 most of the light is absorbed in the first milli-
meter. It appears easier to move a cell into and out of a 1-mm layer than
a 10-mm layer; short flashes of high intensity will be achieved more easily
by turbulence. It would appear that a system to be explored for turbulence
effects would be a layer 10 to 20 mm thick containing 10 to 20 g/1 dry weight.

The data above also allow approximate calculation of the absorption by
a single cell. Consider one cubic centimeter of suspension containing 1.0
g/1 (dry wt.) or 4.0 mm^3/cc (living cells). These cells could be packed

Table 2

Calculated light absorption of a suspension of Chlorella pyrenoidosa
using Beer's law, log IQ/IX = occx

Wave length (Å)	log I_0/lx (c = 0.96 mm³/ml = 0.24 g/1, x = 1.4 cm)	α (c = g/1, x = cm)
4400.,.............	0.62	1.9
5600.,.............	0.12	0.4
6800.,.............	0.49	1.5
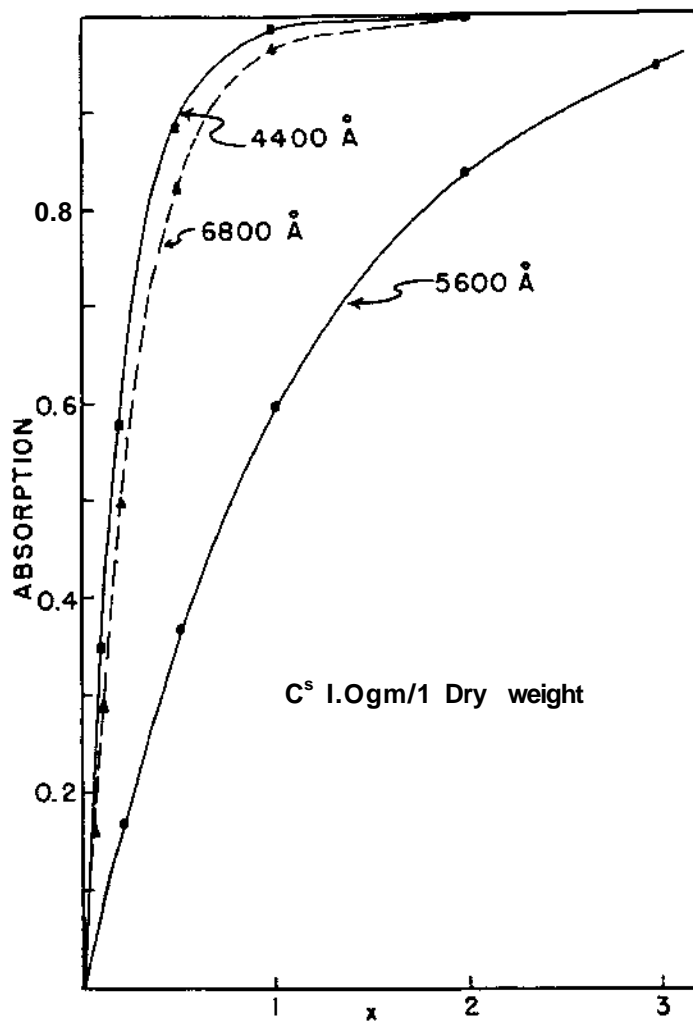
Fig. 4. Absorption of light by a suspension of Chlorella pyrenoidosa as calculated from the data of Emerson and Lewis [217].

against the front square centimeter of surface to give a thickness, x, of 0.004 cm or 40 microns. If each cell were a cube 4.0 microns on edge, this would allow 10 layers of cells, and a layer one cell thick would correspond to 0.1 cm of the suspension. The light absorption in 0.1 cm of suspension, hence in a single cell, would be 30 to 40 per cent of the incident red and blue light.

Unfortunately, the above data provide only rather poor approximations. Because of varying pigmentation, there is no fixed value of the specific absorption coefficient for Chlorella at any one wave length. Scattering corrections were not entirely taken care of by the method of Emerson and Lewis.

An over-all comparison is afforded by unpublished data of Kok. He observed the absorption by a different strain of Chlorella in the 5890 Å sodium lines, using an integrating sphere to minimize scattering errors. His d^ta yielded a value of 0.29 for the specific absorption coefficient at 5890 A, compared with a value of 0.45 from the data of Emerson and Lewis.

Chapter 5

THE EFFICIENCY OF LIGHT-ENERGY CONVERSION IN CHLORELLA CULTURES AS COMPARED WITH HIGHER PLANTS[1]

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A

Introduction

Rabinowitch [265(a)] estimated the efficiency of energy conversion by green plants to be about 2 per cent of the solar radiation which reaches the earth's surface and is usable in photosynthesis (that is, excluding the infrared). This means that, during their growth, they convert about 2 per cent of the usable solar energy into chemical energy (organic matter), Wassink [295] made calculations based on optimal yields of agricultural crops in the Netherlands, and obtained figures of from 1 per cent to about 2 per cent of the usable radiation incident on the cultivated surface. The estimates were based on the total dry weight of the crop, assuming its average composition to be CH_2O. A summary of these data is given in table 1.

For comparison, the efficiency that one would expect to obtain can be calculated from the rate of photosynthesis. Extensive experimental evidence has been collected, e.g. in short-time experiments with Chlorella. Assuming a quantum yield of 0.10-0.12 per mol of oxygen evolved, and quanta of 50 kcal/mol $h\nu$, the calculated efficiency is about 25 per cent [182, 218, 288].

Any attempt at successful mass culturing of Chlorella with good energy conversion has to start with an analysis of the reasons for this discrepancy.

The following points seem important: First, the high efficiency of photosynthesis by Chlorella has been obtained in experiments of short duration, with "resting cells/' Second, under similar conditions leaf disks of higher plants show quantum yields of about 0.07 to 0.1 in sodium light, which means an efficiency of about 20 per cent [294].

55

Table 1

Efficiency of solar energy conversion during the growing season of some agricultural crop plants
(from Wassink [295])

(1) Crop plant	(2) Production of dry matter (g/cm^2)[a]	(3) Chemical energy of dry matter evaluated as CH_2O $(ergs/cm^2)$	(4) Growing period[b]	Solar radiation[c]			(8) Efficiency $= \frac{\text{column 3}}{\text{column 7}}$ (%)
				(5) Total radiation received (cal/cm^2)	(6) Usable in photosynthesis (excluding infrared) (cal/cm^2)	(7) Values of column 6 in $ergs/cm^2$ $\times 10^{12}$	
Onions 3.5		0.55	April-Sept.	58,000	29,000	1.22	0.45
Carrots. 6.86		1.07	May-Oct.	54,400	27,200	1.14	0.94
Potatoes. 9.6		1.5	April-Sept.	58,000	29,000	1.22	1.23
Wheat. 10.45		1.62	Nov.-Aug.	61,000	30,500	1.28	1.26
Rye grass							
(Lolium). 10.2		1.60	March-Oct.	67,500	33,800	1.42	1.13
Beets, mangels 16.0		2.5	May-Oct.	54,400	27,200	1.14	2.20
Maize. 12.8		2.0	May 10-Sept. 10	43,600	21,800	0.92	2.18
Sugar cane_____33.0		5.2	April-March	129,000<[d]	64,500	2.70	1.92

a From agricultural data.

k The months named are included.

c Calculated after Reesinck [266], measurements made at Wageningen (except those for sugar cane).

d Recalculated from recent measurements by Dee and Reesinck at Djakarta [214{a)]. This value is not far from the one used previously [295], derived from data reported by Boerema in 1920 (cf. [295]), viz., 120,000 cal/cm2, yielding an efficiency of 2.05 per cent.

One of the first questions to be answered is whether a fundamental difference exists between the photosynthetic efficiency found in short-time experiments with resting cells and that observed under conditions of active growth. It should be noted that in experiments of short duration a correction for respiration is included in the estimate of the efficiency. On the other hand, it is not easy to make an allowance for respiration losses during growth when efficiency is based on the production of organic matter. This difference, however, cannot explain the large discrepancy observed.

If the low efficiency of higher plants is due to some external limiting factor such as excessive light intensity, low carbon dioxide content of the air, unfavorable temperature or humidity (see [295]), algal cultures might lend themselves more readily to improvement in efficiency. For instance, carbon dioxide could be supplied more easily, effects of too high light intensities could be decreased by sufficient density of culture and by adequate turbulence (cf. [215]), suitable temperatures might be more easily maintained, humidity is no problem, and conditioning of the environment in general seems more feasible.

A brief account follows of the chief results of experiments carried out along the lines Indicated in this laboratory during the past few years. A

more detailed report will appear later. Experiments on the efficiency of
the production of organic matter by Chlorella and higher plants under con-
trolled conditions were started in 1948 by the first author with advanced
students; a few of their results appear below. In 1949 the Netherlands
Organization for Applied Scientific Research (T.N.O.) made regular work
possible. Kok entered the group and then, during his stay in the United
States in 1951, was replaced by Van Oorschot. Kok confirmed the prelim-
inary data obtained on the efficiency of Chlorella growth, making studies
of total balance under controlled conditions [241]. Van Oorschot chiefly
concentrated on the efficiency of mass cultures both indoors and outdoors.
Some additional information on the efficiency of higher plant growth was
also obtained.

<div align="center">B</div>

Efficiency of Chlorella Growth in Small Cultures

Preliminary Observations

Experiments on Chlorella growth in light from incandescent lamps,
with a view to estimation of the efficiency of energy conversion, started
by Miss J. T. de Vries in June 1948, were extended and improved by J. F.
Bierhuizen early in 1949. He cultivated Chlorella vulgaris (strain "Bot-
anie") in 0.5-liter inverted glass-stoppered bottles, each having two holes
with cotton plugs in the bottom and an aeration filter mounted in the neck.
Each bottle was placed in a small light cabinet with an opaline glass and a
filter (BG 17, 3 mm, Schott) in the front. Four cabinets were arranged
around a. water-cooled 300-watt incandescent lamp, the temperature being
22-23° C. Incident and transmitted light were measured at various places
in the light cabinet with a barrier layer photocell, and were converted to
ergs per square centimeter per second by special measurements. The
cultures were aerated with air containing 5 per cent CO_2.

The growth curves showed the normal S-shape. With suitable correc-
tions for light losses, the efficiency of dry-matter production (evaluated
as CH_2O) calculated over the period of active growth, which was about 10
davs, was 12-15 per cent at 5000-8000 lux, 20-24 per cent at 1500-3000
lux (1 lux = 0.0929 f.c). Though preliminary, these experiments led to
the impression that the efficiency in growing cultures is not appreciably
lower than that observed in current experiments on photosynthesis.

Balance Experiments

Kok reinvestigated the question under still better defined conditions.
Chlorella strain "A" 2 was cultivated in a Warburg apparatus with vessels
of about 250 ml capacity (containing 100 ml of nutrient medium) under con-
tinuous illumination with sodium light at 25° C, for about 4 days; the maxi-
mum light intensity was about 2 x 1Q4 ergs/cm2/sec. Six manometers

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[2] This strain looks very much like Chlorella vulgaris. We have not yet been able to compare
it with C. pyrenoidosa grown under the same conditions.

were used at a time, receiving, e.g., different light intensities. During the experiment, air with 3 per cent CO_2 passed through the vessels. When measurements of gas exchange were made, the manometers were closed and the gas phase was in equilibrium with Pardee solution [256] in a central well.

The following data were collected: the rates of photosynthesis and respiration, the light energy absorbed, the amount of dry matter produced, its chemical composition, 3 and in certain cases its heat of combustion. 4 In one experiment 88 mg of dry matter was produced, of the following composition: C, 50.4 per cent; H, 7.1 per cent; N, 9.6 per cent; ash, 6 per cent. The heat of combustion was 5.77 kcal/g, so that 548 cal were fixed. The algae had received 2335 cal light energy; the efficiency thus was 23.5 per cent.

Of about 30 determinations of efficiency in experiments of this type, the majority were between 12 and 21 per cent. These experiments may be considered to have proved definitely that under favorable conditions the efficiency of algal growth is of the same order of magnitude as that of photosynthesis observed in experiments of short duration.

Additional Observations

Kok found indications that at high light intensities carbon fixation surpasses nitrogen assimilation, a condition which results in cells with low protein content (cf. also [151]). Alternation of day and night periods was found to favor protein synthesis [241], The rate of respiration appeared to be correlated with that of photosynthesis; their ratio thus is independent of light intensity. At very low light intensities this correlation does not hold, because some residual respiration remains, which results in decreased efficiency of growth. Nitrogen starvation, aiming at high fat content, tends to give low efficiency.

Our observations as to the effects.of composition of the culture medium are still limited. We observed that Chlorella, in cultures of about 0.5 liter, under continuous light from fluorescent tubes attains a maximum density, in the culture medium used, of about 7 mm3/ml; renewal of solution every 2 or 3 days increases the density to 40 mm3/nil, 5 whereas addition of minerals during growth results in intermediate densities.

C

Efficiency of Chlorella Growth in Mass Cultures

Outdoor Mass Cultures

Outdoor cultures of Chlorella, strain "A," were started late in the summer of 1950 and continued in **1951.** Most of the reported data have been col~

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3 We wish to thank Dr. Van der Kerk, Utrecht, for carrying out these determinations.
4 We wish to thank Professor Coops, Amsterdam, for carrying out these determinations.
5 Dry weight was not determined in these experiments. For some mass cultures 1 g of dry weight (ash included) corresponded to an average amount of 8.4 cm^ of fresh cells.

lected by Van Oorschot. Concrete tanks of 1 m2 area and 300 liters capacity were filled with simple inorganic nutrient solution to a depth of 30 cm and inoculated with 10 liters of pure culture. The nutrient solution used was either Warburg's solution [291], containing 1.00 g Ca(NO3)2, 0.25 g KNO3, 0.15 g NaCl, 0.25 g KH2PO4, 0.50 g MgSO4 '7H2°> °-02 £ FeSO4 * 7H2O, and 0.04 g sodium citrate per liter of tap water, or a modification of the medium of Benson et aL [190], containing 0.50 g KNO3, 0.136 g KH2PO4, 0.50 g MgSQ$_4$- 7H$_2$O, 0.02 g FeSO4 • 7H$_2$O, and 0.04 g sodium citrate per liter of tap water.

The mass cultures were grown under natural conditions in full sunlight. The tank was covered with a glass plate to keep it as clean as possible, "semiaseptic." During growth the cultures regularly received carbon dioxide from a cylinder, and the suspension was stirred strongly with a motor stirrer. After 5 to 7 days of growth the algae were harvested, washed, dried, weighed, and sampled to determine chemical composition.

Data obtained in connection with these experiments are given in table 2. Series A consists of 13 experiments in full daylight. The experiments of series B and C are parallel experiments, made later in the season; series B received full daylight, in series C daylight was weakened to 22 per cent. The efficiency was calculated on the basis of ash-free dry weight, taking into account the R-value [151].

For the full-light cultures (series A and B) the efficiency was 2.6 and 2.7 per cent respectively; in series C (22 per cent light) it was 6.3 per cent. In series A the average percentage of ash is high (table 2), because certain cultures showed a remarkably high percentage. The reason for this is not known, but it may be assumed that the growth of the algae led to the deposition of some chemical, probably calcium phosphate, upon the cells or between them. (Since the efficiency determinations were made on an ash-free basis, this phenomenon did not influence the efficiency values.) The high ash values were encountered only in Warburg's medium, not in that of Benson et al.

Indoor Mass Cultures

Two concrete tanks of the same type as those used for the outdoor cultures were set up in a dark room, and illuminated continuously with eight daylight fluorescent tubes each. In some experiments, a much higher light intensity was achieved with four 1000-watt incandescent lamps. The inoculation and maintenance was similar to that in the outdoor cultures, Chlorella, strain *"A,"* being used.

Data related to these experiments are given in table 3. The temperature was about 24° C in series A. It was about 32° C in series C, owing to the high energy input. In series B, serving as a parallel to series C, the same temperature was attained with the aid of additional electric heating.

The incident energy reaching the surface of the culture was measured at various places with a photocell; these readings were averaged, and converted to absolute units by comparing them at one place for each meas-

Table 2

Some data concerning environmental conditions, yield, chemical composition,
and efficiency of light-energy conversion in <u>outdoor</u>
mass cultures of Chlorella

	Series A [a]	Series B [b]	Series C [c]
No. experiments[c]	13	4	4
Incident energy (kcal/m^2/day)[d]	1757	1222	269
Yield, ash-free dry weight (g/m^2/day)	7.15	4.92	2.70
Temperature (°C) 9 a.m.	16.8	15.5	14.8
Temperature (°C) 5 p.m.	25.7	24.8	22.8
Efficiency *(%)* [e]	2.6	2.7	6.3
Average chemical composition:[f]			
No. experiments[g]	8	2	2
Carbon (%)	41.1	47.4	45.7
Hydrogen *(%)*	5.9	6.5	6.5
Nitrogen (%)	9.2	10.7	9.7
Ash *(%)*[h]	22.4	12.8	15.4
R-value	44.0	44.8	44.8

[a] May 23 to October 8, 1951.
[b] August 23 to October 8, 1951.
[c] Items below are averaged over the mentioned number of experiments.
[d] Exclusive of infrared radiation. Computed from radiation measurements by *the* Physics Department of the Agricultural University, Wageningen.
[e] Computed from ash-free dry weight.
[f] Oxygen estimated as 100% - (C+H+N+ash).
[g] Not all cultures have been analyzed.
[h] See text.

urement with readings of a standardized thermopile. Readings of the thermopile were made with and without the filter (RG8, Schott and Gen). This filter transmits radiation only with wave lengths greater than 7000 Å, which were considered to be inactive in photosynthesis. The difference between the two thermopile readings was used for the conversion of the photocell measurements.

The efficiency was calculated in the same way as for the outdoor cultures. The 9 experiments of series A with low light intensity gave an average efficiency of 13.3 per cent, the maximum being 19.7 per cent. The average efficiency obtained in series B, with low light intensity, was 10.9 per cent; the average efficiency obtained in the parallel series C, with high light intensity, was 4.7 per cent.

Conclusions

(1) Semiaseptic Chlorella cultures in large tanks are capable of producing high efficiency (about 15 per cent or more of the incident light).

Table 3

Some data concerning environmental conditions, yield, chemical composition,
and efficiency of light-energy conversion in indoor

mass cultures of Chlorella

	Series A [a]	Series B [a]	Series C [a]
No. experiments*[3]	9	3	3
Incident energy $(kcal/m^2/24-hr)$[c]	368	300	2650
Yield, ash-free dry weight $(\%/m^2/U$ hr)	7.70	5.20	19.3
Temperature (°C)	24	32	32
Efficiency (%)d	13.3	10.9	4.7
Average chemical composition: [e]			
No. experiments^	7	1	1
Carbon (%)	49.1	43.5	44.2
Hydrogen (%)	6.8	6.2	6.1
Nitrogen (%)	10.6	7.9	10.9
Ash (%)	11.2	19.2	16.2
R-value	46.0	44.2	43.6

[a] Series A and B irradiated continuously with eight daylight fluorescent tubes; series C irradiated continuously with four 1000-watt incandescent lamps; series B and C consist of parallel cultures; duration of each experiment 3-6 days.

" Items below are averaged over the mentioned number of experiments.

[c] Exclusive of infrared radiation. Measured with calibrated photocell (see text).

d Computed from ash-free dry weight.

[e] Oxygen estimated as 100% - (C+H+N+ash).

* Not all cultures have been analyzed.

(2) Under conditions of natural illumination in summer the efficiency was found to be 2 to 3 per cent.

(3) In both indoor and outdoor mass cultures, lower light intensities give rise to higher efficiency.

(4) In proportion to the amount of energy received in 24 hours, the efficiency of the indoor cultures is higher than that of the outdoor ones. This may be due to the more favorable indoor temperatures and to the regular spreading of the energy over the whole 24-hour period. Especially, excessive illumination (far above the limiting range of photosynthesis) around noon may be a factor in the low outdoor efficiency. Indoor "day and night'* experiments have now been started.

(5) The rather vigorous stirring has not shown a clear beneficial effect on the efficiency of outdoor cultures in full light in summer, for the average efficiency so far does not greatly surpass that of the best crop plants. Experiments with flashing light by one of us, reported in chapter 6, point to the necessity of very quick and effective changes of light and darkness-

(6) Improvement of outdoor efficiency may be expected from improved stirring, combined with further studies as to optimal density of culture; from maintenance of favorable temperature; from further studies of the most favorable composition of the medium; and from use of cell material with increased "Blackman" capacity, i.e. ability to use bright light efficiently.

D

Efficiency of Higher Plants

At the side of the outdoor Chlorella tanks a plot of grass, 1 m2 in area, in an existing lawn was harvested. The plot was regularly watered and supplied with nutrient solution. Three successive harvests (between May 29 and October 26) gave an average efficiency of 2.6 per cent of the incident light (without infrared); the material had the following composition: C, 40.1 per cent; H, 5.8 per cent; N, 4.3 per cent; ash, 16.8 per cent; the H-value was 37.9.

Beet seedlings were raised in the laboratory under fluorescent tubes at 20° C, and successive harvests were compared with the energy that had fallen on the leaf surface. Efficiency values of 11 to 15 per cent were recorded. This estimate was based on total dry matter, for which the composition CH_2O was assumed. Similar experiments performed by D. J. Glas and P. Gaastra in this laboratory in 1949 had given efficiency values of 12 to 19 per cent. Thus, the situation in higher plants, especially as compared in indoor and outdoor cultures, seems much the same as in algae. Beetroot field crops have an efficiency of about 2 per cent.

The results of all experiments performed so far favor the supposition that the excessive solar radiation in summer is one of the chief reasons for the low efficiency values observed under natural conditions both in higher plants and in mass cultures of algae.

E
Summary

The efficiency of light-energy conversion in both small- and large-scale cultures of Chlorella is 12 to 20 per cent, provided the intensity of the illumination is not too high. The same holds for cultures of higher plants. Outdoor stirred mass cultures of Chlorella in full summer light show efficiency values of 2 to 3 per cent, as do good field crops of higher plants. Excessive illumination seems an important factor in producing low efficiency under natural conditions.

Chapter 6

EXPERIMENTS ON PHOTOSYNTHESIS BY CHLORELLA IN FLASHING LIGHT[1]

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Intermittence of illumination of an algal culture may provide a means of utilizing a larger fraction of the sunlight shining on a given area, as is explained in section D of chapter 4. A quantitative study of the effect of intermittence on photosynthesis was expected to yield basic data that could be used in designing a system for growing algae under suitable conditions of intermittent illumination, such as might be realized in a turbulently flowing culture.

Although several investigations of this subject have been made in the past [197, 215, 268, 286, 296], the data obtained did not cover a sufficiently wide range of variables for our purpose. The experiments described in the present chapter indicate patterns of intermittence according to which the energy of sunlight may be used with increased efficiency in growing algae.

A
Experimental Technique

General Method

A previous study [241] demonstrated that the rate of photosynthesis is a usable index for growth of algae. Therefore quick and simple manometric measurement of the oxygen evolution that accompanied photosynthesis can be used to characterize the amount of growth to be expected from the absorption of a given amount of light energy.

The measurements were carried out with a volumetric apparatus to be described elsewhere [242], using a thin layer (about 3 mm) of algal suspension in vessels of two sizes, having exposed areas of 30 and 3 cm2, respectively. Oxygen evolution was observed each minute for periods of 10 to 15 minutes; the observed rates were corrected for dark respiration. In this way the same sample of cells could be subjected to a series of dif-

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[1] A more detailed presentation and discussion of this study will appear elsewhere. This will include consideration of its bearing on the problem of the mechanism of photosynthesis.

ferent conditions during one experiment. Since algae, especially when grown in sunlight, are not a very reproducible experimental material, and since photosynthesis rates at high light intensities are not very stable, this quick method was advantageous.

Light Source

A 1000-watt incandescent lamp was used to illuminate the algae with light having an intensity clgse to that of solar radiation. Only light of wave lengths shorter than 7000 Å is active in photosynthesis. Therefore the energy was measured by means of a thermopile, first with and then without a Schott RG8 filter. In this way the infrared radiation (passed by this filter) could be subtracted from the total intensity of the light beam to give the energy having wave lengths shorter than 7000 Å.

The highest intensity used in most experiments was about 25×10^4 ergs/ cm2/sec (equivalent to about 7000 f.c), which was the intensity of direct sunlight (4000-7000 Å) at Palo Alto, California, measured with the same instrument. In experiments with the smaller manometric vessel the intensity could be raised to three to five times this value.

Intermittence

The light-dark cycles were varied both in speed and in ratio of light to dark period, in contrast with the work of earlier investigators who used constant flash times with different lengths of dark interval. This regular intermittence was obtained by using a rotating sector consisting of two blades, each with two 90-degree cutouts, the relative positions of which determined the angular opening and hence the ratio of light to dark periods, which ranged from 1:1 to 1:100. The frequency of illumination was determined by the speed of revolution of the disk, which was varied from 30 to 2300 revolutions a minute.

An image of the evenly illuminated plane of the sector was focused on the thin layer of algal suspension by means of a lens of fair optical quality. In this way erroneous optical effects were reduced to a minimum and we were sure that the algal cells received flashes of light having the intensity of sunlight.

Algal Suspension

The experiments were made with Chlorella pyrenoidosa (Emerson strain) grown in one or the other of two annular growth chambers. These chambers, constructed by Myers [101], were operated without photoelectric control of cell density. One chamber was illuminated with sunlight outdoors and the other with fluorescent tubes indoors. A flow of fresh medium diluted the suspension continuously. By changing the rate of flow it was possible to vary the density of the culture. The outdoor culture was diluted rather rapidly, so that the suspension remained thin enough for all algal

cells to be exposed to nearly the full intensity of sunlight during growth. We thought it of interest to see how far adaptation to strong light could proceed in Chlorella. Table 1 illustrates the differences observable between the two types of cell (data from two experiments at 25° C).

Table 1

Chlorophyll content and saturation rate of Chlorella grown outdoors and indoors at 25° C

	Outdoors	Indoors
Chlorophyll content (y per mm^3 cells)	3	12
Saturation rate ($mm^\wedge$ 02 per 10 min per $mm^\wedge$ cells)	3.2	8.5
Saturation rate ($mm^\wedge$ O2 per 10 min per y chlorophyll)	1.1	0.65

In most of the flashing-light experiments the Chlorella cells, after being harvested from the growth medium, were resuspended in a carbonate-bicarbonate buffer (85/15 NaHC03/K2CO3, 0.1 molar) and the oxygen evolution was measured. Because this buffer mixture has a pH of 9.2, experiments were also carried out in acid media, either by using diethanolamine as a carbon dioxide buffer in a separate compartment of the vessel, or by measuring the gas exchange without any buffer present. In both cases a 3 per cent CO2 atmosphere was used. No consistent differences were found between the different media, either in continuous or in intermittent illumination.

The quantity of cells* used for most experiments (0.6 to 2.5 mm^3/cm^2) was such as to provide 8 y of chlorophyll per square centimeter of irradiated surface, as determined from a measurement of the chlorophyll content of each sample. This value was an arbitrary compromise between low absorption and still measurable rates. Check experiments with higher quantities of cells gave identical results in terms of the relative efficiencies, a fact which indicated that no erroneous effects of too high fractional absorption interfered.

B

Results

Typical Observations

With a single sample about thirty measurements could be made in a day, both with different openings of the disk and with different speeds. The observed volumes of oxygen evolved per unit time were plotted as a function of the amount of light energy incident on the cells during that time, as shown in figure 1. In continuous light the rate Z was observed. When neutral filters were placed between the vessel and the light source to decrease the amount of light, the rate of photosynthesis decreased as shown by the solid curve ZS0. Observed points from an actual experiment corrected for respiration are shown by the crosses in figure L
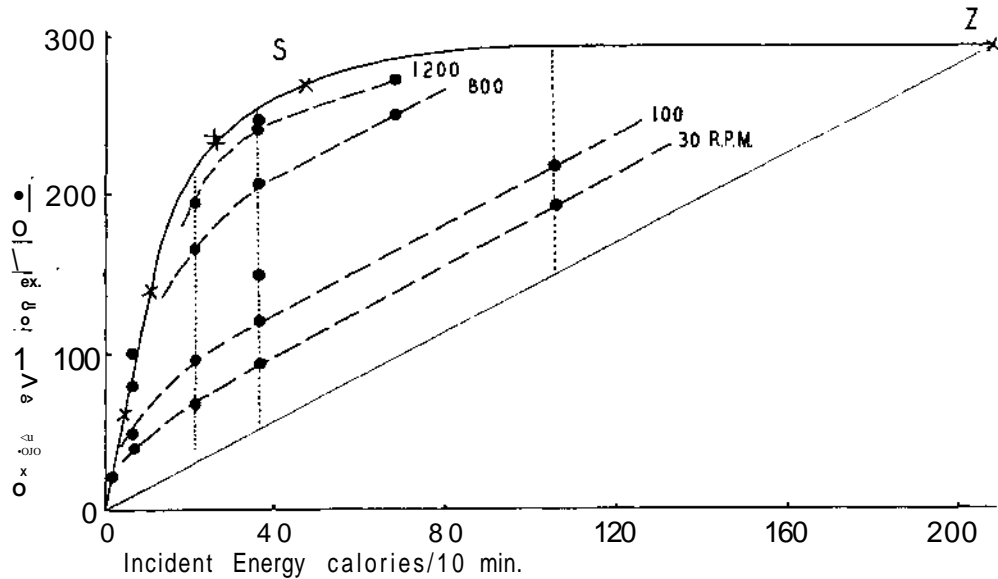
Fig. 1. Rate of photosynthesis (measured by amount of oxygen evolved) by "indoor" Chlorella cells (60 mm3 suspended in buffer mixture, pH 9.2) exposed to different amounts of light energy from a constant light source reduced either by filters (crosses) or by a rotating disk (circles). The numbers indicate the speed of rotation.

In flashing light the rate of photosynthesis depends on the ratio of light to dark periods and their absolute times as well as on the amount of light. For instance, if we keep the speed of the disk constant but change its opening (thus increasing the incident energy), the rate increases in the way shown by the dashed lines in figure 1. If, on the other hand, with a given disk opening, we increase the speed of rotation, the rate will increase as shown by the dotted lines in figure 1. At low speeds the rates approach points on line 0Z, whereas at very fast speeds they approach points on line OSZ.

This increase of the rate with increasing disk speed was studied more accurately in an experiment using a disk opening $(t_0 + t_p)/t; F = 5.5$, where t_D is the "dark time" and tp is the "flash time/' The result is given in figure 2, in which for convenience the flash time (being inversely proportional to the disk speed) is plotted on a logarithmic scale. The rate in continuous light of the same integrated intensity (defined below) was taken as 100. It must be remarked, however, that in this particular case this rate did not represent the optimal efficiency, because of the high value of the integrated intensity.

This effect of increased disk speed is essentially the effect to be expected from increased turbulence in Chlorella cultures.

An experiment involving flashing light of different intensities, obtained by combining the neutral screens with the rotating disk, is shown in figure 3.
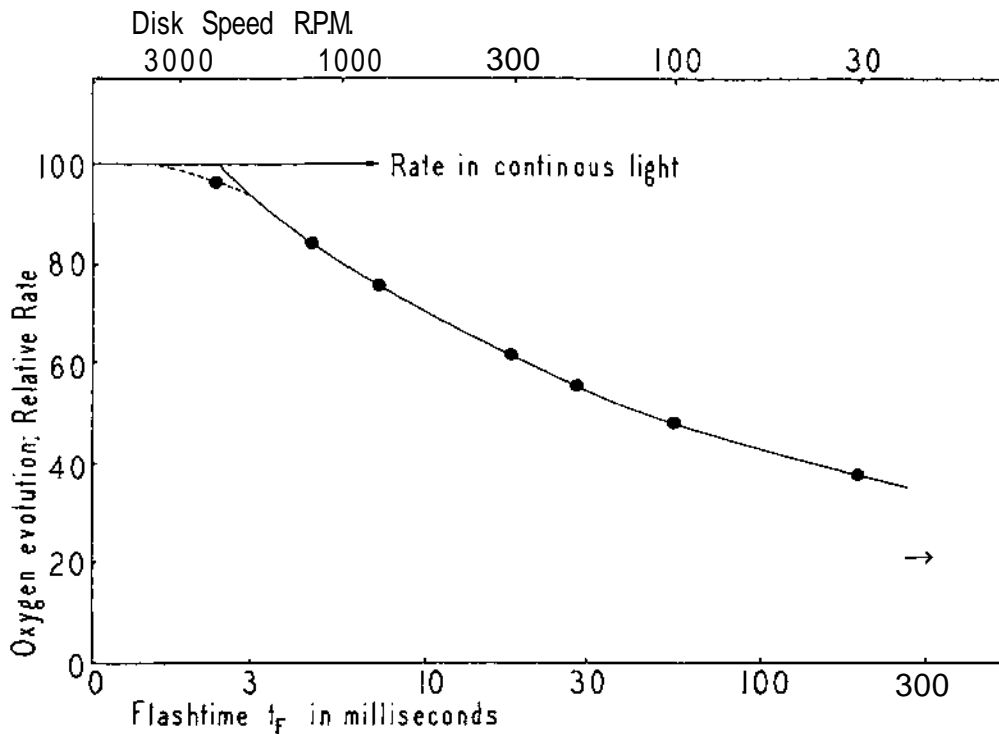
Fig. 2. Relative rates of photosynthesis in flashing light of different flash times, the ratio of flash time to total period being constant. (tj}+ tp)/tp = 5-5- Experimental data same as in figure 1.

The solid line was obtained in the same way as that in figure 1, using an-other sample of Chlorella. The three open circles indicate rates obtained with three flashing-light regimes without filters. Insertion of the filters decreased the rates for these respective flashing-light regimes as shown by the dashed lines that join the observed points marked by solid circles.

Comparative Rates and Efficiencies

A summary of the foregoing observations is presented, in somewhat idealized form, in figures 4 and 5. Along line OS in figure 4, photosynthe-sis is proportional to light intensity, and the light is used with optimal and constant efficiency. From S to Z the efficiency drops, as is shown in figure 5, where the efficiency along OS is taken as 100 per cent. The absolute value of this efficiency had already been studied separately [241], as re-ported in chapter 5,

In these figures the abscissa is the average integrated intensity ($1|$) of the light, which is the average intensity over the period of time during which the rate of photosynthesis was measured, expressed as a fraction of full intensity (I_o) of the **"sunlight"** used in these experiments. ($I_o = 25X1Q^4$
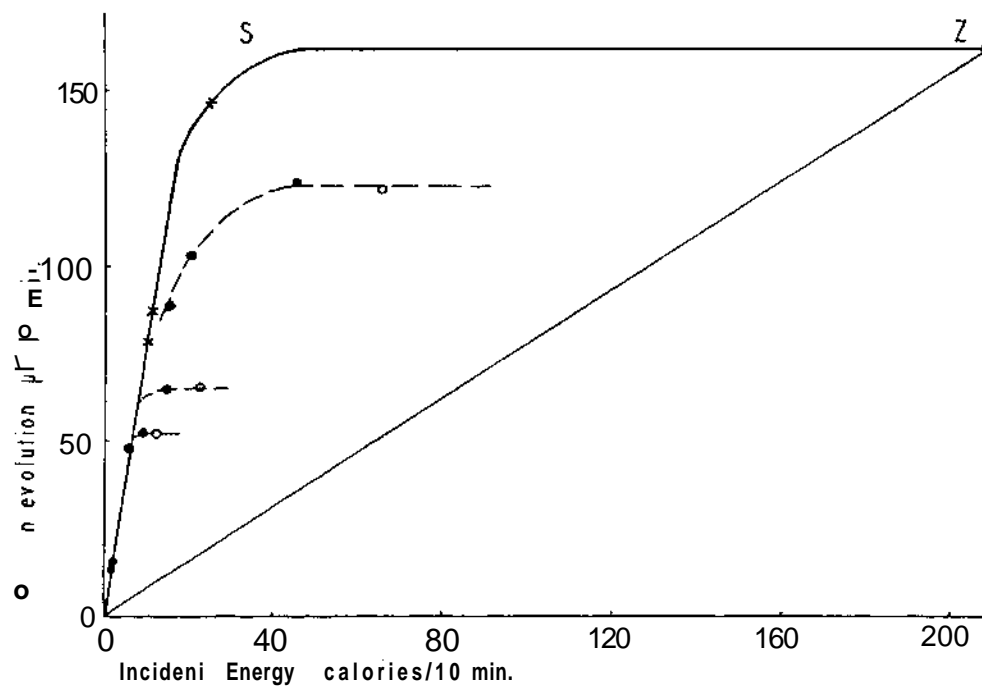
Fig. 3. Hate of photosynthesis by "indoor" Chlorella cells (40 mm^3 in buffer mixture) exposed to different amounts of light energy from a constant light source reduced by combining neutral screens with a rotating disk.

ergs/cm2/sec.) A particular value of I_j could be attained in any one of several ways. For a given intensity I_g of the source of light, the same I_j could be obtained either by means of continuous illumination through a neutral filter or by means of flashing light. For most of the experiments, *Z $^=$ *0> ^ut *ior* some, I_z was greater than IQ.

Point A in figure 4 represents the rate of photosynthesis for a given regime of intermittence, and the corresponding point in figure 5 indicates the relative efficiency. For this point the "flash time" (tp) is 16 msec and the "dark time" (tp) is 84 msec, which gives $I_j = 0.16$ for $I_z = I_0$« The variables studied in these experiments influence the rate of photosynthesis and the efficiency in the ways indicated by the groups of three lines through points A in the two figures.

If we keep tp and *tjy* constant and vary I_z from 0 to IQ, a saturation curve OGA in figure 4 is obtained. Point G indicates the intensity IQ at which the saturation rate of photosynthesis has been reached for this inter-mittence pattern. A value of $I_z = (I_G/*A) *G$ could be used in this pattern with full efficiency. When I_z is increased beyond this value, the rate of photosynthesis remains constant along the line GA in figure 4, but the efficiency decreases, as shown by the line GA in figure 5, Further in-
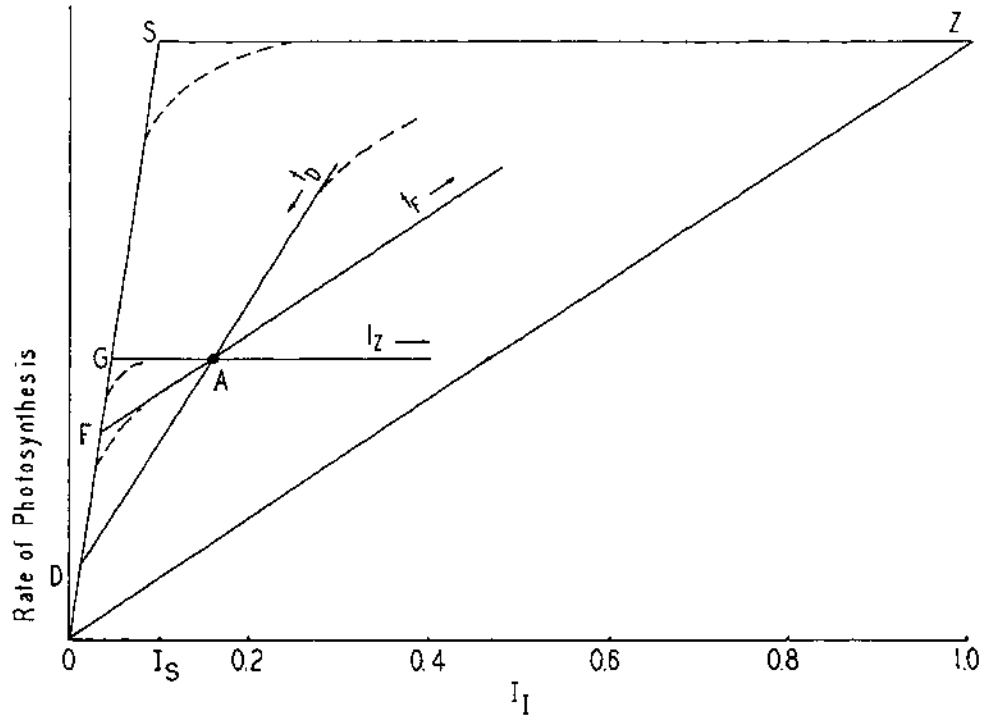
Fig. 4. Rate of photosynthesis by Chlorella cells in flashing light having different patterns of intermittence.

crease of I_z above IQ would give the same rate, as indicated by the horizontal line to the right of A, and a further decrease in efficiency^ as shown in figure 5.

If t_F is varied, while t_D and $Iz = I()$ are constant, the rate of photosynthesis increases along OS to the point F, which indicates the integrated intensity corresponding to the saturating flash time (t'p) for the given dark time. (In the present example t_F is about 3.1 msec.) From F the rate of photosynthesis increases more slowly, following approximately a straight line through A parallel to OZ. Figure 5 shows that the efficiency drops rapidly beyond point F.

Finally, we can consider the effect of varying tjj, with tp and *1%* constant. A slightly curved line from 0 through A is followed as tjj decreases. For shorter dark times the efficiency drops rapidly, as shown by the line DA in figure 5.

Precision

The actual relations were less simple than is suggested by the solid lines in figure 4. Depending on the type of cell studied, the saturation
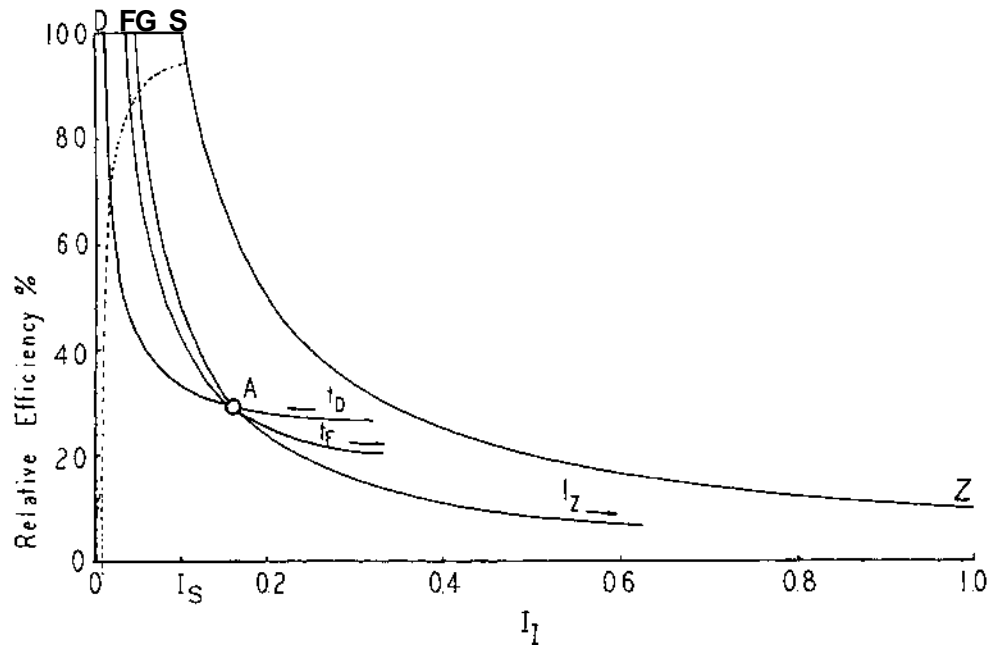
Fig. 5. Relative efficiency of photosynthesis by Chlorella cells in the different patterns of light intermittence shown in figure 4. The dotted line refers to respiration losses; see section D.

levels varied and transition ranges appeared, as indicated by the dotted lines in that figure. For instance, instead of a rather sharp bend at S ($I_j \approx 0.1$), which was usually observed for indoor cells, smoothly bent curves extending to $I_j *7 \, 0.35$ were observed for cells grown outdoors.

For each experiment the efficiency of light utilization was computed for each observation, taking the slope of OS as 100 per cent. The general relations obtained in this way were consistent, although the numerical values of efficiencies varied as much as 20 per cent for some points, especially near transition ranges. For the final compilation of data on which figures 6, 7, and 8 were based, the mean values of several replicate experiments were used.

C

Intermittence Patterns

Ratio of Dark Time to Flash Time

Figures 4 and 5 show that no pattern of intermittence can give 100 per cent efficiency unless $I_j < I_{g_s}$ where I_g is the intensity at which saturation is reached in continuous light. This means that in sunlight algal cells
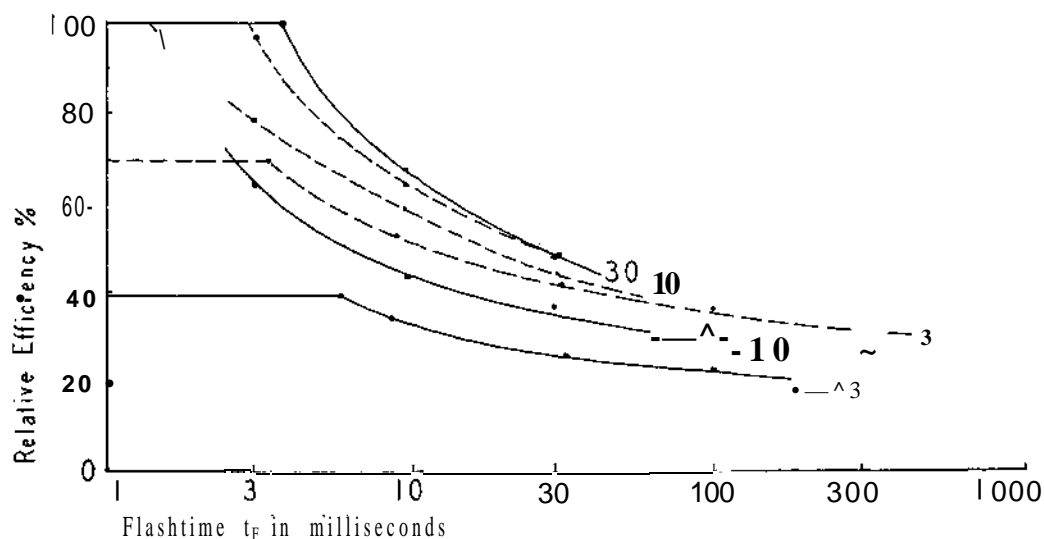
Fig. 6. Effect of length of flash time of flashing light on the relative efficiency of photosynthesis by Chlorella cells grown indoors (solid lines) and outdoors (dashed lines).

such as were used in these experiments have to remain in the dark at least ten times as long as in the light. In general, t_{jy}/t_F must be greater than IQ/IQ in order to obtain maximum efficiency. (For other algae I_g may not approximate 0.1 $IQ_?$ as it does for <u>Chlorella pyrenoidosa</u> at 25° C.)

Absolute Values of Flash Time and Dark Time

In addition to fulfilling the condition expressed in the preceding paragraph, the flash time must be sufficiently short to realize full efficiency. (Compare figure 2.) In figure 6 the efficiency is plotted as a function of the flash time, using 3, 10, and 30 for the ratio $(t_D + t_F)/t_F$ as secondary parameters. This figure shows that the flash time for the cells grown indoors had to be less than 4 msec in order to achieve full efficiency.

Cells Grown Outdoors

Most experiments were performed with cells grown indoors, because their response was more uniform. It is of practical importance, however, to compare the results obtained with those from cells grown **outdoors,** which are given by the dashed lines in figure 6. The difference between
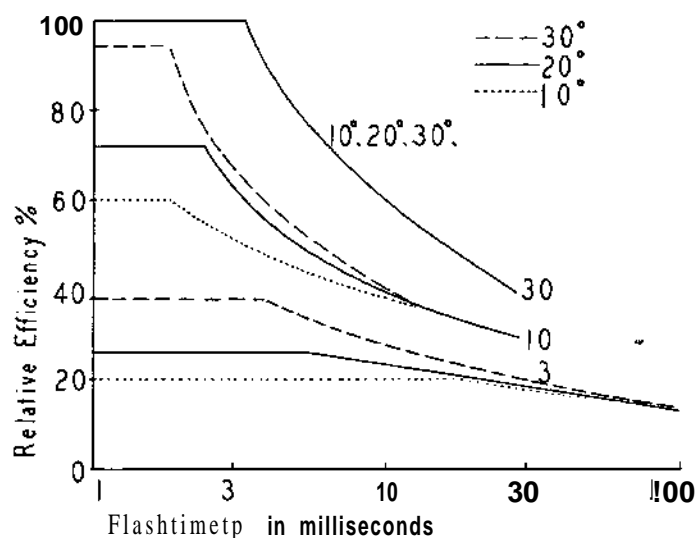
Fig. 7. Effect of temperature on the relative efficiency of photosynthesis by Chlorella cells in flashing light having different patterns of intermittence. The ratio $(tD + tp)/tp$ is given by the number at the end of each group of lines.

the results with the two types of cell is relatively small. The shift of these curves with respect to the solid lines for the cells grown indoors is easily explained by the higher saturation level usually shown by the outdoor cells, which has already been described.

Effect of Temperature

The essential effect of lowering the temperature is to lower the saturation level of photosynthesis, with the slope of line OS in figure 4 remaining constant. Therefore, the saturation intensity (Ig) is simultaneously lowered. In a given set of experiments the saturation intensity was 0.13 IQ at 30°, 0,07 I_o at 20°, and 0.04 IQ at 10° C. The effect on the efficiency achieved at these lower temperatures, which is plotted in figure 7, results mainly from this change in the saturation curve. For ratios of $(tD + ty)/tp = 3$ and 10, the limiting condition $ti)/t_F > IQ/I_s$ is not fulfilled at 10° and 20°.

Effect of Intensity

The results presented so far have been for a light source of a particular intensity. The effect of a much stronger illumination was also studied, with the results shown in figure 8. Extremely short flash times (less than 1 msec) are required to achieve full efficiency. The general effect of varying the intensity *{!%)* of the light source is given in figures 4 and 5.
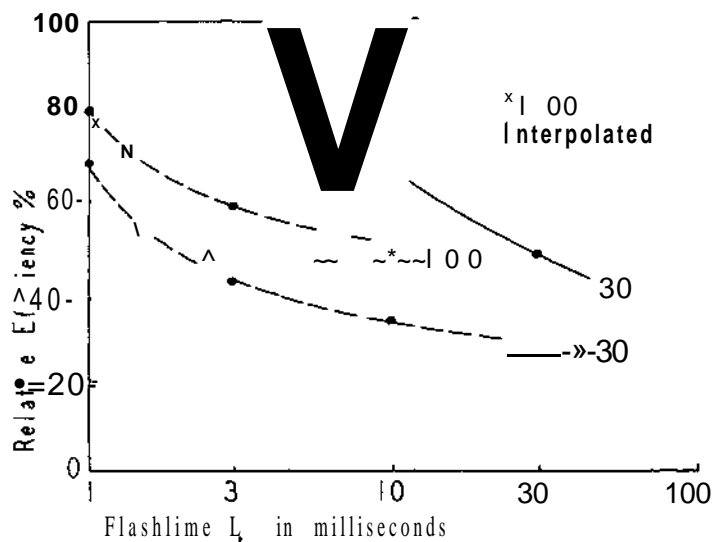
Fig. 8. Effect of intensity of light source (Ig) on the relative efficiency of photosynthesis by Chlorella cells in flashing light having different patterns of intermittence. Solid lines, $I_g = 23 \times 10^4$ ergs/cm2/sec. Dashed lines, $I_z = 110 \times 10^4$ ergs/cm2/sec. The ratio $(t_D + t_p)/tF$ is given by the number at the end of each line.

D

Discussion

Loss Due to Respiration

When drawing conclusions about growth on the basis of measurements of photosynthesis, we have to consider respiration losses. Since a respiration rate observed in a single experiment has no general validity, we have up to this point dealt with total light actions only. A constant respiration rate would diminish the efficiency in the way illustrated by the dotted line in figure 5. That is, the lower the average intensity to which the individual cells are exposed, the greater the fraction of the light required to compensate for respiratory processes, and the smaller the fraction left for use in growth.

For this reason the mean light intensity per cell in a dense suspension may be too low, even with full sunlight, for efficient growth of cells. Hence, the use of too dense suspensions will result in a loss in efficiency of utilization of sunlight.

Under constant conditions the respiratory rate tends to be correlated with the photosynthetic rate (and therefore with the average light intensity) [241]. There is a time lage in the adjustment of the respiratory rate to a

change in the photosynthetic rate. For this reason the inconstancy of out-
door conditions will have an unfavorable influence on the efficiency of light
utilization.

Intermittence through Turbulence

    Going from the basic information described in the preceding sections
toward its practical application, several difficulties will be met. The pat-
tern of intermittence required for a high yield of Chlorella appears to put
rather extreme demands on the turbulence obtainable in a flowing culture.
    We must keep in mind that periods of virtually complete darkness are
necessary between the light flashes. No gain in efficiency of light utiliza-
tion could be expected, for example, if our "indoor[3]* cells were alternately
exposed to full light intensity and one-tenth light intensity. To gain in
efficiency, the cells would have to receive all their light in, say, a layer of
the suspension equal to less than one-tenth of the total depth, then go into
complete darkness in the other nine-tenths of the suspension. The economi-
cal realization of a turbulent flow that will submit individual cells to favor-
able flash patterns, rather than to a random distribution of intensity varia-
tions, probably is a major engineering problem.
    Conditions to which the cells are exposed during outdoor growth are
constantly varying. The higher the light intensity, the more turbulence will
be desirable. As long as optimal intermittence is not realized, only part of
the possible increase in yield will be obtained. Except for pumping costs,
the highest attainable degree of turbulence will be favorable under all con-
ditions.
    In view of *the* complex interplay of varying light intensity and tempera-
ture, the choice of optimal density of suspension will probably be a com-
promise, arrived at by practical experience under actual average operating
conditions. Though the chlorophyll content and light-saturation levels of
thick suspensions grown in sunlight are not known, a rough guess based on
available data is that the amount of cells present per square centimeter of
irradiated culture area would be from 100 to 400 mm3. It would be advan-
tageous to use this quantity of cells in a shallow layer having high cell den-
sity in order to satisfy economically the requirement of a flash time of the
order of milliseconds induced by turbulence.
    So far we have only extrapolated from data obtained with one strain of
Chlorella. These cells show characteristic differences (table 1), depending
on whether they are grown in thin suspension exposed to sunlight, or indoors.
The outdoor cells give more favorable yields in both continuous and inter-
mittent illumination. This advantage is not found when cells grow in thick
suspensions exposed to sunlight, where the mean light intensity is again low.
    It is quite likely that algal strains may be found that have better char-
acteristics in these respects than the strain dealt with here. Use of such
strains would very much decrease the difficulties in realizing efficient
growth in strong light.

Conclusion

The general conclusion from these experiments is that it should be possible to grow high yields of algae in full sunlight, provided that the turbulence and density of the culture can be adjusted to produce the proper pattern of intermittence in illumination of the individual algal cells.

Chapter 7

EFFECT OF DIURNALLY INTERMITTENT ILLUMINATION ON THE GROWTH AND SOME CELLULAR CHARACTERISTICS OF CHLORELLA[1]

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Though the light source to be utilized in large-scale mass culture of unicellular algae is expected to be natural sunlight, the effect of diurnal alternation of light and dark periods upon the growth and various cellular characteristics of algae has not yet been studied systematically. With a view to filling in this gap in knowledge, we performed the following experiments, using Chlorella ellipsoidea as material.

A

Experimental Procedure

Culture Conditions

The experiments with continuous and intermittent illumination were run successively, using the same culture chamber and illuminating system. The cultures were grown in an aspirator bottle of 25-liter capacity (26.5 cm in diameter), using 20 liters of culture solution of the following composition (per liter): KNO_3, 5.6 g; $KH2PO4$, 2.5 g; $MgSO4 \cdot 7H2O$, 5.0 g; $FeSO4* 7H2O$, 0.0028 g; Arnon's A4 solution; 1 ml; B7 solution, 1 ml The culture was constantly aerated with air containing 5 per cent $CO2$, and illuminated by seven 200-watt tungsten lamps placed around the bottle about 13 cm from the bottle wall. The average intensity of the incident light at the bottle wall was estimated to be about 16,000 lux.[3] To avoid overheating of the culture by the lamps, the bottle was constantly flooded from above with tap water during illumination. In the experiment with intermittent illumination, the darkening was effected by turnihg off all lamps and

covering the whole culture system with a black cloth. Except*lor the first light period, which lasted 24 hours, the alternate dark and light periods lasted exactly 12 hours each. The temperature was 22±2° C in the culture with continuous illumination, and 26±2° C in the culture with intermittent illumination.

Measurements

At intervals of 4 or 8 hours, measurements were made of the packed cell volume (by a centrifuging technique using a hematocrit), cell number (using a hemacytometer), percentage frequency of distribution of cell size, statistical average of cell volume, 4 and chlorophyll content of cells.5 For some algal samples the nitrogen content (by the semimicro-Kjeldahl method) and the dry weight per volume of cells were also measured. The latter was found to be in the range 0.252 - 0.263 g/ml with an average of 0.256 g/ml, which did not show any systematic change with the age or maturity of the culture.

B

Growth Curves

The results obtained in the two experiments using different modes of illumination are summarized in figures 1 and 2. In both figures, A shows the logarithmic growth curves, one in terms of packed cell volume per liter (solid lines) and the other in terms of cell number per liter (dashed lines). Logarithmic scales of the ordinates were taken so as to make 1 ml of packed cell volume correspond to a cell number of $5.6 \times 10^{*0}$, which is the ratio usually found in well matured cultures. 6 Since there is a definite quantitative ratio between the packed cell volume and the dry weight of cells, the curves for the latter may be regarded, mutatis mutandis, as identical with those for the former. In B in both figures are given the values of the relative growth rates (k), which were determined both for packed volume (solid lines) and for cell number (dashed lines) according to the formula $k = 1/(t_2 \sim t_i) \log_Q (^\wedge/N_j)$, where N_j and N_2 refer to the packed volume or cell number per liter at the successive times t_i and t_2 respectively, the time being measured in days.

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* For each sample the diameters of 300 individual cells were measured, and the average cell volume (v) was calculated according to the formula $v = 0.524 \, S n_i d^\wedge/S^\wedge i$, in which n_i means the number of cells having a diameter of d_i microns,

5 The chlorophyll in the weighed samples was extracted with acetone and treated with aqueous HCl solution (1:1), and the pheophytin formed was transferred into benzene and its quantity determined spectrophotometrically, using light of wave length 866 millimicrons.

^Considerable variation in this ratio with change of environmental conditions is reported by other investigators. See chapters 9, sections B and C.--Ed.
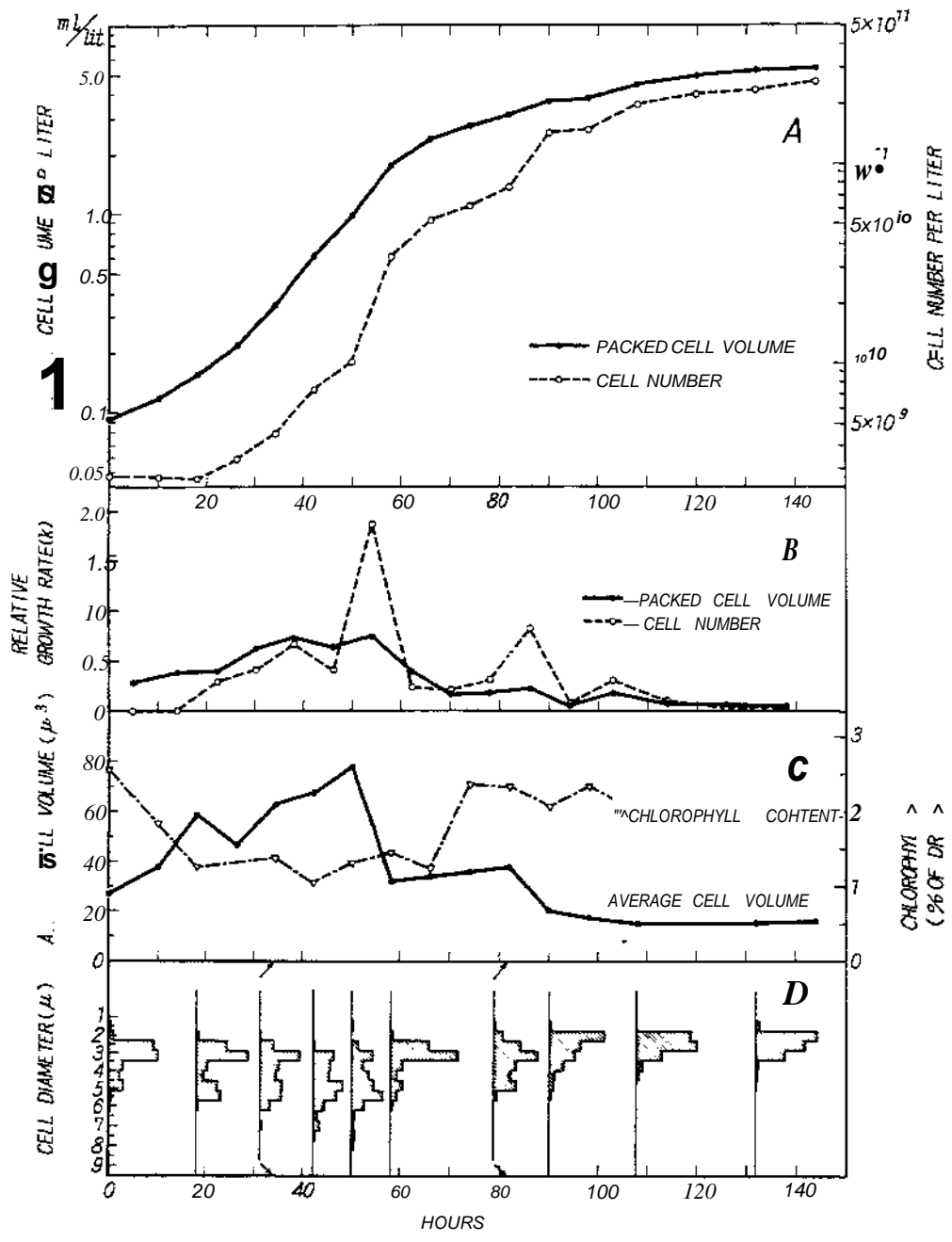
Fig. 1. Growth of Chlorella ellipsoidea under continuous Illumination.

A» logarithmic growth curves in terms of packed cell volume and cell number per liter.

B, relative growth rates in terms of packed cell volume and cell number.

C9 average cell volume (in cubic microns) and chlorophyll content of cells.

D, statistical distribution of cell diameter* (in microns) as represented by frequency polygons at various stapes of culture. The base line (or the arrows attached to it) of each polygon indicates the time of observation.

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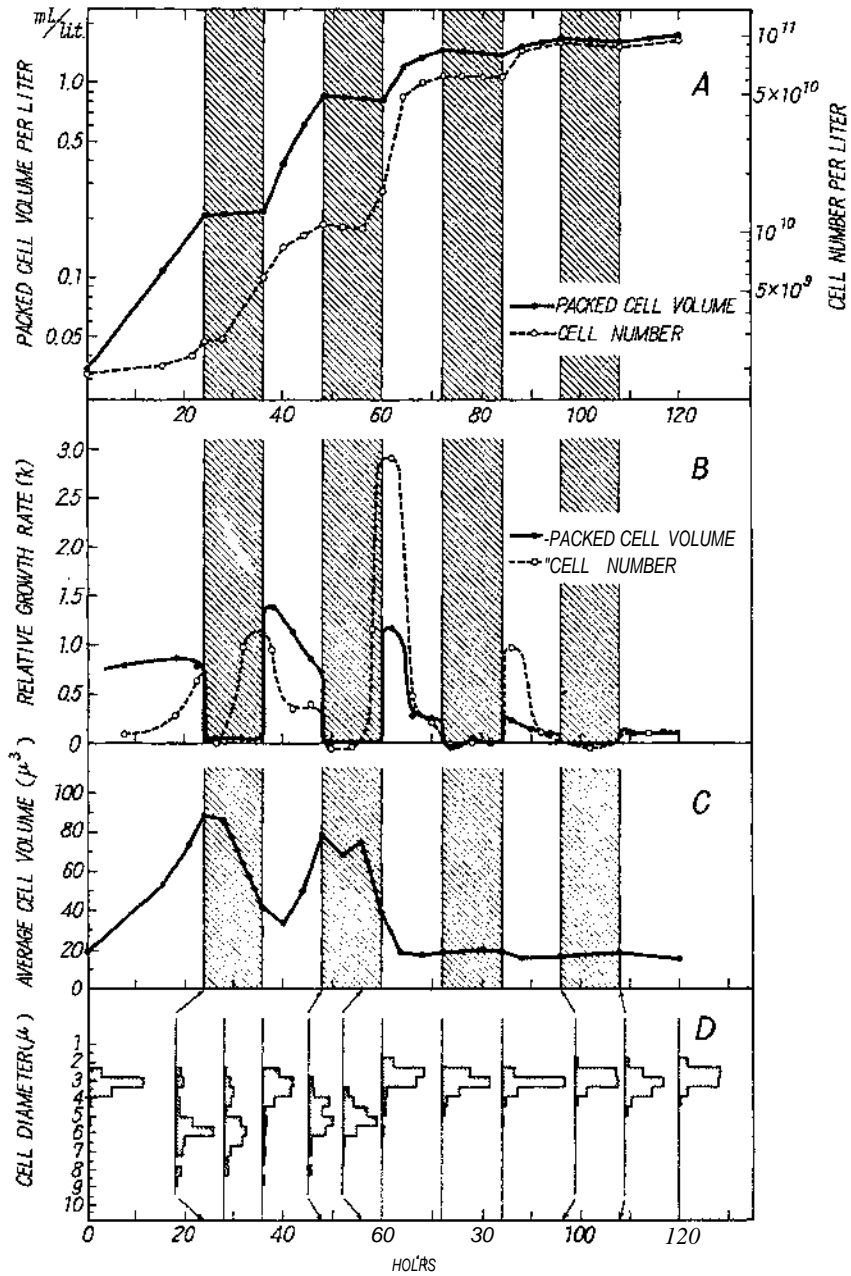
Fig. 2. Growth of Chlorelia ellipsoiriea under diurnally intermittent illumination. The periods of darkening (12 hours each) are indicated by shading.

A, logarithmic growth curves in terms of packed cell volume and cell number per liter.

B, relative growth rates in terms of packed ceil volume and cell number.

C, average cell volume in cubic microns.

D, statistical distribution of cell diameter (in microns) as represented by frequency polygons at various stages of culture.

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Packed Cell Volume

As was expected, the logarithmic growth curve for packed cell volume
followed a zigzag course in the intermittently illuminated culture; the rise
of the curve occurred immediately at the onset of each light period, and
ceased instantly at the onset of each dark period. The tangent of the curve
was always somewhat greater in earlier stages than in later stages of the
light periods. The highest value (k = 1.41) of the tangent observed at the
beginning of a light period corresponded to the formation of 0.136 g of cell
materials per gram dry weight of cells per hour. This value is twice as
large as the maximum growth rate (k = 0.75) observed in the continuously
illuminated culture, and almost equal to the maximum rate of photosynthe-
sis shown by the same alga at 25° C (formation of 0.166 g dry weight of
photosynthate per gram of cells per hour; cf. Tamiya [286]).

In figure 3 are plotted the growth curves obtained in the two experi-
ments, together with one (curve HI) obtained in another experiment with
continuous illumination (using the same culture bottle and illuminating
system; temperature 24-25° C); As may be seen, the diurnal interposition
of a 12-hour dark period caused no retardation in the over-all logarithmic
growth at earlier stages of culture, a fact which indicates that the darken-
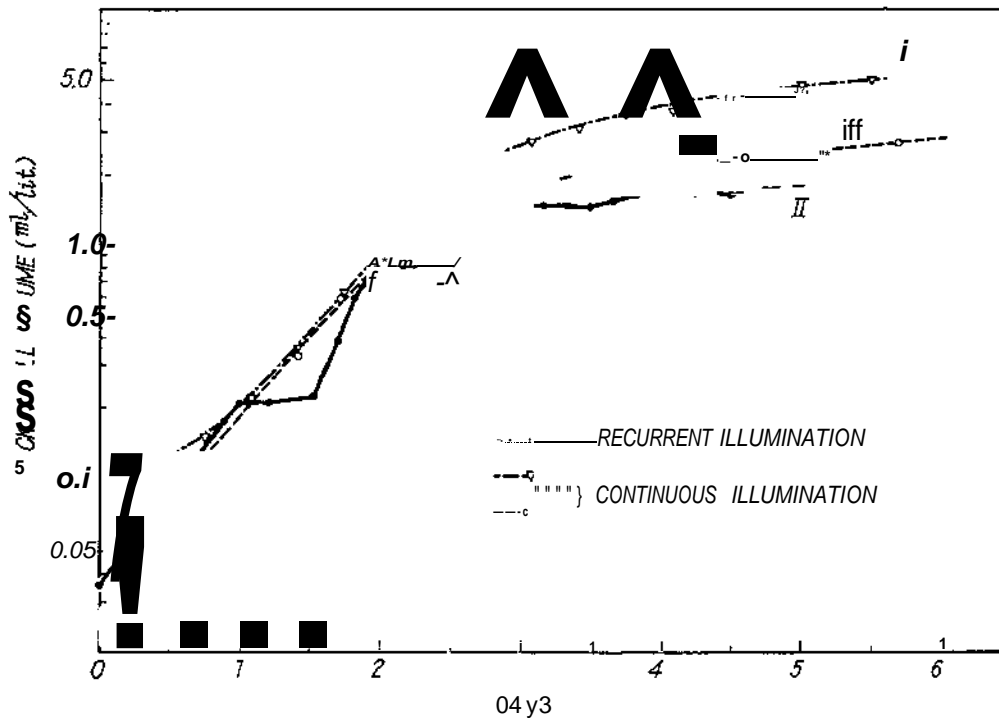
Fig. 3. Comparison of the growth curves (in terms of packed cell volume) obtained in the
experiments with continuous and intermittent illumination.

ing had a favorable effect on the growth in the subsequent light period. At later culture stages, however, when the amount of available light, had decreased considerably owing to the increase of population density, the over-fall rate of growth was decidedly decreased by the interposition of the dark periods.

Cell Number

The growth curves in terms of cell number showed some remarkable features in contrast with the curves for packed cell volume. In both experiments the curves for cell number remained almost flat for about 20 hours at the beginning of the culture, while in that period a distinct increase of packed cell volume took place. As may be seen from figure 2, the cell number increased not only in the light, but--at least at earlier stages of culture--also in the dark. The cell multiplication in the dark took place rather suddenly toward the end of the dark periods; for example, at the end of the second dark period, the cell number increased as much as 1.6-fold within only 4 hours. Noteworthy is the fact that this phenomenon was not accompanied by an increase of the packed cell volume. Experimental evidence showed that division of this type occurred after an induction period lasting about 8 to 10 hours independently of the illumination. Analogous abrupt division of cells seems to have occurred also in the experiment with continuous illumination (once between the 50th and 58th hours and again between the 82d and 90th hours), and is reflected in the two distinct inflections in the growth curve in figure 1A. Inspection of the curves in figure 2 shows that besides this "bursting" division there occurred also a "steady" division which proceeded regularly in the light, but not in the dark, more or less keeping pace with the increase of packed cell volume.

Cell Size

Individual cells in the culture varied considerably in size, and the average size of the cells in the whole population changed in a peculiar manner during the course of culture. As a rule, the average cell size was larger at earlier stages of culture than it was later. In view of the fact that at later stages of culture the intensity of available light was greatly lowered, this finding is in line with the observation of Myers [95] that Chlorella cells grown under weak light are smaller than those grown under strong light. These experiments also corroborated the observation of the same author that the chlorophyll content (see fig. 1C) and nitrogen content (both in percentage of dry weight) are lower in larger cells than in smaller cells. Large cells with an average volume of 87 cubic microns were found to contain 5.64 per cent nitrogen; the corresponding value obtained for small cells with an average volume of 15 to 20 cubic microns was 6.49 to 6.64 per cent.

In figures ID and 2D are presented polygons showing the percentage frequency of the distribution of cell diameter at each stage of culture. In

both experiments the size distribution was more heterogeneous at earlier stages, and with the progress of culture it became more and more homogeneous, showing a predominance of smaller cells. An interesting fact emerges when we examine the mode of change of form of the polygons in the course of culture: there are two main groups of cells by size, one ranging from about 2 to 4 microns with a maximum frequency at about 3 microns, and the other ranging from about 4 to 7 microns with a maximum frequency at about 5.5 microns. The changes in average cell size in the culture may, by and large, be regarded as being determined by the relative abundance of cells belonging to these two groups of sizes. The bursting division mentioned above was a process in which larger cells suddenly changed into a number of smaller cells. For convenience of reference, we may call the larger cells "light cells'' and the smaller cells "dark.cells." It should be remarked that besides these two kinds of cell there were small numbers of intermediate and overgrown cells with diameters of about 4 microns and 7 to 11 microns, respectively. There is ample evidence that dark cells and intermediate cells have stronger photosynthetic activity than do light cells and overgrown cells (cf. Emerson and Arnold [216], Myers [96]).

C

Discussion

Formative Metabolism

Various facts observed in the experiments described above may be explained coherently by considering the nature of the light and dark cells and their mutual relations. It goes without saying that the over-all growth phenomenon in algal culture involves, besides photosynthesis, a series of biochemical and physiological processes such as nitrogen metabolism, and synthesis of organic components and structural elements of cells, culminating in the formation of autospores and their ripening into vegetative cells. We may call all these latter processes en bloc "formative metabolism," and distinguish it from the photosynthetic process. Whereas photosynthesis occurs only in the presence of light, formative metabolism may proceed by itself in the dark, though the presence of photosynthates is a prerequisite for the latter process. Substantial gain in cell materials, as reflected in increase of packed volume or dry weight of cells, can occur only when photosynthesis is in progress, and therefore only in the light.

Dark and Light Cells

The balance between photosynthesis and formative metabolism is of primary importance in determining the physiological features of algal cells. When formative metabolism overbalances photosynthesis—as will occur either in dim light or in cells having weak photosynthetic activity,

even when they are well illuminated--there will result the [s]"formative maturing" of cells. The dark cells as defined above are in such a condition. When, on the other hand, photosynthesis overbalances formative metabolism--as will be the case when formatively matured cells are exposed to strong light--the cells will gradually gain in mass by the accumulation of photosynthates. Such a process will eventually lead to the formation of the large cells which we have defined as light cells and overgrown cells. The dark cells with their well organized photosynthetic apparatus are, we might say, hankering after photosynthesis, whereas the light cells with their more or less disorganized photosynthetic apparatus are strongly disposed toward formative metabolism. The progress of formative metabolism in light cells leads to the formation of autospores, which gradually turn into vegetative cells and eventually separate from one another to become individual dark cells.

The whole process may be expressed in the simplest way by the following formulas:

$$(1)\ D \xrightarrow{k_l} L; \quad \text{and} \quad (2)\ L \xrightarrow{k_d} nD$$
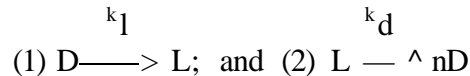
where D and L represent dark and light cells, k_l and k_d the rate constants of the light process (photosynthesis) and the dark process (formative metabolism), respectively. The value n represents the number of daughter cells produced from one mother cell, which is stated to be 2, 4, 8, or 18. It should be remarked that the ratio of diameters of the dark and light cells observed in the present experiments was approximately 1:2, a fact indicating that the n-value predominating in our experiments has been $2^{\wedge} = 8$. In the course of culture, a condition will obtain in which the two processes mentioned above keep step with each other; "steady" division will then take place. Under certain circumstances, however--and indeed for some reason unknown at present--there occurs a disturbance of the equilibrium, which leads to overaccumulation of light cells followed by their bursting division into dark cells.

Effect of Intermittent Illumination

In order for algae to utilize both light and nutrient substances with high efficiency, photosynthesis and formative metabolism must proceed in adequate balance with each other. The high efficiency in utilizing light observed in earlier stages of the intermittently illuminated culture may be explained as follows: At earlier stages in our experiments the amount of light available to the algae must have been more than saturating for the photosynthetic process. In the continuously illuminated culture such a state caused an overpredominance of light cells, which had relatively low efficiency in utilizing light. In the intermittently illuminated culture, the predominance of light cells was adequately regulated by the interposition of dark periods, during which time the light cells could be turned into dark cells by the operation of formative metabolism. At later stages of culture, however, the decrease of available light due to the increase of

population density caused an excess of formative metabolism and consequently a predominance of dark cells. Under such circumstances the over-all growth was limited by the process of photosynthesis, so that the interposition of dark periods entailed a considerable retardation of growth.

Chapter 8

INORGANIC NUTRITION OF ALGAE

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A

Introduction

In any attempt to provide for a given species of alga a medium favorable for its growth, the culturer must condition the environment to meet as many of the intrinsic requirements of that alga as possible. The factors comprising the environment may be conveniently considered as either physical or chemical, though some may play a dual role. The physical factors are light, which is the energy source, and temperature, which is maintained within a limited range suitable to the organism under culture.

The chemical environment, with which this chapter deals, is a dilute aqueous solution from which all the raw materials necessary for structural and protoplasmic synthesis are drawn. Though organic materials may be supplied for various purposes in the culture of algae, the organisms which we are considering here are capable of continued growth in a strictly inorganic medium consisting of over 99 per cent water and less than 1 per cent salts and dissolved gases. The following discussion will review the principal techniques at present available for meeting these requirements of the algae.

Though considerable progress has been made toward solution of the problem, it would be presumptuous to suggest that no unresolved difficulties exist. In fact, adequate information concerning the inorganic nutrition of microorganisms lags seriously behind the progress made in the study of their organic metabolism, as Hutner et aL [61] have pointed out in their review of the role of metals in metabolism.

The demands that the engineers have placed upon physiologists for information leading to the achievement of a steady state as the best method for the continuous maximum production of algae have served to emphasize the gaps in our knowledge. It is the second, and perhaps more important, function of this discussion to present the areas in which our knowledge is most deficient and where we meet the greatest difficulty in applying what information we do have to mass-culture techniques.

B

Water

Pure water must be available for laboratory culture of algae, but water used in mass culture need not reach laboratory standards of chemical and bacteriological purity. A brief review of past experience with what is quantitatively the major constituent of the medium may prove helpful.

Distillation

In the laboratory, distilled water is used routinely. Such water, passing through typical metal distilling apparatus, may collect small quantities of extremely toxic metals, notably copper, which may restrict growth without being detected. For meticulous purification, quartz distilling apparatus is acknowledged to be best, though platinum [137] and tin [233] have been used to good effect. Plastic also has been suggested. Apparatus of Pyrex glass in America or Jena glass in Europe provides water of sufficient purity for routine use, though it is unacceptable in many studies of trace-element nutrition. Research, especially by Steinberg [280, 281], has emphasized the degree of contamination of water by impure chemicals and by leaching of glass in distilling apparatus and culture vessels, especially those in use for some time.

Ion Exchange

In our laboratory at the University of Maryland, water for mass-culture work is passed through a column containing cation and anion exchange resins. Such resins are effective for removal of electrolytes, though organic materials and colloids are not affected. Either large rechargeable beds or small expendable cartridges produced by the Barnstead Still and Sterilizer Company may be used. A continual check on conductivity will ensure water carrying no more electrolytes than are normally present in routinely distilled water. This deionized water has even proved useful for some trace-element investigations. Satisfactory growth rates of the algae we have used indicate that any harmful effects from traces of the resin which may leach from the columns are slight. The objection that the process of ion exchange does not serve to destroy microorganisms may be partially met by passing water coming from heat exchangers near $100°$ C through a glass coil in a cold-water bath prior to ion exchange. Such water has been satisfactory where pure culture is not essential but where any reduction of contamination is desirable.

Natural Sources

The employment of such techniques as are mentioned above for purification of available water supply is indispensable in definitive introductory

work with any organism, but once the nutritional requirements of the organism are delineated, there is no reason why natural waters of determined analysis cannot be employed directly. The analyses of a variety of fresh-water sources compiled by Chu [19] and given in table 1 illustrate the variability of constituents encountered in different waters or even in different parts of the same body of water. Whether or not a given element present in such an analysis is in a form available to the organism must be taken into consideration in determining its possible effectiveness as a supplement to the added nutrients.

Table 1

Concentrations (in parts per million) of important mineral constituents of natural fresh waters
(from various sources as compiled by Chu)
(The figures give the range, the upper being the minimum and the lower the maximum;
when only one figure is given, it represents an average.)

Water	N	P	Fe	Ca	Mg	K	Na	SiO2	Total solids
Lake Superior.........................	0.001	....	0.0007	0.135	0.03	0.03 (K+Na)		0.08	0.6
Lake Superior.........................	0.113	,.........	0.044	13	3.1	3.2 (K+Na)		7.4	60
Byske-elf	....	....	....	2.2	1.15	1.7	0.9	1.5	19.2
Ennerdale	0.016	....	0.01	2	0.72	5.8 (K+Na)		1.0	30.9
	0.08	0.0009 0.01	0.05	10				2.2	
Windermere ....:...........	0.048	0.0007	0.01	2.4	0.84	4.3 (K+Na)		0.2	59.8
	0.2	0.04	0.09	4				1.7	
Amazon.................................	....	....	....	5.43	0.52	1.36	1.15	10.6	37
				12.45	1.53	1.76	1.57	11.1	59
Nile....................................	trace	trace	1.25	15.6	5.25	1.7	5.1	10.3	119
	0.098	0.023	1.52	33.8	9.4	13.4	34	26.7	174
Mississippi............................	0.32	....	0.07	32	8.4	10 (K+Na)		11	166
Mississippi............................	0.61	....	0.61	44	16	21 (K+N^)		24	269
Mississippi............................	0.05	trace	....	....	....	....	....	......	....
	0.91	0.065							
Wharfe.................................	trace	0	....	32	2.1	....	....	0.5	124
	1.58	0.022		72	13.2			14.9	344
Illinois...............................	0.023	....	0.15	55.1	27.2	3.4	11.3	26.1	152.4
	2.82		0.32	107	28.7	6.5	42.2	31.6	398.8
Illinois...............................	9.5	....	0.21	47	20	16 (K+Na)		12	267
	11.3		0.27	50	21	18 (K+Na)			287
Thames at Slough......................	3.5	....	....	86.5	4.8	. . . . . . .15.5		......	332
	8.7 a								
Welland................................	1.98	....	....	99.5	10	. . . .. . .14.05		5.5	370
Trent..................................	1.65	....	....	184	45.5	85 (K+Na)		10	734
Lake Winera	trace	0.02	....	....	....	....	....	......	....
	0.25	0.89							
Wisconsin lakes.......................	....	trace 0.015 0.75 b	....	....	....	....	....	......	....
Texas reservoir lakes	0	0.04	....	....	....	....	....	......	....
	0.44	0.3							
English lakes	0.016	0.0005	0.01	0.2	0.7	....	....	0.1	30.6
	0.2	0.04	0.32	6.3	3.1			2.4	75.9
Selected typical fresh waters	trace	....	....	1.59	0.81	trace	trace	0.3	19.2
	2.0	0.62		184	45.5	5.6	27.8	19	734
20 British waters C...................	0.8	0.02	....	....	....	....	....	5.4	....
	5.0	0.137						13.3	
36 well waters	0.1	0.003	....	....	....	....	....	9.3	....
	11.4	0.072						19.3	
Surface waters d......................	0.8	....	....	1.1	trace	......	31	trace	25
	8.7			158.8	10.7		33.5	13	750
Average of surface waters	0.05	0.064	2.08	37	7	2	5	16.4	152 e
Average of surface waters f.......	0.31	0.2 e		31	5	3	8.8	17.3	152

[a] Ri¥er Thames at Staines.
[b] Maximum P at 20 m depth.
[c] Average for a year.
[d] Excluding **Thames** at Grays.

[e] Figure from Pearsall (1322).
[f] Calculated with 152 as the average salinity.
[&] Including aluminum-

Normally the concentrations of major nutrients in the water supply would not be expected to be great enough to be significant in mass cultures. The range of micronutrients with regard to both sufficiency and toxicity should, however, be known. With a satisfactory natural source available, only sterilization might be required. It would be hazardous to attempt to utilize any source directly without adequate analysis and test cultures. A fraction of a part per million of a toxic ion could mean the difference between success and failure in culturing a given organism with high yield as the objective.

C

Major Nutrients

Active research stemming from the first suggestion that plants may actually require a number of inorganic elements for growth has built a considerable body of knowledge applicable primarily to higher plants. It was ten years after Sachs [271] in 1860 presented his nutrient formula for higher plants that similar solutions were employed for algae, and another twenty years before Beijerinck [187] used bacteriological technique to isolate individual species of algae for study. Reasons for the greater progress in understanding the inorganic nutrition of higher plants are implicit in the greater economic significance of these plants as well as in their adaptability for study.

Replacement Technique

Investigation of the nutritional requirements of the algae was not neglected entirely. A series of workers, Artari [184], Chu [19, 20], Urhan [162], Pirson [120], Rodhe [142], Gerloff, Fitzgerald, and Skoog [224], and many others, using the replacement technique employed so-successfully with higher plants, found the nutritional requirements of the algae to be similar to those of the more complex phanerogams.

Solutions similar to Sachs[5] [271] or Knop's [240] were employed, all ions but one being allowed to remain constant and the remaining one being supplied in a series ranging from zero to a concentration well above what might be expected to be optimum. These solutions, inoculated with cells of the aiga under consideration, were placed under natural or artificial light and observed periodically for growth by photometric means, cell count, or dry weight of harvest. The concentration showing the best growth was selected as optimum for subsequent work. Table 2, compiled from Chu **[19],** illustrates the range of nutrient concentrations thus determined as acceptable to several species of fresh-water algae. A review of work of this sort indicates that the composition of a nutrient solution for the algae would not differ materially from that used for higher plants, providing for nitrogen, phosphorus, potassium, sulfur, magnesium, calcium,, and iron. Only in the apparently low or nonexistent **requirement** for calcium are the algae distinct.

Table 2

Suitable concentrations (in parts per million) of elements required for the growth
of seven species of algae (after Chu)

Element	Chlorophyta			Chrysophyta			
	Pediastrum boryanum	Staurastrum paradoxum	Botryo-coccus	Nitzschia palea	Fragilaria crotonensis	Asterionella gracillima	Tabellaria flocculosa
N	2.1-5	2-10	1.4-14	5-10.5	1.65-10.5	1.7-17	....
P	0.09-18	0.1-1.8	0.09-18	0.2-1.8	0.2-1.8	0.18-9	0.2-1.8
Ca	0.2-2	0.2-19	0.02-9.4	0.9-18	0.02-93.5	0.18-91	10-91
Mg	2.4	4-7	0-3.8	0.1-48	0.1-9.6	0.01-7.6	1-9.6
K	13.6-24	14.1-34.5	13.6-34.5	13.6-24	13.6-66	13.6-18.7	13.6-34.5
Na	0.04-2	0-24	0.04-19.5	....	....	....	....
Si	0.9-1.8	0-9.0	0.02-9.1	4.6-18.2	9.1-18.2	4.6-9.1	0.9-9.1
Fe	0.02-0.8	....	....	....	....	....	0.3-0.4

Inorganic Analysis of Algae

The knowledge thus derived has provided phycologists with sufficient
information for preparing adequate culture solutions both for taxonomic
work and for considerable physiological investigation, notably that on
photosynthesis in Chlorella. It has, however, been only in recent years,
as a result of work done primarily by Scott [149], Ketchum [64, 65],
Ketchum and Redfield [68], and Spoehr and Milner [151], that the algae
have begun to be subjected to the scrutiny of inorganic analysis for their
own sake. It is significant that the work of Osterhout [253], Blinks [193],
and Hpagland and Davis [230] analyzing the cell sap of large-celled fresh-
water and marine algae should be concerned not directly with the nutrition
of the algae, but with their adaptability as tools for study of the mechanism
of ion absorption.

A comparison of an analysis of a typical higher plant with those of two
fresh-water algae (table 3) reveals the basic similarity of their elemen-
tary composition. If we accept the premise that the major nutrient require-
ments of algae are similar to those of higher plants, research should be
directed to refining our knowledge with regard to the special needs of the
algae, particularly with a view to attaining a steady-state system such as
is desirable in any attempt to produce them in bulk.

Comparison with Higher Plants

Several fundamental differences are apparent between the cultivation
of higher plants and that of algae. The growth of the higher plant is to a
degree determinant, in that a point is reached somewhere in its life history
when it or its fruit or both are harvested for their total value. In the ap-
plication of nutrients, the time and quality of the harvest are the governing
factors. The objective in algal culture is to produce continuously a maxi-
mum quantity of cells of a given quality.

Table 3

Comparison of elementary analysis of fresh-water algae and of a typical higher plant

| Element | Per cent total dry weight in | |
	Corn [a]	Algae
carbon	43.6	49.51-70.17 [b]
Oxygen	44.4	17.40-33.20 [b]
Hydrogen	6.2	6.57-10.20 [b]
Nitrogen	1.5	1.39-10.98 [b]
Sulfur	0.67	0.91 [c]
Phosphorus	0.20	0.94-1.51 [d]
Calcium	0.23	0.00-1.55 [d]
Potassium	0.92	0.04-1.44 [d]
Magnesium	0.17	0.26-1.51 [d]

[a] W. L. Latshaw and E. C. Miller, Elemental composition of the corn plant, J. Agri. Res. 27: 845-860 (1924).

[b] Spoehr and Miner [151].

[c] Krauss[243].

[d] Scott [149].

The composition of the cell body, however, has been shown by Spoehr and Miner [151] to depend on the amount of nitrogen supplied, the protein content varying from 8.7 to 58.0 per cent. There is now reason to believe that variations in the other elements can produce as striking a variation in analysis. What then is to be the "quality" of the yield? This question is one of the first to be answered in developing a nutrient formula which will meet the requirements of the culture. Whether simply optimum growth, or carbohydrate content, or protein content, or sterol content, or some other constituent is to be the desired objective in the yield will in a large measure determine the inorganic medium employed. Although at first this variability in content (far greater than that in higher plants, especially in fruit) may seem a disadvantage, the possibility of directing the metabolism of the algae along widely divergent lines by so simple an expedient as varying the inorganic background promises remarkable versatility in products obtained from such a source.

Uptake of Individual Ions

Attempts to find a so-called "best" solution for the culture of higher plants have resulted in the conclusion that there is no such solution, but rather that plants are able to grow well over a considerable range in the quantity of ions supplied as well as in the composition of the salts provided. Data of Myers [97], using growth as a criterion, indicate that the situation may be similar in the algae. The uptake of nutrients is not necessarily correlated with actual metabolic requirements. Any analysis of algal cells designed to give information as to the nutrient solution to be used must take this into account. Figure 1, from Scott [149], giving the mineral composi™
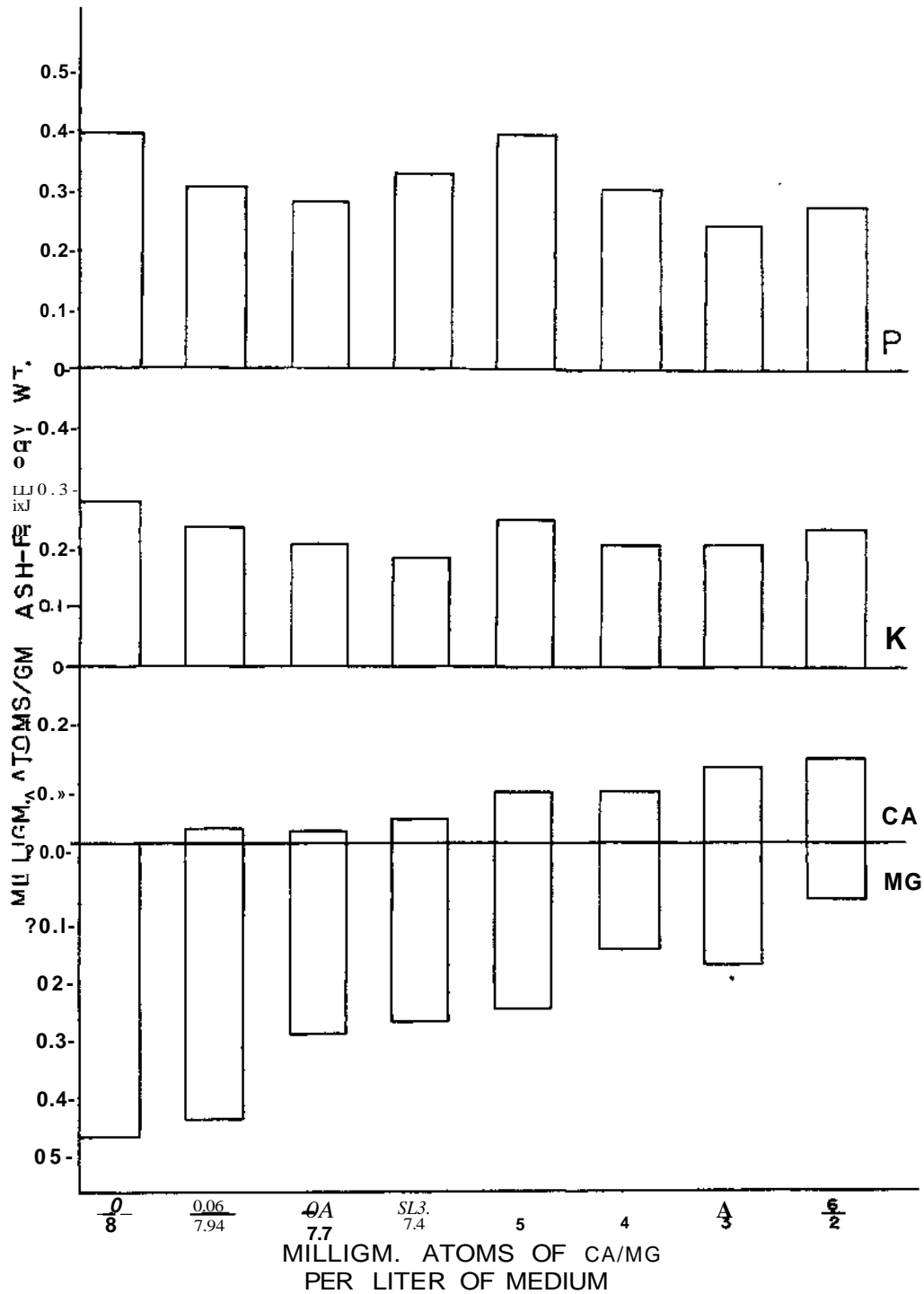
Fig. 1. Mineral composition of Chlorella cells grown in culture media containing varying ratios of calcium and magnesium. (From Scott [149].)

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tion of cells grown in varying ratios of calcium and magnesium, illustrates the point. In spite of the demonstration by Hopkins and Wann [58] that calcium is not essential for the growth of Chlorella, considerable uptake of this ion is indicated.

Not only may ions nonessential to the plant cell be accumulated, but under certain conditions luxury consumption of an ion may occur. Figure 2, from Krauss [243], shows the difference in nitrogen accumulation by cells with and without the three micronutrients boron, zinc, and manganese. It is apparent that the uptake of nitrogen proceeded almost as rapidly in the cells where growth was limited by micronutrient deficiency as in those growing at a normal rate. This resulted in a very high nitrogen content per cell. Further work is in progress to determine the nitrogen fractions in cells grown under these conditions.

It seems reasonable, therefore, to assume that within genetic limits there can be considerable variation in cellular organization of a given species, affecting profoundly the nature of the organism being cultured. Understanding of these phenomena awaits a more comprehensive understanding of ion absorption.

The variations in inorganic uptake brought about by modifications of the culture medium are exceeded by the variations observed when different species are compared. Osterhout [253] determined the potassium uptake by two species of a marine alga in the same medium. Halicystis ovalis was found to absorb 0.32 mole of potassium, in contrast with the very slight uptake of 0.006 mole by H. Osterhoutii. Similar differences in absorption of potassium, sodium, and chloride have been observed for Nitella and Chara and reported by Broyer [289], The performance of different species in different media may also be conditioned by the previous history of the cells. For instance, prior conditioning in a low-salt medium can greatly increase the rate of ion absorption when cells are transferred to a high-salt medium [289]. Coupling the use of a synthetically advantageous variation from the normal with the attainment of maximum growth will in most cases pose a serious problem.

Continuous Culture Experiments

In seeking an inorganic medium that will produce cells with certain desired characteristics during continuous culture and harvest, if is important to know how variations in the medium will affect the quantity of cells produced. The continuous culture technique of Myers and Clark [101] provides the most accurate means of measuring growth in differing media while maintaining all other environmental conditions constant and the culture pure. Their device maintains a constant population in an annular glass tube by means of a photoelectrically actuated solenoid valve. When the cell concentration increases above a predetermined point, fresh medium is automatically added, and the amount of overflow during a given period can then be used as a measure of growth. Using this apparatus with the culture solution given in table 4, Myers [97] demonstrated that growth of
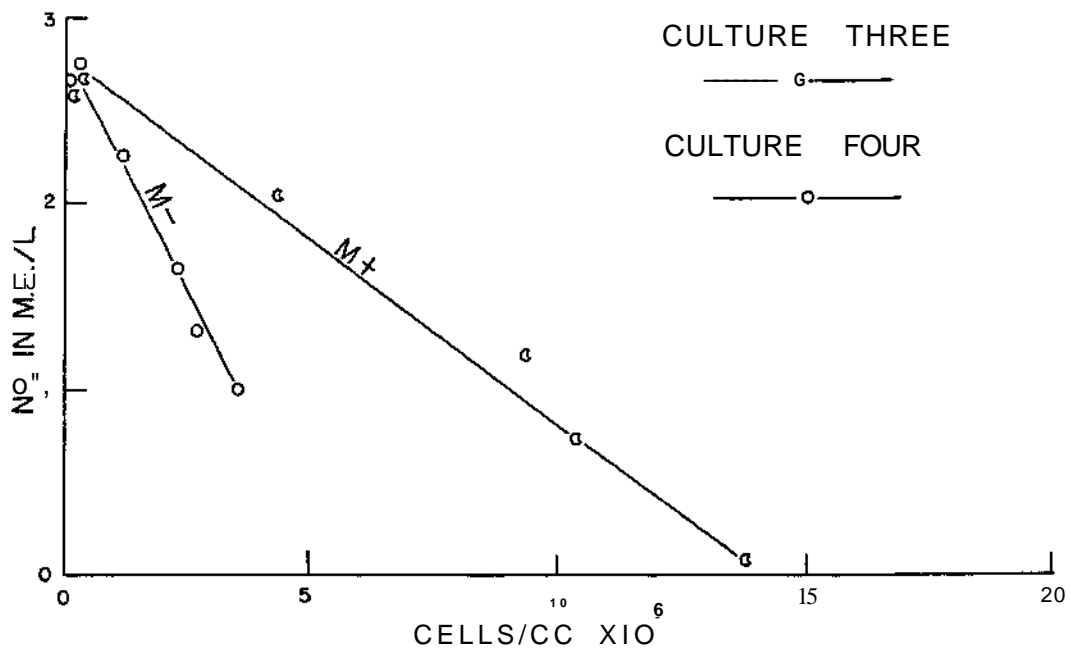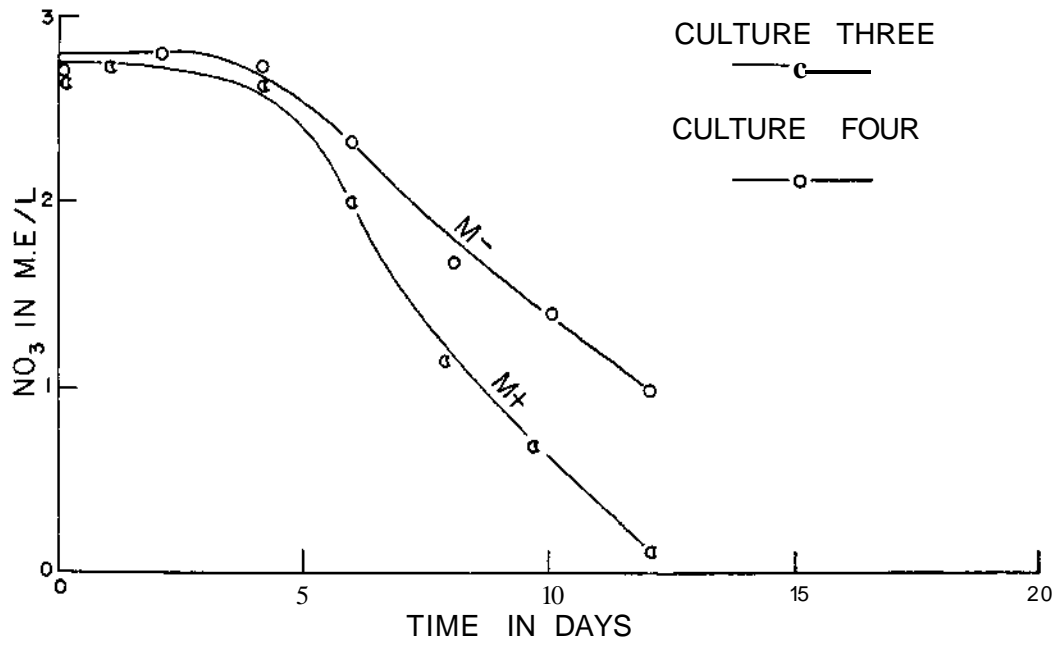
Fig. 2. Rate of removal of nitrate from nutrient media of two mass cultures of Scenedesmus, showing the effect of deficiency in manganese, boron, arid zinc in culture four as contrasted with the nondeficient culture three. Removal has been plotted against time (above) and cell count (below). (From Krauss [243}.)

Table 4
Myers' nutrient solution for Chlorella

Major nutrients		
corr	Concentration in culture	
	g/l	Molarity
KNO$_3$	1.21	0.012
MgSO$_4$-7H$_2$O	2.46	0.010
KH$_2$PO$_4$	1.23	0.009
Fe$_2$(3O$_4$)$_3$	0.052	0.00013
Na citrate	0.195	0.00056
Micronutrients (Arnon A4)		
Element	ppm element	Molarity
B as H3BO3	0.5	46×10^{-6}
Mn as MnCl2-4H2O	0.05&	92×10^{-8}
Zn as ZnSO4 $^{7H}2°$	$°-^{05}$	77×10^{-8}
Cu as CuSO$_4$ • 5H$_2$O	0.02	32×10^{-8}
Possible micronutrients (Arnon B7)		
Mo as MOO3	0.01	10×10^{-8}
V as NH$_4$VO$_3$	0.01	20×10^{-8}
Cr as Cr$_2$K$_2$(SO4)$_4$-24H$_2$O	0.01	97×10^{-9}
Ni as NiSO$_4$-6H2O	0.01	11×10^{-8}
Co as Co(NO3)$_2$-6H2C	0.01	17×10^{-8}
W as Na$_2$WO$_4$-2H$_2$O	0.01	55×10^{-9}
Ti as TiO: (COO • COOK)$_2$ • 2H$_2$O	0.01	32×10^{-8}

[a]Used in 10 times this concentration by Arnon [183].

Chlorella pyrenoidosa continued at a log^o rate of 0.47 between twice the
original concentration and one-tenth the original concentration of salts.
At concentrations below this, there was an indication that primarily nitro-
gen had become limiting.

Such data as Myers' are needed to establish upper and lower limits of
nutrient utilization. They are distinctly superior to those from the earlier
work, where light was not controlled and the carbon dioxide supply from air
(0.03 per cent), well below optimum (2.0-5.0 per cent), was introduced by
continuous bubbling or simply allowed to diffuse through the surface. It
would be expected that the nutrient solutions giving maximum growth under
optimum **conditions** would be more concentrated than those formulated for
use in such a **suboptimum** environment.

Relative Rates of Nutrient Removal

In an effort to determine relative rates of nutrient removal from mass
cultures in an open system, **Krauss** [243] grew **Scenedesmus** obliquus in

300-liter polyethylene-lined vats supplied with 5 per cent CO2 in air under constant light of 1500 foot-candles in controlled temperature chambers. Samples of the medium large enough for analysis of all major ions were taken every second day. Nutrient uptake in such a culture is shown in figure 3.
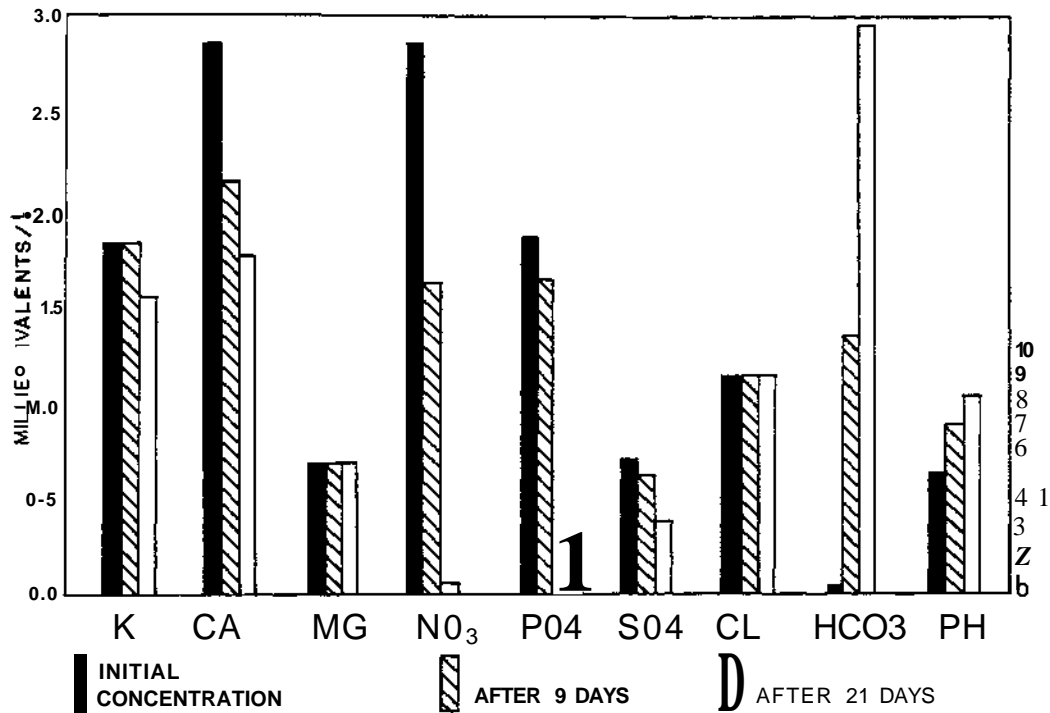
Fig. 3. Levels of principal ions in solution after the first, ninth, and twenty-first days of a mass culture of Scenedesmus. (From Krauss [243].)

Several features of the analysis are of interest. Nitrate is removed most rapidly and is completely utilized in the 21-day period. The other ions are not depleted so rapidly. (Above pH 7 much of the calcium and phosphate loss may be due to coprecipitation.) The considerable preponderance of anion utilization resulting from the rapid nitrate uptake is balanced by the increase in the bicarbonate ion* which serves to maintain the electrostatic neutrality of the system. (Analysis for the magnesium ion by several methods proved unsatisfactory, and undoubtedly more magnesium is removed than is indicated.) As nitrate is removed, the solution becomes strongly alkaline. Removal of the carbon dioxide supply, moreover, results in a prompt increase in pH to as high as II.

Such a picture is likely to be fairly typical of cultures with nitrogen supplied as nitrate. Rise in pH can be prevented by utilization of ammonium nitrate as described by Trelease and Trelease [180] for higher plants,

and by Milner (section B of chapter 9 of this monograph) for the algae. Removal of NH3+ will tend to lower the pH progressively. On the other hand, the introduction of the ammonium ion results in the formation of magnesium ammonium phosphate, which is completely insoluble at pH 7, a property useful in quantitative analysis but not in culture media. The gradual rise in pH in cultures using nitrate only, though troublesome because of reduction in the solubility of phosphate salts at the higher pH, provides a convenient guide to the status of the nitrogen supply, which can be supplemented periodically with nitric acid.

Carbon Dioxide Supply

The formation of bicarbonate provides a buffer to the pH rise and can supply certain species of algae with a utilizable source of carbon. Osterlind [108, 109, 110] has demonstrated that though Chlorella is unable to utilize bicarbonate, Scenedesmus does so readily. Though evidence in the literature is conflicting [265], it seems likely that either the bicarbonate ion or the undissociated salt does pass through some cell membranes. Nevertheless, membranes are more permeable to carbon dioxide, and a nutrient balance developing a pH low enough to maintain gaseous carbon dioxide in solution is desirable. Emerson and Green [33] have indicated that 0.1-0.5 per cent CO_2 in the medium is sufficient for optimum photosynthesis. 1 The equilibrium shift of gaseous carbon dioxide, bicarbonate, and carbonate ions with increasing pH as graphed by Emerson and Green [33] is given in figure 4. Some traces of free carbon dioxide are always supplied by the reverse reaction in equilibria at even the highest pH. It seems unlikely that these traces, though continuously replaced, could be absorbed rapidly enough to account for the rates of assimilation observed by Österlind [109]. Tolerance of a relatively high pH by certain species may be accompanied by a cellular mechanism for the utilization of bicarbonates or carbonates.

According to the law of Henry, continuous bubbling of an excess of a carbon dioxide-air mixture into the medium will tend to produce an equilibrium where the concentration of the carbon dioxide in solution is proportional to its partial pressure in the gas phase. This will in turn depress the pH to match the equilibrium attained. Continuous bubbling can therefore provide any desired amount of free carbon dioxide in the solution, regardless of the initial pH of the medium. The amount of bicarbonate formed when the equilibrium concentration is attained will depend on the initial pH.

Periodic Replenishment of Nutrients

The analysis of nutrient solution by Krauss [243] indicates a removal of the major ions in approximately the ratio given on a milliequivalent

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*New data for Chlorella are given in section C of chapter 9.--Ed.
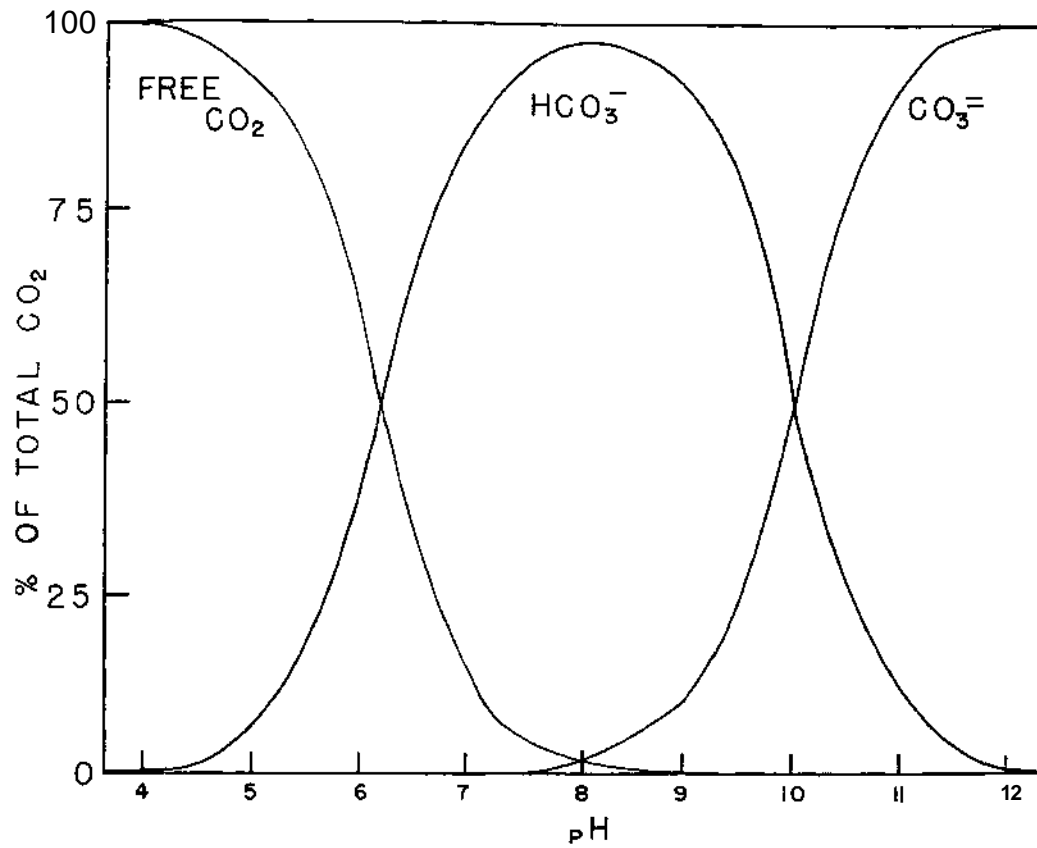
Fig. 4. Proportion of total carbon dioxide in each of the three forms, free carbonic acid (CC2 and H2CO3), bicarbonate ions, and carbonate ions, as a function of the hydrogen-ion concentration of a solution. (After Emerson and Green [33].)

basis in table 5. Also given are the approximate ratios supplied in tjie formula adopted as a result of these analyses (see table 6).

Table 5

Ratios of uptake of major ions from the medium compared with the ratios supplied in the formula for fresh-water Chlorococcales

Ion		Uptake	Supply
NO_3^-	....	10	10
$SO_4"$	....	2	2
$PO_4\sim$	....	2	5
cr	....	0	0
K^+	....	2	12
Mg^+	....	0	2

Further analyses are under way leading to a balance sheet for both the organism and the medium, employing the formula in table 6 as a starting point.   These should determine the amount of the major salts to be added at periodic intervals to maintain the inorganic medium at the level necessary for optimum growth. Success has been obtained with periodic addition of nitrate as dilute nitric acid, with less frequent supplementation of

Table 6
Nutrient solution for fresh-water Chlorococcales [a]

Compound	Ion	Wt. (g/1)	ppm	me/1
KNO_3		1	1000	9.9
	K	0.387	387	9.9
	NO_3	0.613	613	9.9
$MgSO_4-7H_2O$		0.25	250	2.01
	Mg	0.0246	24.6	2.01
	SO_4	0.0975	97.5	2.01
KH_2PO_4		0.25	250	5.50
	K	0.0717	71.7	5.50
	PO_4	0.174	174	5.50

Micronutrients (modified from Arnon [183])

Compound	Wt. (g/1 in stock soln.)	0.1 ml/1 gives ppm of the metal
H_3BO_3	2.860	0.05
$MnCl_2*4H_2O$	1.810	0.05
$ZnSO_4-7H_2O$	0.222	0.005
$CuSO_4- 5H_2O$	0.079	0.002
MoO_3	0.015	0.001
$Ca(NO_3)_2-4H_2O$	59.0	1.00
$CoCl_2 6H_2O$	0.04	0.001

Iron solution (modified from Rodhe [142])

Compound	Wt. (g/1 in stock soln.)	1 ml/1 gives ppm of Fe
Ferric citrate ($FeC(3H_5O_7-3H_2O)$)	5.30	1.0
Citric acid	5.30	

[a]pH may be adjusted to the desired range with either potassium hydroxide or hydrochloric acid.

the other ions in proper ratio. This may obviate the difficulty of the shifting pH resulting from preferential anion absorption. Routine determination of pH could then serve as a prime index to the inorganic status of the culture, leading to a steady state in the nutrient supply.

Though the techniques for the replacement of major ions without loss of water seem to be in sight, the supply of iron and the micronutrients provides another problem no less important than that presented by the more abundant ions.

D

<u>Iron</u>

Though additional effort should be devoted to the problem of iron nutrition, bearing on the method and form of introduction of iron into the nutrient solution, some agreement does exist on several critical points. Even though iron may have a quantitatively minor role, more difficulty has been encountered in satisfying this requirement than any other. Initial addition of iron to the nutrient solutions in a range from 0.18 to 7.22 ppm is a general practice. Myers [97] was able to demonstrate that Chlorella received an adequate iron supply from amounts ranging from 13.3xl0"5 M to as little as G.020X1CT5 M.

The maintenance of this amount in solution in a form available to the plants at the pH encountered at inception of the cultures and as the medium is modified by the growth of the algae is a major problem. It has been attacked with some measure of success by essentially three distinct techniques, all having some undesirable features.

Pringsheim [263] is of the opinion that the salutary effects of extracts of garden soils added to media are primarily due to the effect of natural organic substances, often called humates, in preventing iron precipitation or solation. Though the use of nondefinitive material such as "soil extract" may be anathema to the chemist, much of Pringsheim's remarkable success in the culture of algae he attributes to the <u>Erdabkochung</u>. Although Algeus [2] points out in his review that certain hormonelike substances may be contained in such materials, it seems highly probable that the prevention of iron precipitation is the major contribution. Indeed, from the pragmatic point of view the utilization of such a substance in mass-culture technique may be advisable, whatever the final agreement on the active fraction in the extract.

A second approach, used in reproducible growth studies, has been the addition of certain organic acids or their salts to establish soluble iron complexes. The more useful have proved to be tartrates and citrates, with preference for the latter. The effect of citric acid on a solution containing ferric citrate appears to be that of mass action preventing the breakdown of the ferric citrate to hydroxide and citric acid. The picture is somewhat complicated, however, by the fact brought out by Peltz and Lynn [257] that ferric salts and hydroxides oxidize citric acid to carbon dioxide and ferrous citrate, which in turn can be oxidized to ferric citrate. The citric acid, therefore, acts as a complexing agent, a reducing agent, and a pH buffer. Rodhe [142] in turn showed that sodium and potassium salts are not effective either in reducing the ferric ion or in stabilizing the iron citrate complex, but most probably accelerate the production of colloidal ferric hydroxide, itself unavailable to the plants. Myers [97], however, has demonstrated excellent growth on the part of Chlorella, using ferric sulfate and sodium citrate. Irrespective of the compound in which the citrate may be added, there is no doubt that a readjustment in the complex

must take place in the presence of the other competing ions of the nutrient solution. This presents a problem similar to that met in the discussion of chelating agents in the next section. It must also be recognized that citrate is itself a metabolite for many microorganisms, and iron may be released as a result of the utilization of the citrate. This could be a major drawback, for excluding metabolizable organic material from mass culture is unquestionably desirable for reasons pointed out in other parts of this monograph.

The third possible solution of the problem rests in the use of chelating agents, discussed in the next section. It is likely that the use of metabolically inactive complexing agents, once well worked out, may provide the ideal solution to the problem of supplying iron and other trace metals.

One other possibility, though remote, should perhaps be explored. Analysis of media in which active growth is occurring may reveal complexing materials liberated into solution by the algae themselves. The amount of organic matter secreted by the algae has been shown to be less than 5 per cent of their total weight under conditions of good growth. The nature of the secreted material is relatively unknown, although Pratt and Fong [133] have found one called chlorellin which appears to be a growth inhibitor. In view of the fact that different species of algae seem capable of utilizing differing amounts of inorganic material to maintain good growth, the effectiveness of secreted complexing or chelating agents, postulated in membrane structure by Jacobson and Overstreet [236], cannot be entirely ignored.

E

Micronutrients

Trace Elements in Algae

No medium for higher plants or algae can be considered adequate without provision for micronutrients. That the success of earlier workers using salts supplying only the major elements was due to *(he* impurity of these compounds is well established. The review by Myers [99] cites research establishing requirements of various species of algae for manganese, zinc, calcium, boron, and possibly copper. Robert [270] presents considerable evidence for the inclusion of copper in the list. These findings are in agreement with the work with higher plants [183].

Molybdenum has been established as a stimulant to growth for several species of higher plants, but not as yet for green algae. It may, however, be expected to play a role in nitrate reduction there, and is generally included in micronutrient solutions (see tables 4 and 6). Additional micronutrients in the form of Arnon's B7 solution, containing vanadium, nickel, cobalt, titanium, chromium, and tungsten, have been added by Myers [97] without showing evidence of stimulation of growth, and are still under scrutiny in nutrition of higher plants as well.

Other trace elements have been identified in algal cells without regard to their possible niche in the metabolic mechanism. These include antimony, arsenic, beryllium, germanium, iodine, lead, manganese, molybdenum, nickel, rubidium, silver, strontium, tin, titanium, tungsten, uranium, vanadium, and yttrium [209]. Notable accumulation above that in surrounding media has been recorded for such elements as manganese [261], strontium [278], yttrium [278], and uranium [231]. The functions that these trace elements may have in the physiology of the organism have not been demonstrated, nor do any necessarily exist. Nevertheless it would be surprising if their absorption did not result in some physiological aberrations.

Chelating Agents

Though the addition of trace -elements is axiomatic, the method of keeping them available to plants, by preventing precipitation, is anything but settled. In artificial media, with the constant removal of cells and the elements they have absorbed, such micronutrients can rapidly become limiting, not only as a result of absorption, but through precipitation or solation as well. To prevent this, Hutner et al. [61] have pioneered in the utilization of complexing compounds, called chelating agents, of which ethylenediamine tetraacetic acid (EDTA) is the best known. Myers [104] has employed this agent successfully in Chlorella culture.

Essentially the chelating agents, as originally classified in 1920, are represented by a class of cyclic organic compounds resulting when a single group becomes attached at two points to the same metallic atom, much as the two prongs of a cheliped may clasp a nut. Such compounds, called inner complex salts, are exceedingly stable, retaining the metallic atoms primarily by co-ordinate valence. The organic part of the complex is heavier than the metallic, and may be from one- to four-toothed rather than exclusively bidentate in structure, as has been emphasized by Yoe and Sarver [297]. A typical chelating reaction of EDTA is as follows:
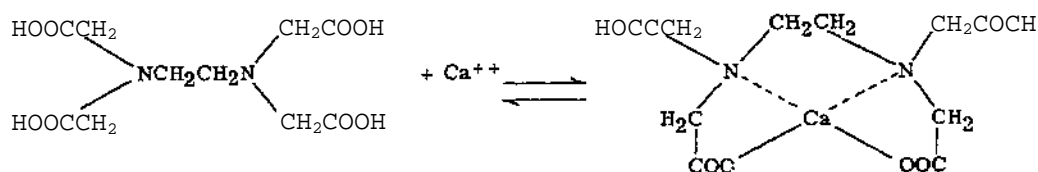
Introduction of a given amount of such a compound will prevent precipitation (oxalate will not precipitate calcium complexed by EDTA), but will release enough of the ion through mass action to provide for the need of the cells. Jacobson and Overstreet [236] have postulated chelate compounds in the structure of the cell membrane, and if there can be ample ionic exchange between colloidal soil particles and plant roots, as discussed by Overstreet and Jenny [254], it may also be possible that ionic exchange as a mechanism for absorption occurs directly between the algal cell membrane and the chelate complex.

Before complete freedom in the utilization of the chelating agents in mass culture can be achieved, several questions need investigation. Chelating agents are highly selective, complexing certain metals to the exclusion of others. In a culture medium replete with metallic elements, it is important to know which will be complexed by a given chelator and what the equilibria will be. Schwarzenbach [272] has determined equilibrium constants for a number of metals and EDTA in unimetallic solution. What the equilibria become in mixed solution is uncertain. From the chemist's point of view such information may be essential. Conversely, a highly efficient system for supply of micronutrients may be worked out biologically, using serial concentrations of the chelating agent, without a determination of either the equilibria attained or the mechanism of ion exchange.

F

Conclusion

There are many problems still to be solved in applying present knowledge about nutrition to the technology of mass culture. Furthermore, much basic work lies untouched, especially with regard to the composition of the cells produced under varying inorganic regimens. It would be surprising if added information in this field did not increase the feasibility of mass culture. The residual problems of reconciling a given qualitative objective with maximum yield will probably always be present, as they are in the production of the major crop plants of the world. Analytical techniques for examination of the synthetic processes of cells growing in different media can be applied more readily to the large samples available periodically from mass cultures. Though revealing many inherent problems, the present interest in mass culture may in turn makfc possible answers to basic physiological questions.

PART m

GROWTH OF ALGAE IN MASS CULTURE

Chapter 9

LABORATORY EXPERIMENTS ON CHLORELLA CULTURE AT THE CARNEGIE INSTITUTION OF WASHINGTON DEPARTMENT OF PLANT BIOLOGY

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A

Introduction

C. S. French and H. A. Spoehr

As a result of experiments initiated in 1942, it was discovered by Spoehr and Milner [151] that the growth of Chlorella may be controlled to produce at will cells of either a high fat or a high protein content. Experience with algal culture in large quantities was obtained in this laboratory from work on the antibiotics produced by Chlorella [152]. In these experiments several kilograms of dried Chlorella were obtained by harvesting cultures grown in 5-gallon bottles in a greenhouse with some control of illumination and temperature and with a supply of 5 per cent CO_2. This work was completed in 1947.

The possibility of using methods of culture that would lead to a product of controlled composition stimulated frequent discussions as to the practical use of such procedures in the mass culture of algae as a source of food or of industrial raw materials. Following numerous discussions between the Department of Plant Biology staff and Dr. Frederick G. Cottrell and Mr. William A. Woods of the Research Corporation of New York, arrangements were made by the Carnegie Institution of Washington for an engineering study of the practical aspects of algal culture, to be carried out by the Stanford Research Institute under contract with the Research Corporation. The Department of Plant Biology acted as informal consultant during the planning and progress of this program. The results of the Stanford Research Institute study were such as to give encouragement to the idea of building a pilot plant; they also showed the need for many more laboratory-scale experiments on the growth of algae under controlled as well as outdoor conditions.

Some new laboratory experiments were started here early in February 1951 with a view to meeting this need. Then when a Chlorella pilot plant 1 was constructed for the Carnegie Institution by Arthur D. Little, Inc. in Cambridge, Massachusetts, the experiments here were oriented with a view to furnishing information that would be of immediate value in its operation.

Sufficient information was not available concerning the effect of form or design of culture chambers on yields and growth rates of Chlorella. Accordingly, a number of algal culture chambers of different design were operated outdoors under natural conditions of illumination with the minimum necessary control of growing conditions. It was intended that these experiments should provide information as to the practicality of the principles underlying the different designs, so that it could be determined which of the culture chambers could profitably be expanded in scale. The model culture chambers actually operated were: (a) a 4x8-foot rocking tray; (b) a vertical, slightly tapered glass tube, 5 inches mean diameter; (c) a 7/16-inch plastic tube, 40 feet long, and a similar tube of glass; (d) a sloping, corrugated glass plate, like a washboard, about 36 inches square.

With the exception of (d), which for various reasons proved to be impractical, the different culture devices illustrated in figure 1 all gave roughly the same daily yield of Chlorella per unit area of intercepted sunlight. *

Very soon the need for a more fundamental approach to the study of the factors that determine the rate of growth of Chlorella, and hence the harvestable yields, became obvious. Comparison of our growth rates with those that could be obtained if all the absorbed sunlight were used effectively showed an efficiency of only about 10 per cent. The standard was the experimental rate of Chlorella growth in weak light under optimum conditions by Kok in Holland before he joined our group, as reported by him in chapter 5.

Some of the questions raised by the low rates obtained in these experiments are: Is the fact that bright light is used less effectively than weak light primarily responsible for this low rate? If so, can it be increased by producing intermittent illumination of each cell through turbulence of the culture? Is the growth rate of a culture limited by the potential rate of some other limiting process when photosynthesis is proceeding rapidly? Do both lar^e and small cells increase in weight at an equal rate per gram of cell material? A search for the answers to these questions has led to some of the experiments to be described in this chapter.

A few of the other fundamental problems of Chlorella culture that have been of interest may also be mentioned here. Cook [279], on the basis of work carried out at the Stanford Research Institute, reported a maximum yield at the low density of 0.36 g dry weight of Chlorella per liter, with a rapid drop in yield both above and below this cell density; but this

*This pilot plant Is described in chapter 17.--Ed,
^ The yield reported in chapter 17 for the pilot plant was approximately the same.--Ed.
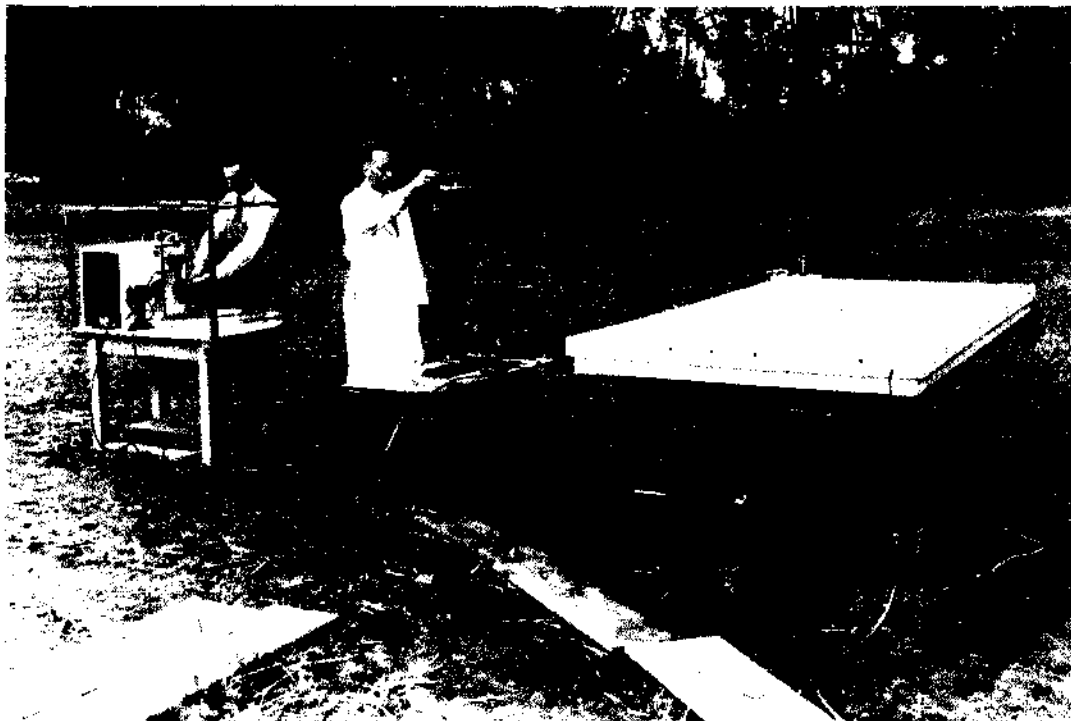
Fig. 1. Three of the outdoor culture units: from left to right, the plastic tubing, the vertical sedimentation tube, and the rocking tray, being worked on by Davis and Milner.

maximum may have been caused by the experimental set-up he used. High cell densities, if they could be used without reducing the growth rate, should greatly reduce harvesting costs* We therefore wished to see if high rates could be maintained under other conditions in cultures of much higher densities. It was also hoped that it might be possible greatly to increase the density of that part of the culture to be harvested, simply by settling. A study of the sedimentation rates of Chlorella and of a continuous production process based upon harvesting by settling was therefore carried out.

One rather extreme illustration of the lack of quantitative knowledge of the interrelations of the factors influencing Chlorella growth is that at the time the pilot plant at Arthur D. Little, Inc. was placed in operation we did not know the temperature for optimum growth in sunlight or the influence of the night temperature on growth.

In order to accumulate quantitative data on the effects of various factors on growth rates in cultures of high as well as of low density, several different types of apparatus were tried out. Outdoor thermostats with the Chlorella growing in nearly horizontal glass tubes, or in vertical test tubes,

were used for the temperature optimum experiments and for bleaching studies. Myers constructed several vertical columns in which the medium could be added slowly and continuously. He also made a lucite cell with photoelectric control of cell density for studies of growth rate at low cell density. Other experiments were carried out in flasks cooled by running water.

One of the difficulties frequently seen in outdoor experiments was the complete bleaching of the cultures when exposed to full sunlight. Later work has shown that this may be avoided by using a heavy inoculum of cells that have been grown outdoors, by partial shading of the young cultures, and by avoidance of extremes of pH.

The following sections of this chapter will summarize the major results that have been obtained and will point out the environmental conditions that now appear to be critical in relation to the mass culture of algae, and the lines of investigation that still need to be followed to place the art of algal culture on a quantitative scientific basis.

B

Outdoor Mass-Culture Units

Large Bottles (H. W. Milner)

The mass production of Chlorella in 5-gallon bottles has been described [152]. Forty-eight bottles, each containing 15 liters of culture, were kept in a greenhouse. The average daily yield of Chlorella from 720 liters of culture was 25 g dry weight. On the basis of illuminated area of the cultures, this yield was 4.8 g/m^/day.

The same kind of culture in 5-gallon bottles outdoors produced 16 g/m^/day dry weight under the most favorable weather conditions and at cell densities of 0.031 to 0.31 g/L When the cell density increased to 1.5 g/1 or more, the yield dropped to about 8 g/m^2/day.

The yield of Chlorella per unit area from cultures in the 5-gallon bottles was about the same as the yield obtained from other types of culture apparatus to be described. Per liter of culture handled, however, the yield from the 5-gallon bottles was much poorer than the yield from other units.

Rocking Tray (H. W. Milner)

In order to utilize effectively the high intensity of sunlight for growing Chlorella, there should be enough cells in the culture to absorb all the light. This may be accomplished by having either a deep culture with a low cell density, or a shallow culture with many cells per unit volume. The latter condition offers the advantage of requiring less nutrient medium for the growth of a given quantity of cells. No individual Chlorella cell should be exposed to the full intensity of sunlight. A high degree of turbulence maintained in an outdoor culture of Chlorella would in effect

subject each cell to a flashing-light type of illumination. The turbulence would also provide for thorough aeration of the culture and would prevent settling of the Chlorella cells. In designing an apparatus for growing a dense Chlorella culture in a thin turbulent layer illuminated by the sun, it appeared desirable to have a unit capable of producing several hundred grains of cells for use as experimental material. With these points in mind, the rocking tray was built.

A panel of plywood fastened to a frame made of two-by-fours formed a tray 235x113x9 cm inside dimensions. The area of the tray was 2.65 m^2, and its capacity was 2.65 liters of culture per millimeter of depth. The tray was coated with white Tygon paint, with a final coat of clear Tygon inside. The cover was made of thin, wire-reinforced plastic, supported by a wooden frame that fitted over the tray. The plastic transmitted 80 to 90 per cent of the sunlight and also served to retain air enriched with 5 per cent CO2 over the culture. The gas inlet was placed near the center of the tray and the gas escaped around the edges. Sixty feet of quarter-inch copper tubing, coated with clear Tygon paint, was coiled on the bottom of the tray. Tap water running through the coil cooled the culture. The tray was supported on its short axis by a piece of pipe, which in turn rested on two V-shaped bearings. A crank attached to one end of the tray was driven by an electric motor through a reducing gear and adjustable pulley train. Both the frequency and the amplitude of rocking were adjustable. This apparatus is illustrated in figure 1.

A 10-second rocking period imparted maximum turbulence to the culture. The 5 per cent CO2 in air passed through the tray at 2 liters per minute. The culture medium had the same composition as that used in the 5-gallon bottles [152]. Water evaporated from the culture medium was replaced daily. Growth of the Chlorella was measured once or twice daily by making cell counts and by determining in a sedimentation tube the packed volume of cells per milliliter of culture. This packed volume in cubic millimeters per milliliter divided by 4 was found to agree closely with determined values of the dry weight of the cells in grams per liter of culture. All the weights of Chlorella reported for the rocking tray arfe dry weights.

In the first experiments 7 to 15 liters of culture were used, giving a mean culture depth of 0.26 to 0.56 cm in the tray. When the inoculum of 0.1 to 0.3 g/1 had grown to about 3 g/1, part of the culture was removed from the tray and was replaced by fresh medium. This operation was repeated every few days. As the Chlorella used nitrogen from potassium nitrate, the culture became alkaline. The growth of about 50 g of cells changed the pH of the medium from 4 to 8. After each harvest, when fresh medium was added, the supply of potassium nitrate in the unharvested culture was replenished and the pH of the medium was adjusted by addition of phosphoric acid. In one such run five harvests were made during 18 days. The daily yield of Chlorella varied with the weather from 3 to 7 g/m2. The average for the 18-day period was 4.8 g. In figure 2 the solid lines show the daily total dry weight of Chlorella in the culture.
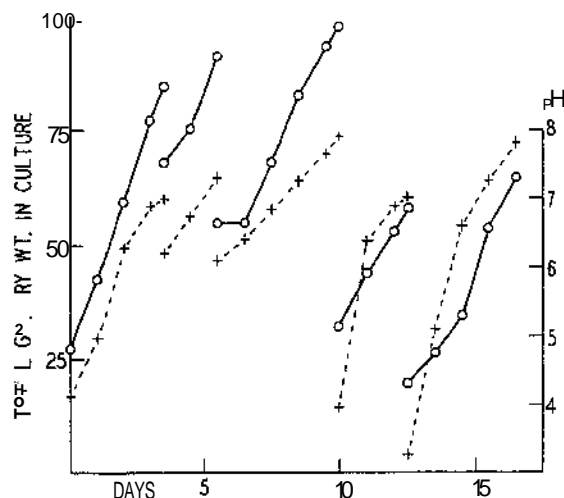
Fig. 2. Increase in cell mass and change in pH of a Chlorella culture in the "rocking tray with intermittent harvesting. Circles and solid lines indicate the cell mass present in the culture; crosses and dashed lines indicate the pH.

the breaks indicating harvests. The dashed lines show the pH of the culture each day.

The foregoing data were obtained during cool and partly cloudy spring weather. The Chlorella cells in several subsequent cultures bleached and died soon after inoculation. This trouble was largely due to overexposure of the cells to sunlight. Because the end of the tray had a vertical path of 4 cm on each rocking cycle, the culture drained from the high end, and the cells in the remaining thin film of culture were exposed too long to direct sunlight. Even dense cultures bleached slowly when exposed to the summer sun. Only when the rocking was adjusted to expose no part of the bottom of the tray was it possible to grow a culture for a long time without bleaching.

The volume of the culture was increased to 45 liters and the amplitude of rocking was reduced to 2 cm (±1 cm from level) at the end of the tray. This gave a mean depth of 1.7 cm of culture, with a minimum depth of 0.5 cm at the high end of the tray. After inoculation, the tray was shaded with muslin, a shorter time each day as the Chlorella grew. When the cell density reached 2.7 g/1, the culture was exposed to full sunlight all day. The density of 2.7 g/1 is considered that of an inoculum of a culture starting under the newly established set of environmental conditions. The culture then grew without harvesting until the increase in mass of Chlorella ceased.

To avoid the large changes in pH encountered previously, and to maintain the supply of fixed nitrogen near the original level during the heavy growth of Chlorella, a new procedure was adopted. When about 10 per cent of the original supply of nitrogen from potassium nitrate had been

used and the pH of the culture had changed from 5.6 to 6.5, the calculated quantity of ammonium nitrate was added to restore the fixed nitrogen concentration to its starting value. After this the Chlorella used ammonium nitrogen while the pH of the 'Culture decreased a few tenths of a unit; then, the ammonium nitrogen having become exhausted, the cells used nitrate nitrogen while the pH again increased to 6.5. At this point ammonium nitrate was added again. This procedure was repeated many times, keeping the pH between 6.0 and 6.5 and the supply of fixed nitrogen within 10 per cent of its original value. The only other nutrient added was 0.02 g $FeSO_4$« $7H_2O$ following each 150 g increase in mass of Chlorella in the culture.

The pH range from 6.0 to 6.5 was favorable to the growth of Chlorella and at the same time was about halfway between the pH values favoring bacteria and those favoring molds. Only bacterial contaminants were observed in the Chlorella culture under this pH control. The mass of bacteria was only 2 to 5 per cent that of Chlorella.

During 20 days the cell content of the culture increased from 2.7 to 11.5 g/1. Aside from daily fluctuations readily attributable to the weather, the increase in mass of Chlorella was linear with respect to time. The daily determinations of cell mass are plotted as circles in figure 3, with a straight line showing the average values. The average yield for the 20 days was 8.2 g/m^2/day, roughly double that obtained before (fig. 2).

In the early stages of growth of this culture there was a daily increase in both cell count and weight of cells. As the cell density increased from about 2 g/1, cell division became a stepwise instead of a continuous process. There were periods of 2 to 5 days without significant (±5 per cent) change in the cell count, followed by a material increase. This stepwise increase in cell count is shown by the broken line in figure 3.
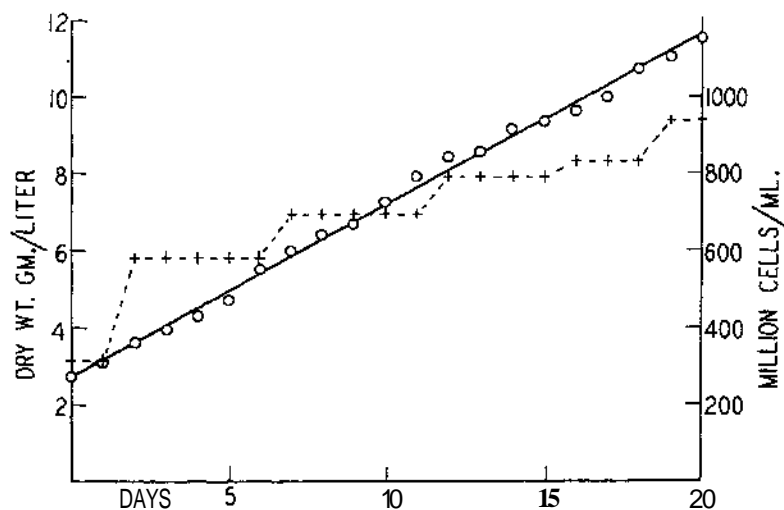
Fig. 3. Growth of a Chlorella culture in the rocking tray without harvesting. Circles and straight line show increase in cell mass; crosses and broken line show daily cell counts.

During the 10 days following the period covered by figure 3, the yield of CMorella gradually decreased to zero. At the time growth ceased, the culture contained 13.6 g/1 and $10^\wedge$ cells per milliliter. After the .culture had remained static 7 days, all the essential microelements were added. No effect of this addition appeared in the ensuing 4 days. A possible exhaustion of micronutrients seemed not to be the only cause for the static condition of the culture.

Two-*thirds of the culture was removed from the tray and was replaced with fresh medium. Growth of CMorella immediately started again. For the next week the daily increase in mass of cells was the same as that shown by the straight line in figure 3. The yield of cells from the harvested portion of the culture was 480 g. All together, 966 g of dry CMorella was obtained during four months' experimentation with the rocking tray.

We may compute a statistical cell size by dividing the centrifuged cell volume by the cell count per milliliter. In young CMorella cultures, where nearly all the cells are small, a statistical volume of 1.8xl0"H ml per cell has been found. According to the data presented in figure 3, the volume of a cell varies from 2.5 to $5.2X10^{-**}$ ml, with a very heterogeneous distribution of individual cell sizes. When growth ceased in the rocking tray (after 30 days) the statistical volume per cell was $6.3X1Q^{-11}$ ml. A 60-day-old culture in a 5-gallon bottle had nearly uniformly large cells with a statistical volume of $9.1X10"^{11}$ ml.

Extremely high cell densities, such as the 55 g/l dry weight reported by Myers et al. [104], were not achieved in experiments with the rocking tray. It may be significant that the depth of culture exposed to sunlight was 0.5 cm in Myers' apparatus and a mean of 1.7 cm in the rocking tray. It may be assumed, other conditions being favorable, that the mass of CMorella cells per unit area of illuminated culture will not increase beyond a value which is probably determined by mutual shading of the cells. The maximum was 13.6 g/l in a depth of 1.7 cm in the tray. This same quantity of cells in the same tray area, if contained in a layer oMy 0.5 cm deep, would have corresponded to a density of 46 g/l, compared with Myers' 55 g/1.

It was found that the largest daily increase in grams of CMorella in the tray was obtained, and that this increase was uniform, between cell densities of 2.7 and 11.5 g/L This is in contrast with Cook's finding [22]. that the maximum yield was at a cell density of 0.36 g/L Cook's apparatus had a 10.2-cm (4-inch) light path. Suppose that the greatest number of grams of CMorella per day will be grown at some fixed mass of cells per unit area of illuminated culture. Then 0.36 g/I in a depth of 10.2 cm would correspond to .2.2 g/l in a depth of 1.7 cm, a value almost within the range found with the rocking tray.

In this discussion, the terms "growth" and "yield" are used to denote the increase in grams of CMorella in the culture, usually expressed with respect to unit volume, time, or area illuminated. They are not to be confused with the term "growth rate," which applies only to the increase in CMorella per unit time with respect to the quantity of CMorella present

in the culture at the beginning of that unit time. The maximum growth rate and maximum yield of a culture may or may not occur at the same time in the life of the culture.

In the rocking tray the maximum yield of Chlorella (in grams increase per day) was not obtained at the point of highest growth rate (per cent increase per day of the cells present in the culture). Growth rates of 100 to 126 per cent per day were found with cultures of low cell density, but at the same time the daily increase in mass of Chlorella was only 1.6 to 2.3 g/nA The greatest daily increase in grams of Chlorella in the tray was the average of 8.2 g/m^2 for 20 days at high cell density (fig. 3). Figure 3 does not illustrate the growth rate of the culture. The growth rate during this same 20 days fell from 18 per cent per day at the start to a mere 4.2 per cent per day at the end. In other words, the same yield of Chlorella was obtained with a 4.2 per cent per day growth rate at a cell density of 11.5 g/1 and an 18 per cent per day growth rate at a cell density of 2.7 g/1. Both these yields were much larger than those obtained with 100 to 126 per cent per day growth rates at cell densities of about 0.1 g/1.

It has been found practicable to grow Chlorella in pound quantities in outdoor cultures exposed to direct sunlight. A thin layer of culture having a high cell density absorbs all the light and gives a good yield. Turbulence of the culture prevents the cells from settling, ensures their aeration with 5 pfer cent CO_2, and may increase their utilization of bright light by providing intermittent illumination.

Plastic and Glass Tubing (E. A. Davis)

Small-scale plastic and glass tubing apparatus were constructed for the continuous growth of unicellular algae. They were designed to permit the culturing of dense suspensions and the harvesting of even denser suspensions as obtained by settling.

The method of harvesting by settling will be discussed in a later section. In the experiments to be described in this section the cells which settled at night, during which time pumping was discontinued, were resuspended each morning.

The experiment had two main purposes: first, to find whether Chlorella could be grown in plastic (Tygon) tubing, and, second, to measure the daily increase in the growth of Chlorella outdoors under nonaseptic conditions in both plastic and glass tubing.

Each apparatus consisted of two main parts: a tubing section for growth, and a settling chamber (a wide-stemmed separatory funnel) from the bottom of which concentrated suspensions could be withdrawn. The'tubing of one apparatus was Tygon of 8 mm inside diameter, and that of the other was glass of 7.5 mm inside diameter. The plastic tubing formed a helix and the glass tubing a grid. In each case there were approximately 40 feet of tubing. The culture capacity of each apparatus was 1 liter, of which the settling chamber contained 350 ml and the tubing section 650 ml.

The top of each settling chamber was equipped with a rubber stopper through which passed four glass tubes, two for the culture and two for the gas stream, as shown in figure 4. The gas stream, consisting of 5 per cent CO2 in air, passed through a cotton filter before entering the gas inlet tube. The orifice of this tube inside the reservoir was adjacent to the intake of the culture outlet tube. As a consequence, gas bubbles were drawn into the culture outlet tube and circulated with the culture. Each culture was circulated by means of a Sigmamotor finger pump, at a rate of 540 to 620 ml/min or 40.1 to 46.1 ft/min. The path of a culture was from the settling chamber through the pump tubing into the long section of tubing exposed to sunlight, and then back to the settling chamber. During warm weather, water was circulated between the outside of the settling chamber and a glass jacket. The overflow passed over the glass plates which supported the tubing. Culture samples were obtained at intervals through the gas outlet tube.

It was found that when gum-rubber tubing was used in the pump, growth was stopped. Tygon was found to be satisfactory. Because of wear, it was necessary to install a new piece of tubing about every ten days.
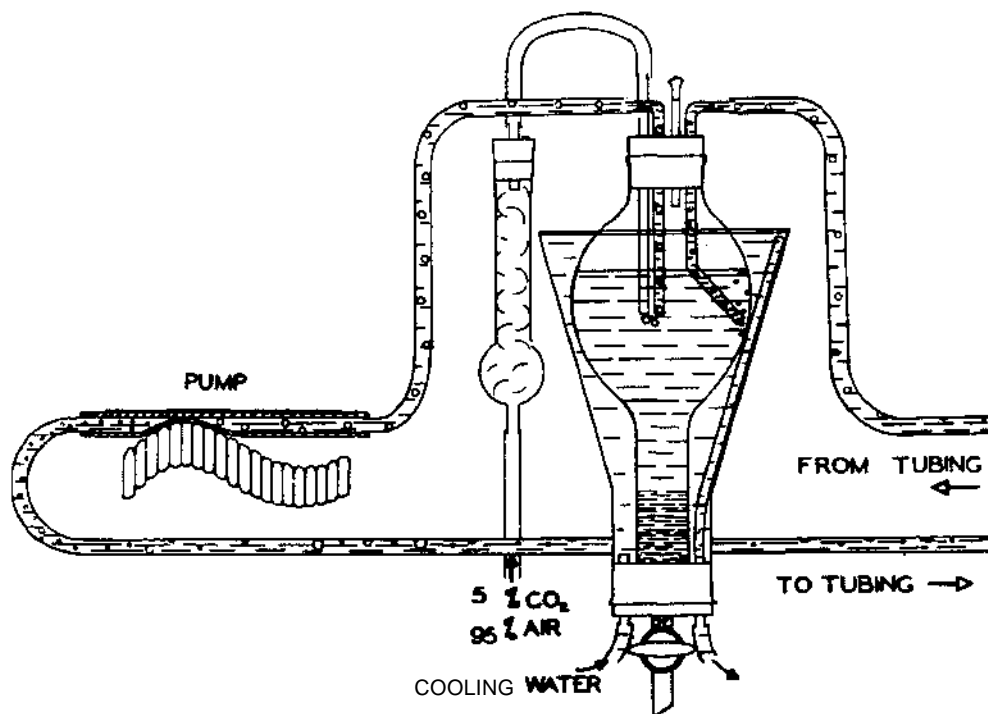
Fig. 4. Settling chamber, pump, and gas-inlet arrangement of the plastic and glass tubing culture apparatus.

The standard medium used contained 0.025 M KNO$_3$, 0.018 M KH2PO4, 0.02 M MgSO4 • 7H2O, 10""^{5} M FeSO4 • 7H2G, 1Q-^{5} M potassium citrate, and 1 ml of Arnon's A4 microelement solution [183] per liter of medium. During the experiment it was frequently necessary to add extra nitrogen and iron. No attempt was made to keep the composition of the medium constant with respect to all the elements during growth.

Because potassium nitrate was used as the source of nitrogen, the pH of the medium increased during growth. This pH increase was used to monitor the addition of nitrogen, in the form of 6N nitric acid, to the medium. The amount of nitric acid to be added to lower the pH to about 6 was determined from a titration curve.

The growth of Chlorella in the plastic and the glass tubing apparatus was compared on the basis of increase in fresh weight and cell number (fig. 5). For 21 days the growth in both units, measured by the two methods, was essentially the same. It was therefore concluded that Chlorella could be grown satisfactorily in Tygon tubing.

The cells in the glass tubing unit were then resuspended in fresh medium while those in the plastic tubing unit were left in their original medium. A discussion of this phase of the experiment will be reserved for the subsection on "Culture Medium." It need only be stated here that growth in terms of fresh weight was the same in both units.

During the experiment, which lasted for 49 days, there was considerable variation in the amount of growth per day. The average daily increase over the entire period was 1.2 g/1 fresh weight for the culture in the plastic tubing unit and 1.3 g/1 for the culture in the glass tubing unit. These values are low because of poor growing conditions on many of the days. The range of the 37 largest daily increases was 2.0 to 5.2 g/1 fresh weight; this is expressed in various units in table 1. This range more nearly represents

Table 1

Range of the 37 largest daily increases, in plastic and glass tubing apparatus, expressed in various units

Unit	Range
g/1, fresh weight	2.0- 5.2
g/1, dry weight	0.5- 1.3
g/ft^2, dry weight	0.4- 1.1
g/m^2, dry weight	4.5-11.7

the maximum daily growth to be expected in the tubing apparatus than the lower values mentioned above. It is believed that growth in this range could be maintained by providing a more adequate medium, in which better provision would be made for maintaining ample supplies of nitrogen and available iron.

Although the percentage increase in growth declined markedly after the first few days, as shown in figure 6, yet the daily **increases** remained about the same over the 49-day period, during which time the density in-
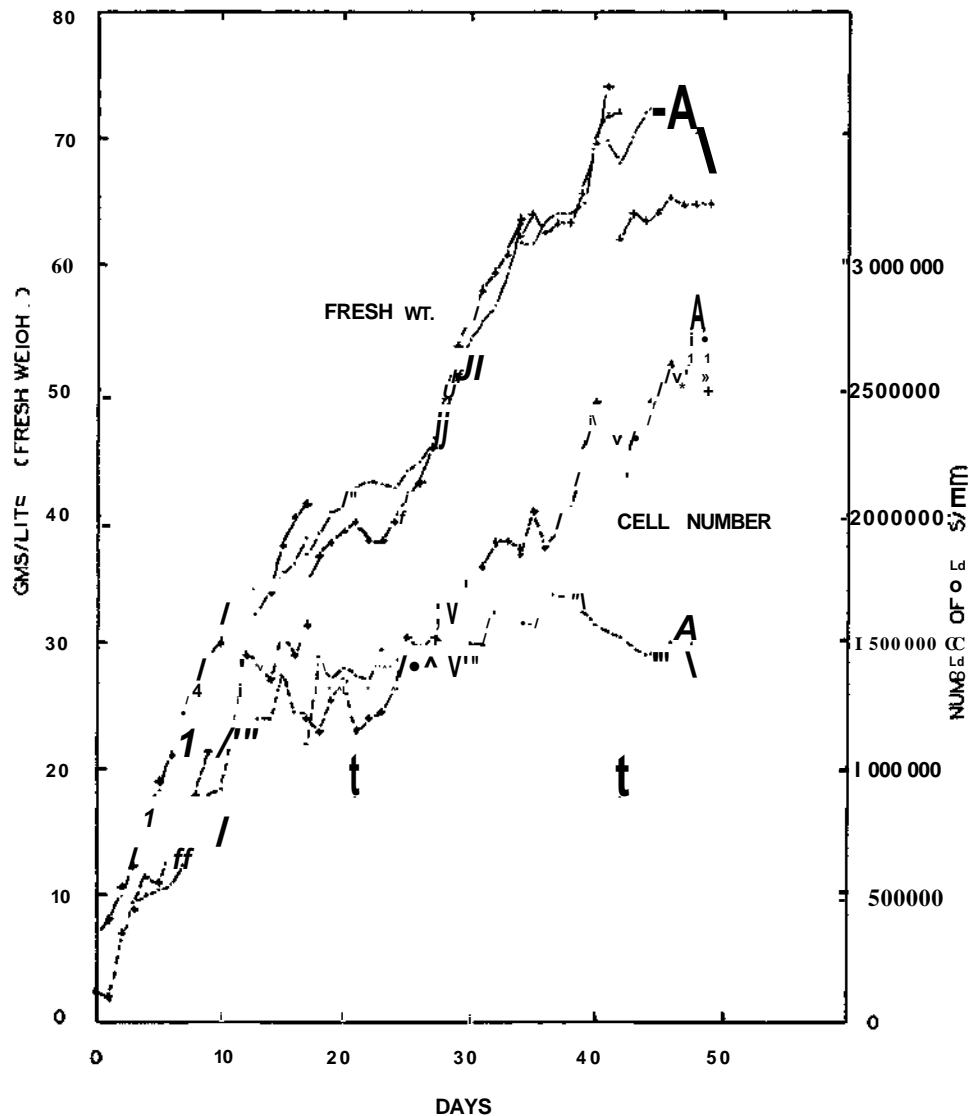
Fig. 5. Growth of Chlorelia pyrenoldosa cultures in the glass and the plastic tubing apparatus outdoors, measured in terms of both fresh weight and cell number. At the arrows, the cells in the glass tubing apparatus (crosses) were resuspended in fresh medium, while those in the plastic unit were left in the old medium.

creased from 7 to a maximum of 74 g/1 fresh weight. It would seem distinctly advantageous, in culturing Chlorelia on a mass scale, to operate at the highest culture density that still gives the maximum daily yield, regardless of the percentage increase in growth, thereby decreasing the quantity of culture to be handled during harvesting.
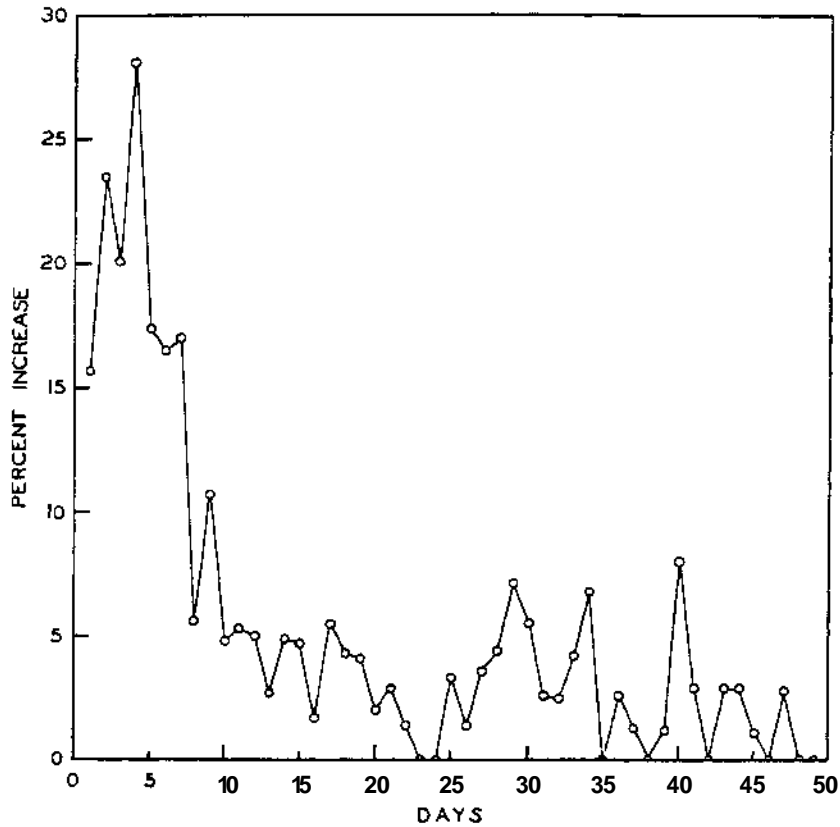
Fig. 6. Growth rate of a *Chlorella pyrenoidosa* culture in the plastic tubing apparatus, expressed as per cent increase per day.

C

Quantitative Studies in Controlled Experimental Culture Units

Carbon Dioxide Concentration (E. A, Davis, Jack Myers, and Jean Dedrick)

The lowest concentration of carbon dioxide that supports maximum growth of Chlorella has been a matter of considerable doubt. Experiments therefore were performed to determine the effect of the partial pressure of carbon dioxide on the growth rate of Chlorella.

For these experiments a constant-density culture apparatus designed by Myers was used. It comprises a lucite growth chamber and a photo-electric system for maintaining the culture at a given density. When the culture density reaches a certain level, a solenoid valve is activated and
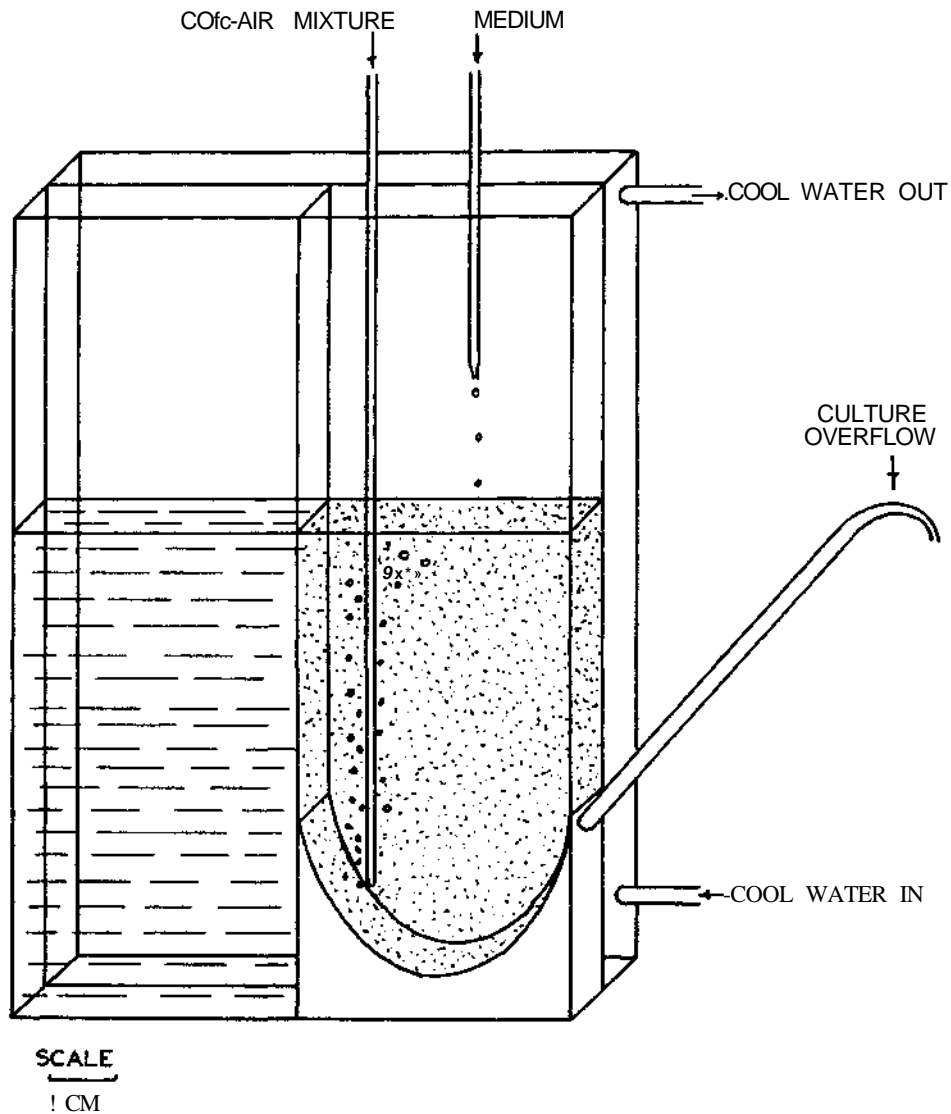
Ftg* 7. Lucite chamber of the constant-density cultare apparatus used for measuring growth rate 0! Chlorelia.

the culture is diluted with fresh medium. The volume of culture is kept constant by means of an overflow tube.

The growth chamber consists of three Incite cells cemented together, as shown In figure 7. The large cell facing the light source (a 200-watt

bulb) contains circulating water thermostatically controlled for cooling purposes. The half cell in front with the rounded bottom contains 40 nil of algal suspension. A carbon dioxide-air mixture is bubbled through the culture, providing excellent circulation as well as supplying carbon dioxide. An-outside overflow tube sealed in at the bottom of the culture cell extends up to the level of the culture. The other half cell contains water. Two photocells, electrically opposed, face the light source: one looks through the water blank, the other through the culture. The circuit is such that line voltage fluctuations causing variations in light intensity do not influence the operation of the solenoid valve.

For the purpose of determining the effect of different carbon dioxide concentrations on growth, it was essential that the apparatus be efficient in maintaining the culture at constant densities. Experiments showed this to be possible. The influence of carbon dioxide concentration on growth was then examined.

The light intensity for these experiments was approximately 350 f.c. at the face of the growth cell. The intensity of the light transmitted by the culture was approximately 150 f.c. The temperature of the culture was maintained at $25\pm2°$ C. The culture density was maintained at 1.5 mm^ of cells per milliliter (a relatively thin suspension). The volume of overflow and the concentration of cells in the overflow were measured periodically. The medium used was regular Knop's solution containing microelements and iron (5 ppm) added as ferric potassium EDTA (see the next subsection, "Culture Medium"). Each experiment lasted at least 24 hours, the volume of overflow being used as the measure of growth. The concentration of carbon dioxide dissolved in the culture medium was measured with a Van Slyke gas-analysis apparatus.

The concentrations of carbon dioxide tested were 4.43, 2.IS, 1.02, and 0.56 per cent. Growth was found to be essentially the same at all these concentrations, as shown in figure 8. The variations lie within experimental error. Under the experimental conditions, then, the growth rate of Chlorella was not significantly influenced by differences in concentration of carbon dioxide from 0.56 to 4.43 per cent, as long as the culture medium was kept in equilibrium with the lower concentrations of carbon dioxide.

Culture Medium (E. A. Davis and Jean Dedrick)

From the standpoint of the commercial production of algae, the question concerning the recycling of medium is important. Accordingly, the growth of Chlorella was compared in fresh and physiologically old media. The experiment was carried out in the plastic (Tygon) and glass tubing culture units, previously described, in conjunction with an experiment to find the daily yield of Chlorella. The growth curves for the experiment are shown in figure 6 in the subsection "Plastic and Glass Tubing."

After it was found that Chlorella grew equally well in the plastic and glass tubing units, the cells in the glass tubing unit were resuspended in
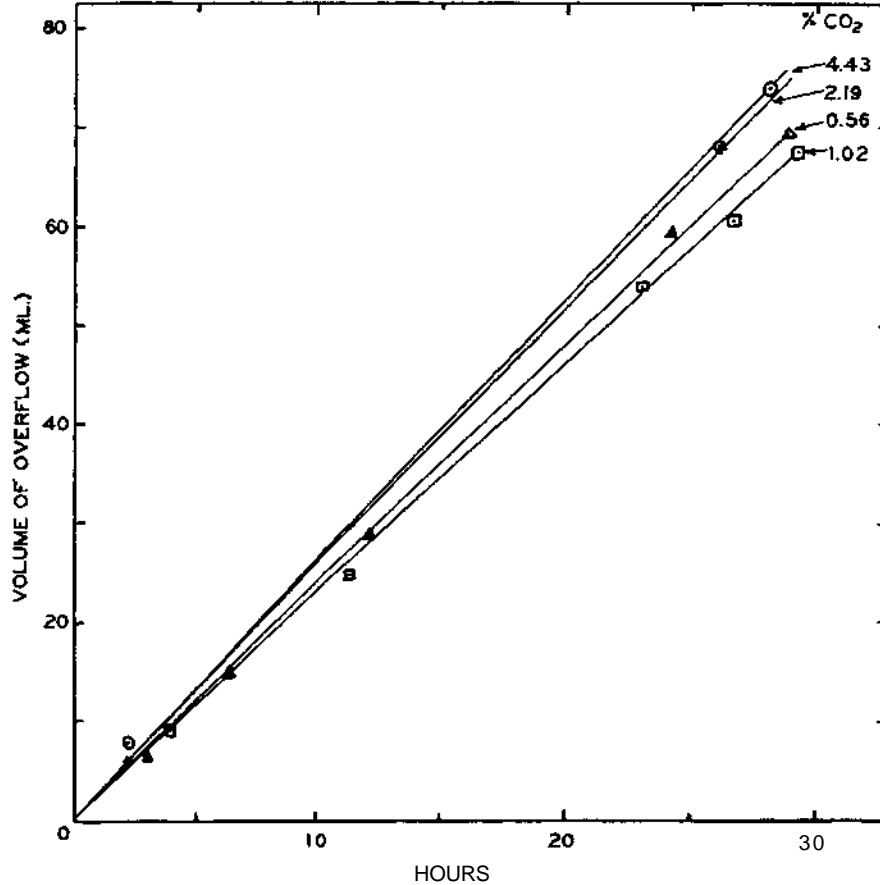
Fig. 8. Growth of Chlorella pyrenoidosa in the constant-density lucite chamber with various concentrations of carbon dioxide in equilibrium with the medium.

fresh medium on the 21st day and again on the 42d day. Those in the plastic tubing unit were left in their original medium. This medium will be referred to as the physiologically old medium. The fresh weight of cells in this medium increased about equally with that of the cells in the fresh medium. Cell number, on the other hand, leveled off in the physiologically old medium, whereas it continued to increase in the fresh medium. Frequent additions of nitrogen and iron were made to each.

The several breaks in the growth curves represent losses of culture due to technical difficulties. When these are taken into account, the results appear more striking.

Figures illustrating the changes which occurred in fresh weight and cell number are given in table 2." At the end of the experiment (49 days)

Table 2

Comparison of cell division and fresh weight increase in fresh and in physiologically old media

(Glass tubing unit received fresh medium on the 21st and 42d days;
Tygon unit did not receive fresh medium.)

No. days	Culture unit	Cell count (no. cells/mm³)	Fresh wt. (g/1)	Av. cell size (X10⁻⁸ ml)	Dry wt. (g/1)
21	Tygon	1,365,000	42.2	3.1	
	Glass	1,150,000	39.6	3.4	
49	Tygon	1,375,000	67.2	4.9	18.1
	Glass	2,510,000	64.8	2.6	17.4

there were about twice as many cells in the fresh medium in the glass tubing unit as there were in the physiologically old medium in the Tygon unit. Fresh weight of cells, however, was essentially the same. Although the cells in both units were of about the same size after 21 days' growth, after 49 days those in the physiologically old medium were about twice as large as those in the fresh medium- At the end of the experiment the quantity of Chlorella in terms of dry weight in each unit was,,the same. The production of organic matter was not, therefore, affected by the physiological age of the medium. In the production of organic matter, cell size is unimportant; it is the total quantity of material produced that counts. Recycling of medium therefore appears to be a practical procedure.

When cells are grown in a physiologically old medium, it is extremely important that adequate supplies of nutrients, especially iron and micro-elements, be kept available to the cells. Because of the difficulty of maintaining soluble iron in a medium containing ferrous sulfate or ferrous citrate, when aseptic precautions are not taken, a medium containing an effective iron-chelating agent not utilized by microorganisms was desired. A medium developed by Hutner for Euglena and used by Myers for Chlorella [104], containing ethylenediamine tetraacetic acid (EDTA) as the chelating agent, was tested. Over a period of several days, in experiments carried on outdoors with the glass tubing apparatus, growth in the EDTA medium was 68 per cent greater than that in the regular medium. This improvement recommends the use of an EDTA medium*

In laboratory experiments an EDTA medium, prepared by adding ferric potassium EDTA to Knop's solution containing microelements, has been used with satisfactory results.

The ferric potassium EDTA stock solution was prepared according to Jacobson [235] by dissolving 26.1 g EDTA in 268 ml of 1.0N KOH, then adding 24.9 g $FeSO_4 \cdot YH_2O$ and diluting to 1 liter. The solution is aerated overnight to produce the stable ferric complex. One milliliter of this solution per liter of nutrient solution supplies 5 ppm of iron. The formula for the ferric potassium EDTA Knap's solution is as follows: KNO3, 1.21 g;

$MgSO_4$-$7H_2O$, 2.46 g; KH2PO4, 1.22 g; A4 microelement solution [183], 1 ml; ferric potassium EDTA stock solution, 1 ml; distilled water, 998 ml.

The tolerance of Chlorella for ferric potassium EDTA was studied, using flask cultures suspended in a water bath and illuminated from below with a 200-watt lamp. Air containing 5 per cent CO2 was bubbled through the cultures. The concentration of ferric potassium EDTA was varied from 0.25 ml to 16 ml per liter of nutrient, supplying from 1,25 to 80 ppm of iron. Chlorella was found to be tolerant of very high concentrations of iron supplied in this form (table 3). Inhibition of growth was

Table 3

Tolerance of Chlorella pyrenoidosa for ferric potassium EDTA

Iron cone. (ppm)	Cell count after 6 days (no. cells/mm^)	Fresh wt. after 6 days (g/1)
1.25	260,000	10.0
2.5	460,000	16.0
5.0	530,000	14.1
10.0	510,000	17.0
20.0	660,000	15.0
40.0	510,000	14.0
60.0	590,000	13.6
80.0	520,000	14^5

not noted up to 80 ppm. Therefore a culture to be grown continuously can be supplied initially with enough iron to last for a considerable time.

As a culture grows, the pH of the medium changes greatly when either nitrate or ammonium ion is used as the nitrogen source. In many investigations, however, it is desirable to maintain a relatively constant pH. A search was therefore made for organic compounds which would serve as suitable nitrogen sources for Chlorella without causing drastic pH. fluctuations in the medium. To be of practical importance, the nitrogen supply should not serve as a source of carbon for contaminants.

Two excellent organic sources of nitrogen which do not serve as energy sources for Chlorella were found. They are glycine and urea. Urea has the advantage over glycine that it does not support the growth of culture contaminants.

Both glycine and urea were compared with potassium nitrate as nitrogen sources. The cultures were grown in flasks as described before. Enough of each compound was added to supply 0.1 g nitrogen. As figures a and 10 indicate, both glycine and urea compare favorably with potassium nitrate as nitrogen sources. Both the fresh weights and the login of the fresh weights are plotted. The slope of the logarithmic curve represents growth rate· Growth in both the glycine and the urea media was greater in nltrate meMum * With glycine tte o/TOwth rate was initially lower than it was with nitrate, but gradually increased until it became greater.
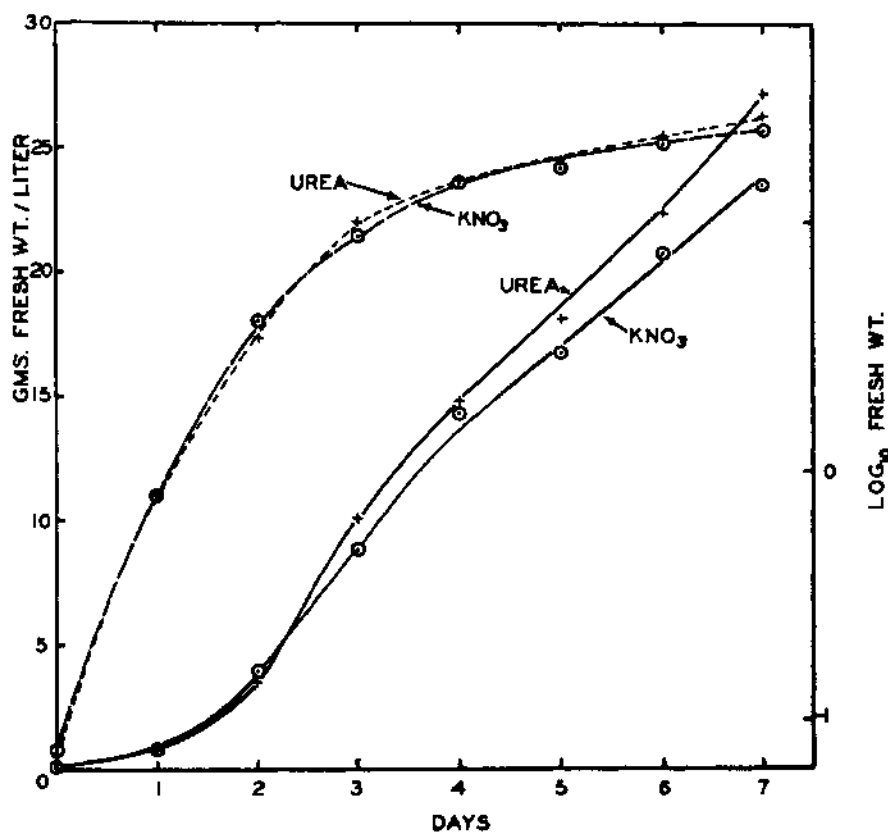
Fig. 9. Growth of Chlorella pyrenoidosa in nasks, with urea compared with potassium nitrate as a nitrogen source. The upper (dashed) curves show the lo&xc *of* the fresh weight of cells present.

The pH changes which occurred in the media are shown in figure 11. The initial pH of all these media was 5.0. With potassium nitrate the pH increased to 8.1, whereas with urea it increased only to 6.5, The final pH of the glycine medium was 4.9. Both glycine and urea, then, serve as excellent nitrogen sources and cause but minor changes in the pH of the medium during growth. Urea was studied further, since it does not support the growth of culture contaminants.

In the culturing of dense suspensions of Chlorella, the nitrogen supply of the medium is depleted very rapidly. It is therefore necessary to make frequent additions of nitrogen or to supply the medium with a large enough quantity to last a longer time. To do the latter, it is necessary to know what concentration can be used without inhibiting growth. The tolerance of Chlorella for high concentrations of potassium nitrate and of urea was therefore studied. The two compounds were compared on an equivalent nitrogen basis. The concentration of potassium nitrate was varied from 1.8 to 14.4 g/1, supplying 0.25 to 2.0 g nitrogen. The concentrations of urea necessary to supply the same quantities of nitrogen were 0.52 to
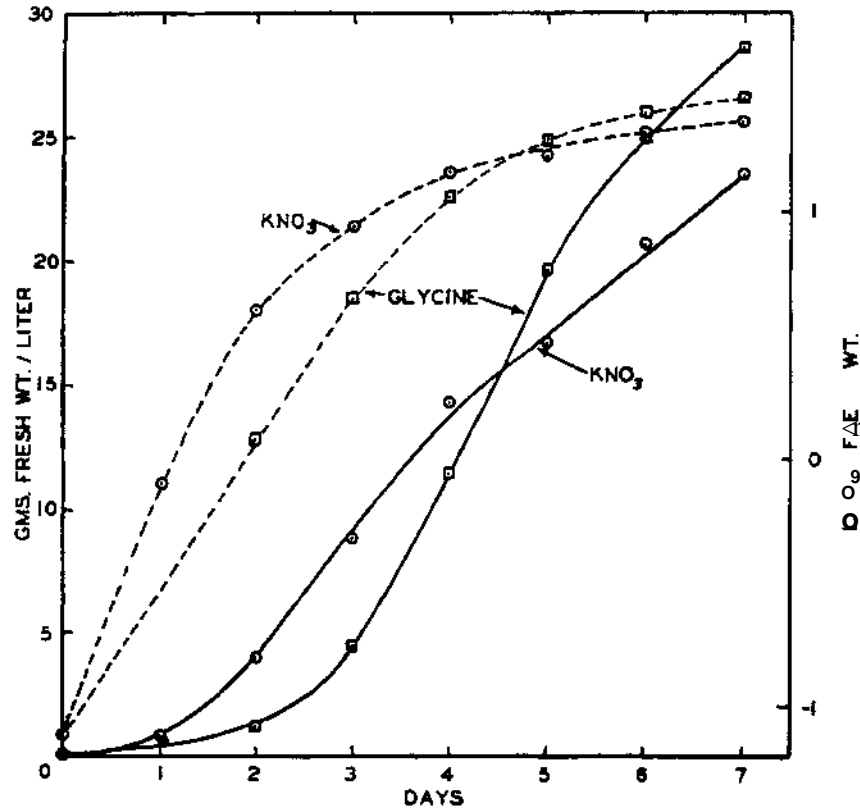
Fig. 10. Growth of Chlorella pyrenoidosa In flasks, with glycine compared with potassium nitrate as a nitrogen source. The upper (dashed) curves show the lo-g^g, of the fresh weight of cells present.

4.2 g/L Cell number, fresh weight, and pH changes which occurred during growth are recorded in table 4.

At all concentrations tested, growth as measured by cell number and by fresh weight was considerably better with urea than with potassium nitrate. Growth was poorer at the highest concentration of both nitrogen sources than at the lower concentrations. However, in the medium containing the highest concentration of urea, equivalent to 2.0 g nitrogen, growth was considerably better than it was in the medium containing the corresponding concentration of potassium nitrate. Also, cells in the most concentrated nitrate medium were badly clumped, while those in the urea medium were normal.

The initial pH of these media was adjusted to 6.0. During the 5-day growth period the maximum to which it increased in the urea cultures was 6.42, whereas it increased to 8.29 in one of the nitrate cultures.

Urea therefore has several advantages over potassium nitrate as a nitrogen source for Chlorella: it gives greater yields,, it causes only
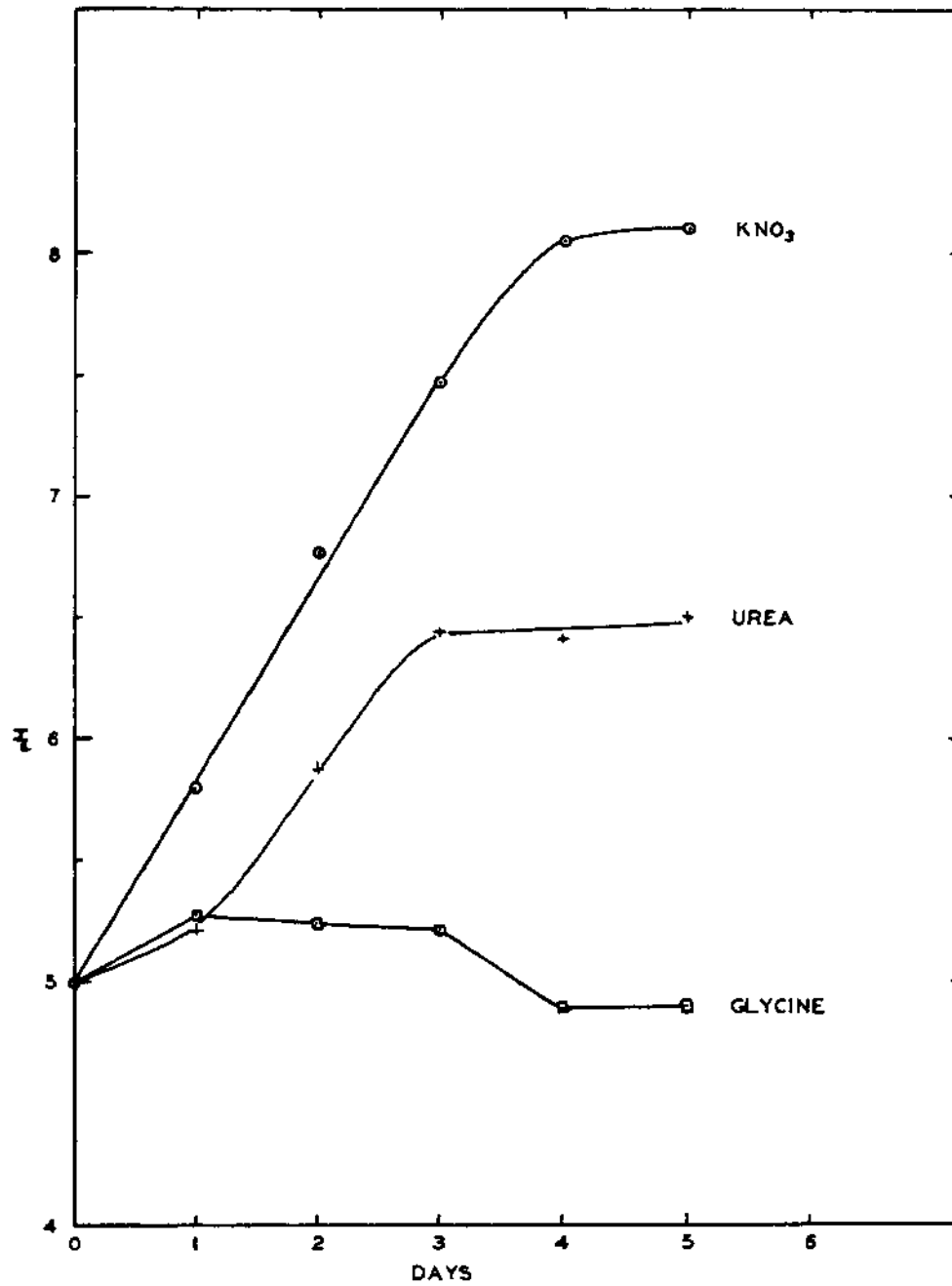
Fig. 11. Changes in pH accompanying growth of _Cftl.orella pyrencáctosa_ when nitrogen is supplied in three different forms.

Table 4

Tolerance of Chlorella pyrenoidosa for potassium nitrate and urea (initial pH = 6.0)

Concentration		Cell count a after 5 days (no. cells/mm^3)	Fresh wt. b after 5 days (g/1)	pH
g/1	g nitrogen/1			
		Potassium nitrate		
1.8	0.25.	355,000	15.0	7.89
3.6	0.5.	305,000	16.4	7.91
7.2	1.0.	295,000	14.6	8.29
14.4	2.0.	145,000	9.6	8.00
		Urea		
0.525	0.25.	380,000	18.5	6.13
1.05	0.5.	410,000	20.9	6.28
2.1	1.0.	355,000	17.6	6.41
4.2	2.0.	230,000	14.6	6.42

[a] Initial cell count was 5175 cells/mm^3,
[b] Initial fresh weight was 0.2 g/1.

minor pH fluctuations during growth, and it furnishes a greater supply of nitrogen without seriously decreasing growth.

Night Temperature and Aeration (E. A. Davis and Jean Dedrick)

Night temperature is known to influence the growth of many plants. Laboratory experiments under controlled conditions were therefore undertaken to study the effect of night temperature on the growth of Chlorella.

Cultures were grown in 200-ml Erlenmeyer flasks. During the day the flasks were suspended in water baths at 25±2° C and illuminated from below with 200-watt lamps. The light intensity at the level of the flasks was approximately 1000 f.c. Five per cent CO_2 in air was bubbled through the cultures. At night the flasks were transferred to constant-temperature chambers and aerated with air. The periods of illumination and darkness were each of 12 hours duration. Packed cell volumes (converted to grams per liter) and cell numbers were determined daily. The night temperatures ranged from 5 to 35° C.

The increases in fresh weight are plotted in figures 12 and 13. Final results, in terms of fresh weight and cell number, after 6 days' growth are recorded in table 5. They are expressed as percentages of the growth of the control cultures held at 25° C during both day and night. Increase in fresh weight was not found to be greatly affected by night temperatures of 10, 15, 20, or 25° C. The greatest growth occurred, however, with a night temperature of 15° C, and declined with night temperatures above and below this value. Night temperatures of 5, 30, and 35° C were definitely unfavorable. The influence of night temperatures on cell number was somewhat more pronounced at the lowest and highest temperatures. The greatest cell numbers occurred in cultures grown with night temperatures of 15, 20, and 25° C. Temperatures of 5, 10, 30, and 35° C were inhibitory.
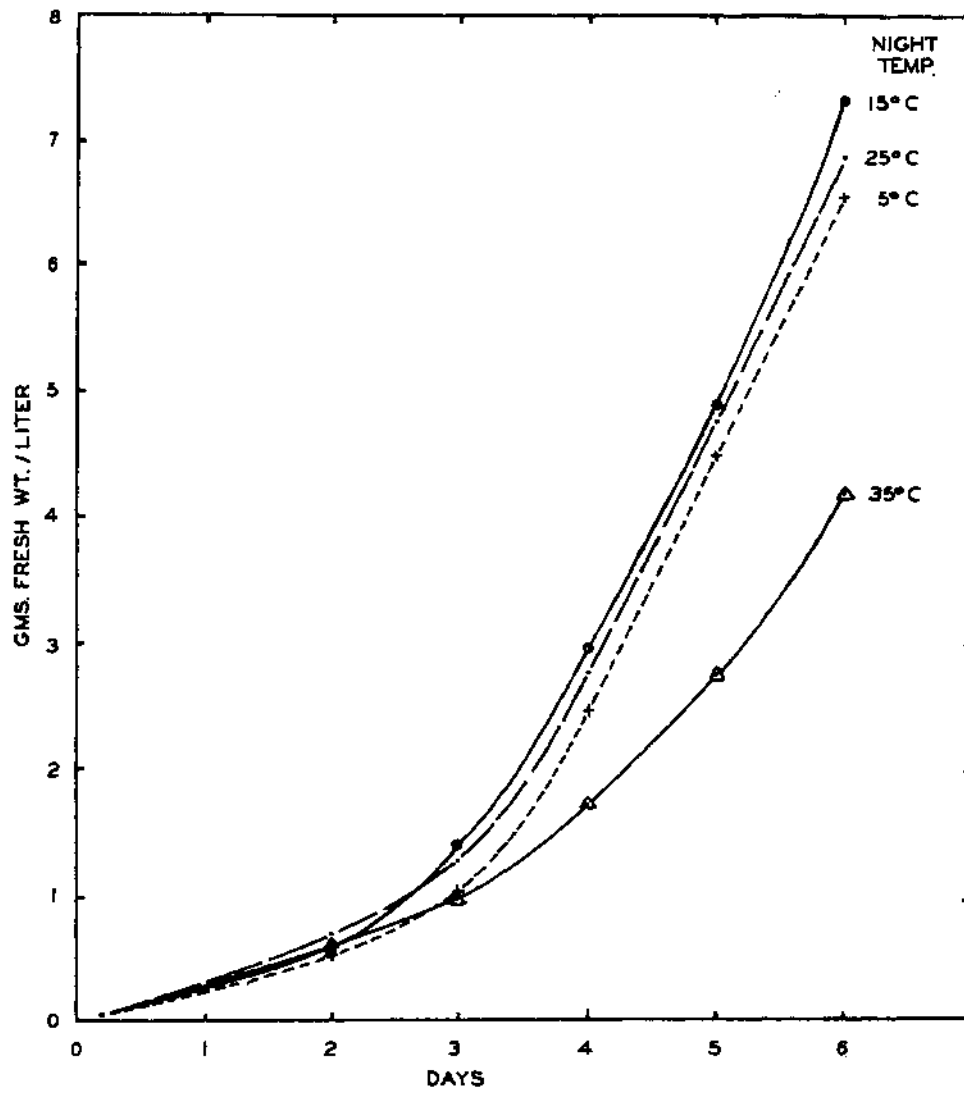
Fig. 12. Growth of Chlorella pyrenoidosa cultures in flasks kept at 25° C during the day but at different night temperatures. The remainder of the data are plotted in figure 13.
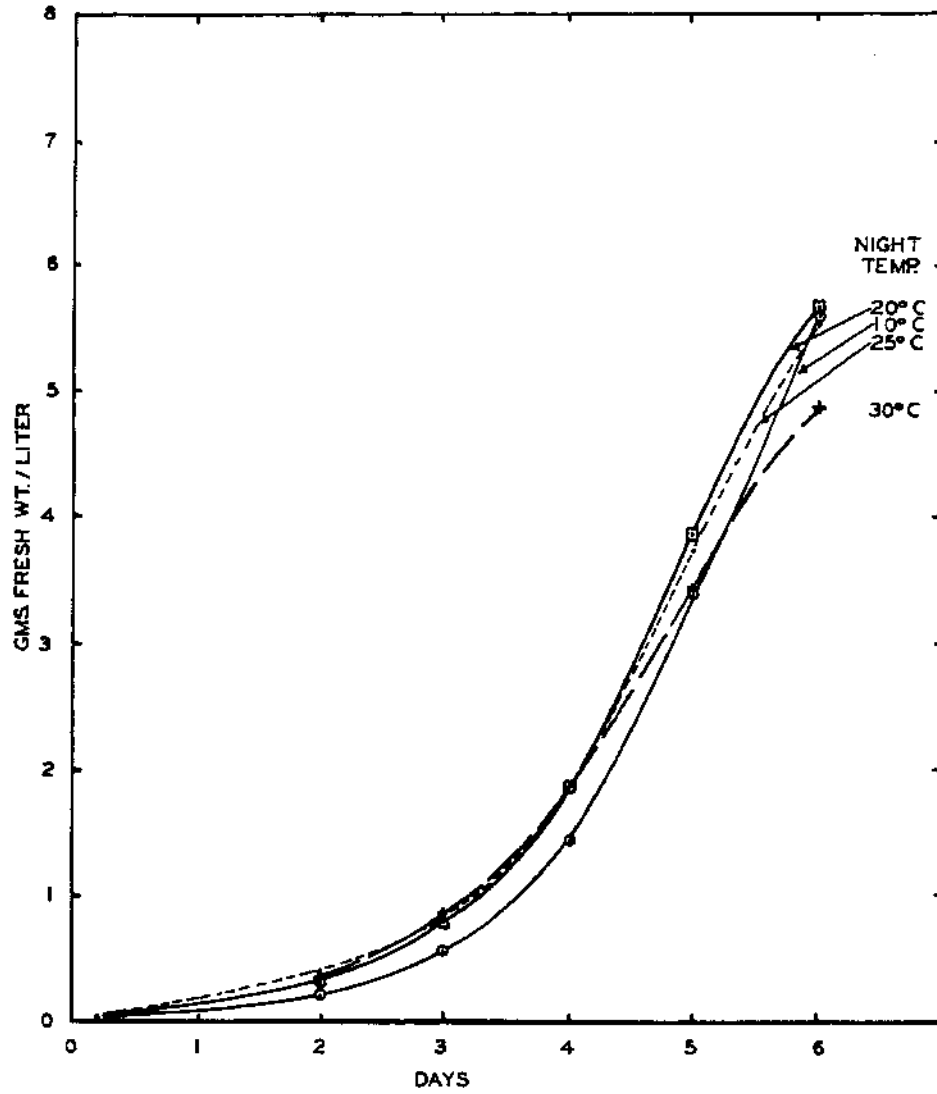
Fig, 13, Growth of <u>Chlorella pyrenoidosa</u> cultures in flasks kept at 25° C during the day but at different night temperatures. The remainder of the data are plotted in figure 12.

Table 5

Influence of night temperature on growth of Chlorella grown at 25° C during the day

(Control at 25° C day and night) .

Night temp. T C)	Fresh wt. after 6 days (% of control)	Cell count after 6 days (% of control)
5	94.8	88.6
10	101.8	86.6
15	106.1	100.0
20	103.3	96.7
25	100.0	100.0
30	88.7	80.6
35	60.8	19.9

fif respect to fresh weight, these results are qualitatively similar to those obtained with cultures grown outdoors at a day temperature of 25° C, reported in the next subsection.

The influence of aeration at night on increase in fresh weight and cell number was also studied. During the day the cultures were illuminated from below with a 200-watt lamp. The temperature both day and night was approximately 25° C. Each period was of 12 hours' duration. All cultures received 5 per cent CO_2 in air during the day. At night air was bubbled through one of the cultures and 5 per cent CO_2 in air through another. A third culture was not aerated. Fresh weights and cell numbers were determined after 6 days.

Night-time aeration was found to have little effect on increase in fresh weight (table 6); all the cultures grew about equally well. The influence of night aeration on cell number was more pronounced. There were considerably fewer cells in the culture which was not aerated at night. Little

Table 6

Influence of night-time aeration on increase in fresh weight and cell number

Night aeration	Cell count after 6 days (no. cells/mm3)	Fresh wt. after 6 days (g/l)
Air	240,000	0.0
5% CO_2 in air	256,000	7.0
None	160,000	5.9

difference was found between the cultures aerated with air and with 5 per cent CO_2 in air. There is indication, therefore, that night aeration does pot greatly influence growth of a batch culture as measured by fresh weight. With respect to cell number, however, it exerts considerable influence.

Day and Night Temperatures with Full Sunlight and with Partial Shading
(EL W. Milner)

During much of the time when the rocking tray was operated, the culture conditions were not ideal for maximum growth of Chlorella. To use
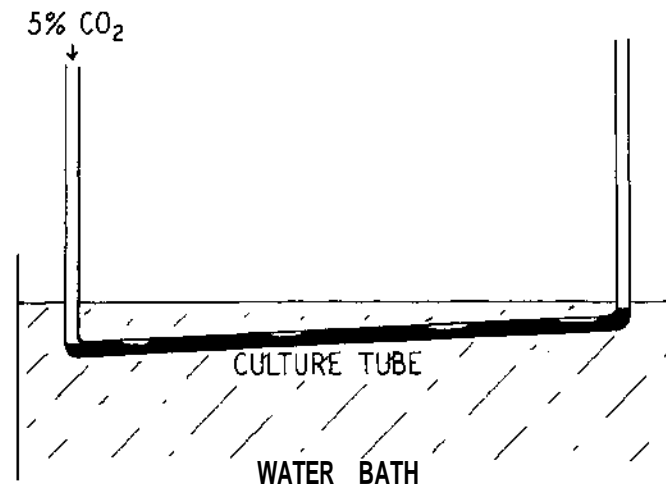
Fig. 14. Apparatus for outdoor growth of Chlorella at controlled temperatures

one unit as large as the tray for a study of different conditions would take a very long time. It was decided to measure the effect of temperature and illumination on the yield of several outdoor cultures of Chlorella growing at the same time.

Three thermostatically controlled water baths were placed outdoors. From one to four Chlorella cultures contained in glass tubes were put in each bath. Figure 14 is a simplified sketch of the apparatus. The culture grows in a half-inch glass tube, bent as shown and supported in the constant-temperature water bath. The cultures were aerated and stirred by bubbles of air containing 5 per cent CO_2. After 4 to 6 days' growth the yield (that is, the increase in cell density) of the cultures was determined. Several sets of cultures were grown under various conditions of temperature and illumination.

When cultures are grown in this manner the yields are influenced by uncontrolled variables, mainly light intensity, so that the results of one experiment may not be directly comparable with those of another. Therefore, In each set of cultures one control was grown in a bath maintained continuously at 25° C. The yield from the control culture is taken to be 100 per cent, and the other yields are expressed as percentages of the yield from the control in each experiment.

In all but the first experiment, duplicate cultures were grown under each of the conditions tested. The yield from duplicate cultures differed by less than 10 per cent, and the yield under the same conditions in different experiments also agreed within 10 per cent.

A Chlorella culture growing outdoors in a 5-gallon bottle provided cells for inoculation of the experimental cultures. The cells were centrifuged from the stock culture and were **resuspended** in fresh medium at the desired cell density.

In the first experiment, bath temperatures of 15, 25, and 35° C were maintained continuously. Three cultures, inoculated with 0.425, 1.45, and 7.50 g dry weight per liter, were started in each bath. The least dense culture allowed much of the sunlight to pass through initially, the intermediate density was approximately that required for total light absorption, and the densest culture was illuminated only part way through. After 6 days' growth the cell densities of the cultures were measured again. Partly cloudy weather probably reduced the yield of Chlorella in this experiment.

The yields are listed in table 7. From the fourth column it is clear that the percentage increase of cells falls off rapidly with increasing size of inoculum. The yield in grams of cells produced, however, is greatest

Table 7

Growth of Chlorella in sunlight at different cell densities and at different temperatures

Experiment 1 (6 days)

Temp. (°C)	Inoculum[a] (g/1)	Increase (g/1)	Percent increase	Yield[a] (g/m2/day)	Relative yield (%)
15	0.425	1.50	353	2.47	24
	1.45	1.85	112	3.29	31
	7.50	2.05	27	3.55	34
25	0.425	3.30	778	5.69	54
	1.45	6.23	378	10.5	100
	7.50	4.00	53	6.78	65
35	0.425	0.20	47	0.33	3.1
	1.45	1.25	76	2.11	20.0
	7.50	0.575	8	UH	9.8

[a]Dry weight.

with the intermediate density of inoculum, except at 15°. At 25° the culture of medium density had about half the growth rate of the thin culture, but produced nearly twice the quantity of Chlorella. For this reason it was decided to use an inoculum of 1.45 g/1 in the following experiments.

In the second experiment, temperatures of 20, 25, and 30° C were maintained continuously; also a 25° and a 30° day, each with a 20° night, were used. The change from day to night temperature was made by moving the culture tube from one bath to another at dusk. The reverse change was made at dawn. The cultures all received direct sunlight during 5 days' growth. The yields are listed in table 8.

A third set of cultures grew 4 days with sunlight at 25 and 35° continuous temperature, and also at these temperatures during the day with a 20° night. These yields are also listed in table-8. At both 30 and 35° day temperatures with a 20° night the yield of Chlorella was more than double that obtained at the higher temperature maintained continuously. Even with a cool night, the 35° day appears too warm for the production of a high yield. The 30° day and 20° night gave the highest yield obtained in this series of experiments.

Table 8

Growth of Chlorella in sunlight at different day and night temperatures

Temp. TC)	Yield[a] (g/m^2/day)	Relative yield (%)
Experiment 2 (5 days)		
20° cont	12.7	95
25° cont	13.4	100
30° cont	8.5	63
25° day ⎫ 20° nightj	15.6	116
30° day ⎰ 20° nightj	17.8	133
Experiment 3 (4 days)		
25° cont	15.3	100
35° cont	3.76	25
250 day ⎱ 20° nightj	17.0	111
350 daY X 20° nightj	10.7	70

[a] Initial density was 1.45 g/1 dry weight.

Using the data from tables 7 and 8 for all the cultures started with an inoculum of 1.45 g/1 dry weight, we can construct two curves showing yield of Chlorella in relation to temperature. The solid line in figure 15 shows the yield at various temperatures maintained continuously. The dashed line shows the yields when the daytime temperature is that on the graph and the night temperature is 20° in each case. The ordinate is the percentage yield for each condition, the respective control at 25° continuous temperature being taken as 100 per cent.

It is a matter of practical importance in the outdoor culture of Chlorella to know how the yield is affected by different degrees of illumination: sunlight, skylight, and shade or cloudiness. For skylight, a movable
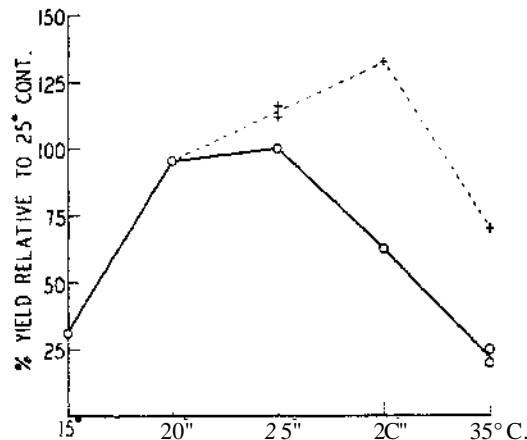
Fig, 15, Effect of temperature on yield of Chlorella grown outdoors in sunlight. Solid line, **continuously** maintained temperatures; dashed line, indicated temperature in daytime, 20° at night in **each** case.

Table 9

Growth of Chlorella outdoors at different light intensities and at different temperatures

Illumination	Temp. (°C)	Yield a (g/m²/day)	Relative yield (%)
	Experiment 4 (5 days)		
Sun	25° cont.	11.95	100
	25° day } 15° nightj	14.8	124
Skylight	25° cont.	10.6	89
	25° day } 15° nightj	10.6	89
	Experiment 5 (5 days)		
Sun	25° cont.	14.2	100
	25° day } 10° nightj	12.9	91
Skylight	25° cont.	10.25	72
	25° day } 10° nightj	10.45	74
	Experiment 6 (5 days)		
Shade	25° cont.	7.85	55
	25° day } 15° nightj	6.96	49
	30° cont.	5.32	37
	30° day } 15° nightj	7.28	51

[a]Initial density was 1.45 g/1 dry weight.

vertical white board was fastened to the edge of the bath to keep the sun off the culture tubes but to cut off very little skylight. For shade, a sheet of muslin was stretched over the entire bath.

The fourth experiment compared the effect of skylight and sunlight, using 25° continuous temperature and a 25° day and 15° night for both conditions of illumination. The yields of Chlorella are listed in table 9. The 15° night caused a 24 per cent increase in yield with sunlight, but no difference with skylight. This experiment is considered unsatisfactory because, on account of partly cloudy weather, the sunlight cultures received only about half the sun they would have received in an equal number of clear days.

The fifth experiment was a repetition of the fourth, with the exception that a 10° night was used instead of 15°. The yields are listed in table 9. The 10° night caused no increase in yield over that at 25° continuously. During this experiment the weather was clear, so that the sunlight cultures received all possible sun.

From Abbot's [181(a)] data on the relative intensity of sunlight and skylight, it is likely that at the latitude of Stanford and with the elevation of the sun in November, the skylight cultures received about two-thirds as much light energy per day as did those in the sun. It had been observed earlier

that the yield of Chlorella in electric light was proportional to *the* product of light intensity and time, provided that the cultures had sufficient cell density to absorb all the light. Perhaps the same principle applies with skylight and sunlight. The actual yield in skylight was 73 per cent of that in sunlight. The estimate of 67 per cent light intensity and the observed 73 per cent yield support the idea that at a high level of illumination also, the yield of Chlorella is proportional to light intensity.

In both experiments with skylight it was evident that the cultures were darker green than the ones in sunlight. Chlorophyll analyses of the cells grown in the fifth experiment showed, on the basis of dry weight, 1.6 per cent chlorophyll in the sunlight Chlorella and 3.5 per cent in the skylight sample.

In the sixth experiment all the cultures were grown under the shade of muslin. The temperatures were 25 and 30° continuously, also these temperatures in the day with a 15° *night.* Experiment 6 was made immediately after experiment 5, and during both the weather was cloudless. Therefore, the control of experiment 5 is used for experiment 6 also. The yields are shown in table 9. The yield with shade and 25° continuously was 55 per cent that of the control. With the same daytime conditions, but a 15° night, no increase in yield was noted. With shade, a 30° day, and a 15° night, however, the yield was 137 per cent of that obtained at 30° continuous temperature.

Summary. At 25° continuously and under three different conditions of illumination, the yields of Chlorella in grams dry weight per square meter per day and the relative percentage yields were: sunlight, 14.2 g or 100 per cent; skylight, 10.25 g or 72 per cent; shade, 7.85 g or 55 per cent.

With sunlight and 25° in the day, but with different night temperatures, the relative yields were: 25° night, 100 per cent; 20° night, 113 per cent; 15° night, 124 per cent; 10° night, 91 per cent.

Yields of 14 tò 18 g/m^/day dry weight (tables 8 and 9) were obtained in November when the cultures received only 8 to 9 hours of sunlight daily, but were growing at favorable temperatures. Yields of about 30 g/m2/day might reasonably be expected in June and July with 12 to 15 hours of sunlight. Therefore the best average yield of 8.2 g obtained from the rocking tray in the summer was much lower than it might have been. With adequate temperature control and optimum culture density the tray could have produced from three to four times the yield actually found.

High-Temperature Strain of Chlorella (H. W. Milner)

Strains of Chlorella that thrive at considerably higher temperatures and have higher growth rates than Chlorella pyrenoidosa have been isolated ai the University of Texas (see chapter 4? pages 45, 51). Time was not available to *test* one of these high-temperature strains under outdoor conditions of growth as described above. But, to obtain some idea of how a high-temperature strain might perform in comparison with ordinary Chloreiia under the high cell density conditions visualized for mass culture of algae, preliminary experiments were conducted with flask cultures.

Two liters of culture in a 3-iiier Fernbach flask gave a culture depth of 7,5 em. The cultures were illuminated continuously from below with

incandescent lamps and were liberally supplied with 5 per cent CO2 in air. Cultures of the high-temperature strain Tx-S-11-5 were maintained at 39±1° C, and C. pyrenoidosa cultures at 25±1° C. The culture medium was the same as that used in the outdoor cultures described above.

The logarithmic growth rate constant for the high-temperature strain exceeded the constant for ordinary Chlorella only in the range of cell density where the cultures were virtually transparent. As soon as the density of the cultures became great enough to cause absorption of an appreciable fraction of the 1300 or 4000 f.c. of incident light, both strains of Chlorella yielded the same number of grams of cells per unit area per day. That is, complete light saturation of all the cells appears necessary for the high-temperature strain to show a higher growth rate than ordinary Chlorella.

From these preliminary experiments it appears that the greater growth rate of the high-temperature Chlorella in very dilute cultures will be of no advantage in mass culture, where cell densities at least large enough for complete light absorption will be employed.

For mass-culture operations the high-temperature strain has the tremendous advantage, however, that it should at high cell densities produce the same yield at 39° C as can be obtained at 25° C with ordinary ChlorelJa. The requirement for cooling on a large scale is thereby greatly reduced.

Before the relative advantages of the two strains for mass culture can be stated more definitely, more extensive and quantitative tests are needed, particularly under the actual operating conditions to be employed.

Turbulence (E. A. Davis)

At present it appears that the most promising way to increase Chlorella yields in sunlight may be to supply the bright light to the individual cells in short flashes followed by dark periods of about ten times the length of the light flashes. In order to increase light utilization, the light, when not falling on a particular cell, must be used by other cells and not merely be cut off by a shutter. The most practical way to achieve this end seems to be by turbulence. To provide short flashes on an individual cell, less turbulence should be required with a dense culture, which absorbs nearly all the light in a thin layer, than with a low-density culture. The thickness of the culture at which 99 per cent of the light is absorbed should presumably be about one-tenth of the total thickness of the culture, as is suggested in chapter 6. Under such conditions increase of growth, probably due to the intermittence effect caused by turbulence, has been measured.

For these experiments the device illustrated in figure 16 was constructed. A round-bottomed annular chamber with water-jacketed lucite outside walls had suspended in it a stainless steel rotor. The space between the rotor and the chamber wall was everywhere 1/4 inch. All surfaces in the growth chamber were polished. Carbon dioxide in air entered at the bottom center through a Incite needle valve. The rotor was driven by a belt from a motor and adjustable speed reducer. A bank of six 300-watt reflector spot lamps lighted the outside of the annulus to about 7000 f.c. By regulating the flow of water in the lucite jacket and steel rotor it was possible to maintain the culture at a temperature close to 25° C.
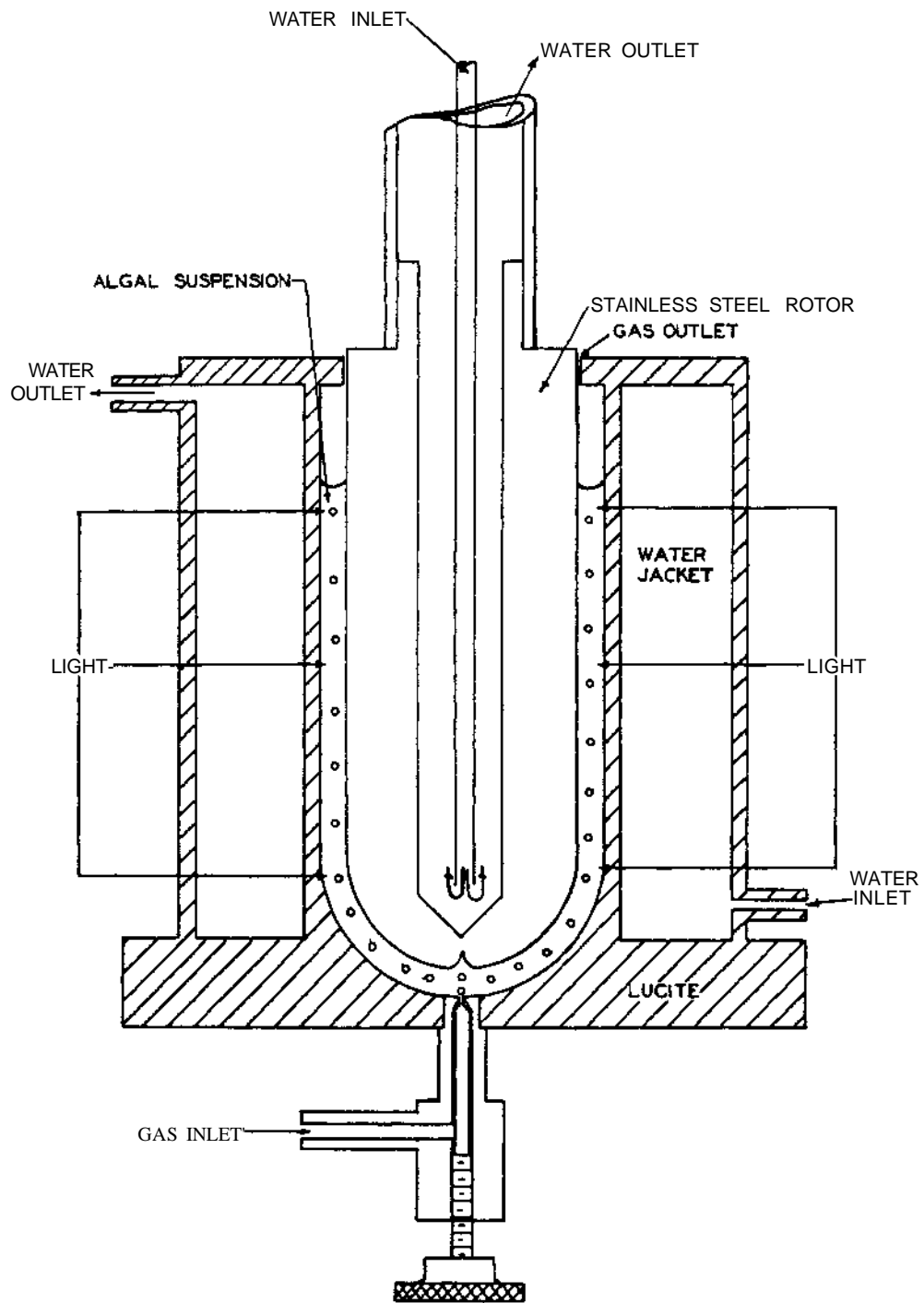
Fig. 16. Culture chamber for growth of CMoreila with variable turbulence. Culture is intro-
duced and withdrawn for sampling at bottom through lucite needle valve, which also controls
bubble sĩze. Turbulence of various decrees is achieved by spinning rotor at different speeds.
Rotor is supported by bearings not shown in figure.

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In order to fill the apparatus, a culture of Chlorella was added to a round-bottomed glass container, fitted with a rubber stopper through which passed a gas inlet tube, a thermometer, and a tube which extended to the bottom of the container. The outside end of the latter tube was connected to the gas inlet of the needle valve attached to the bottom of the growth chamber. The culture was forced up into the growth chamber by air containing 5 per cent CO2, which then bubbled through the culture. The rate of bubbling could be controlled with the needle valve. Under operating conditions the apparatus contained 100 ml of culture.

The medium used in these experiments had the following formula: KNO3, 10.0 g; MgSO4 • 7H$_2$O, 2.46 g; KH2PO4, 1.22 g; A4 microelement solution, 1 ml; ferric potassium EDTA stock solution, 4 ml; distilled water, 995 ml. A liter of medium contained 20 ppm of iron and enough potassium nitrate for the growth of about 55 g fresh weight of cells. All weights given hereafter will be fresh weights unless otherwise stated.

It was proposed to start a culture at a low density with continuous illumination, letting it increase until it reached about 150 g/1, a density considered high enough to absorb all the light in the first millimeter of culture. An initial culture of very low density (0.1 g/1) bleached because of high light intensity. Another culture of greater density (0.6 g/1) and exposed to weaker light grew rapidly. As the culture grew, the light intensity was increased until the culture was illuminated by all six lamps. During the first 5 days the cells were changed to fresh medium once every 24 hours. Thereafter they were changed every 12 hours. This provided ample nutrients for growth.

A culture density of 150 g/1 was reached in 11 days. During this time the rotor speed was about 200 rpm. After the density reached 150 g/1 the effect of turbulence on growth was studied.

Every 12 hours the culture was drained out of the annular growth chamber. The density of the culture was determined by measuring the packed volume of cells in a given volume of culture. This was readily accomplished with centrifuge tubes graduated in cubic millimeters. Packed cell volumes were converted to grams fresh weight. To restore the culture density to 150 g/1, a calculated volume of culture was removed; the cells in the remainder of the culture were collected by centrifugation and resuspended in 100 ml of fresh medium, after which the culture density was again measured. It was therefore possible to measure growth starting with a culture density close to 150 g/1. The pH of the medium usually increased from 5.4 to 7.7.

Various degrees of turbulence were obtained by altering the speed of the rotor. Speeds tested were 0, 16, 208, and 475 rpm- The culture was subjected to considerable turbulence at the highest speeds. At 0 rpm the culture was stirred only by gas bubbles. This was used as the control condition. Since the gas bubbles imparted considerable motion to the culture, however, this condition did not lack turbulence.

In order to make sure that growth was not limited by the availability of carbon dioxide, the concentration of CO_2 was increased to 10 per cent

for a complete growth period. In no case was growth greater with 10 per cent than with 5 per cent CO2.

Ten or more measurements were made at each rotor speed. Table 10 gives the averages. With a rotor speed of 0 rpm, 25.2 g dry weight of

Table 10

Influence of turbulence on the growth of Chlorella

Rotor speed	Growth expressed in various units			Per cent increase
	Fresh wt., g/1/12 hr	Dry wt.,[a] g/1/12 hr	Dry wt., g/m2/12 hr	
0	15.1	3.8	25.2	....
16	20.5	5.1	34.1	35.3
208	25.6	6.4	42.8	69.8
475	25.9	6.5	43.2	71.4

[a] Ratio of dry weight to fresh weight arbitrarily taken as 0.25.

Chlorella were grown per square meter of illuminated surface in 12 hours. This was increased 35.3 per cent at 16 rpm, 69.8 per cent at 208 rpm, and 71.4 per cent at 475 rpm. The yield increased up to 208 rpm, above which no significant increase was found.

At 0 rpm the dry-weight yield of 3.8 g/1/12 hr or 25.2 $g/m^2/12$ hr is at least twice that found with the outdoor culture units described in this chapter. This suggests that at the high culture density used, the stirring caused by the flow of gas bubbles was great enough to subject many of the cells to flashing light of the correct pattern for the intermittent light effect, thus increasing yield. If the yield under conditions not favorable for the intermittent light effect is considered to be approximately 11 $g/w?/12$ hr dry weight, approximately that found with the outdoor culture units, then at the rotor speed of 208 rpm the yield of 42.8 $g/m^2/12$ hr represents an increase of 289 per cent. For purposes of comparison it will be necessary to determine yields under conditions of less turbulence than those yet worked with.

The investigation therefore indicates that yield can be increased at least 70 per cent and perhaps 300 per cent by culture turbulence. It may be that a still greater increase will be found with greater turbulence or at other culture densities.

Diurnal Fluctuations in Cell Division and Enlargement [3]
(E. A. Davis and Jean Dedrick)

In the course of experiments with the rocking tray it was observed over a period of several days that cell division occurred at night and increase in volume of cells during the day. The phenomenon was very striking for a while, but was not lasting.

[3] An independent investigation of this subject is reported in chapter 7.--Ed.
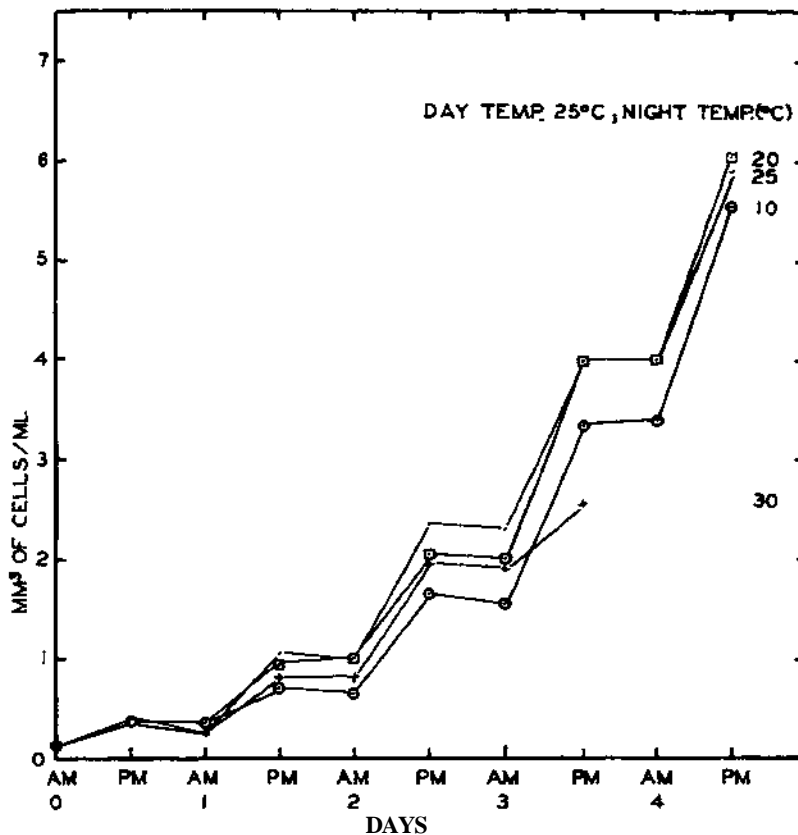
Fig. 17. Diurnal fluctuations in cell volume of Chlorella pyrenoidosa cultures grown at 25° C during the day and at 10, 20, 25, and 30° C during the night. Day, 9:00 a.m. to 9:00 p.m.; night, 9:00 p.m. to 9:00 a.m.

Laboratory experiments were undertaken to find whether such diurnal fluctuations as were noted under uncontrolled conditions could be reproduced and the factors responsible determined.

Cultures were grown in 200-ml Erlenmeyer flasks. During the day (9:00 a.m. to 9:00 p.m.) the culture flasks were suspended in circulating water contained in glass bowls and illuminated from below with 200-watt lamps. Each culture unit contained four flasks illuminated by one lamp. The light intensity at the level of the bottoms of the culture flasks was approximately 1000 f.c. The temperature of the water baths could be controlled by adjusting the flow of water. A humidified gas mixture of 5 per cent CO_2 in air was bubbled through the cultures. At night (9:00 p.m. to 9:00 a.m.) the cultures were transferred to constant-temperature chambers and aerated with air. It had previously been found that although night aeration had little influence on yield, it increased cell division. Night aeration is
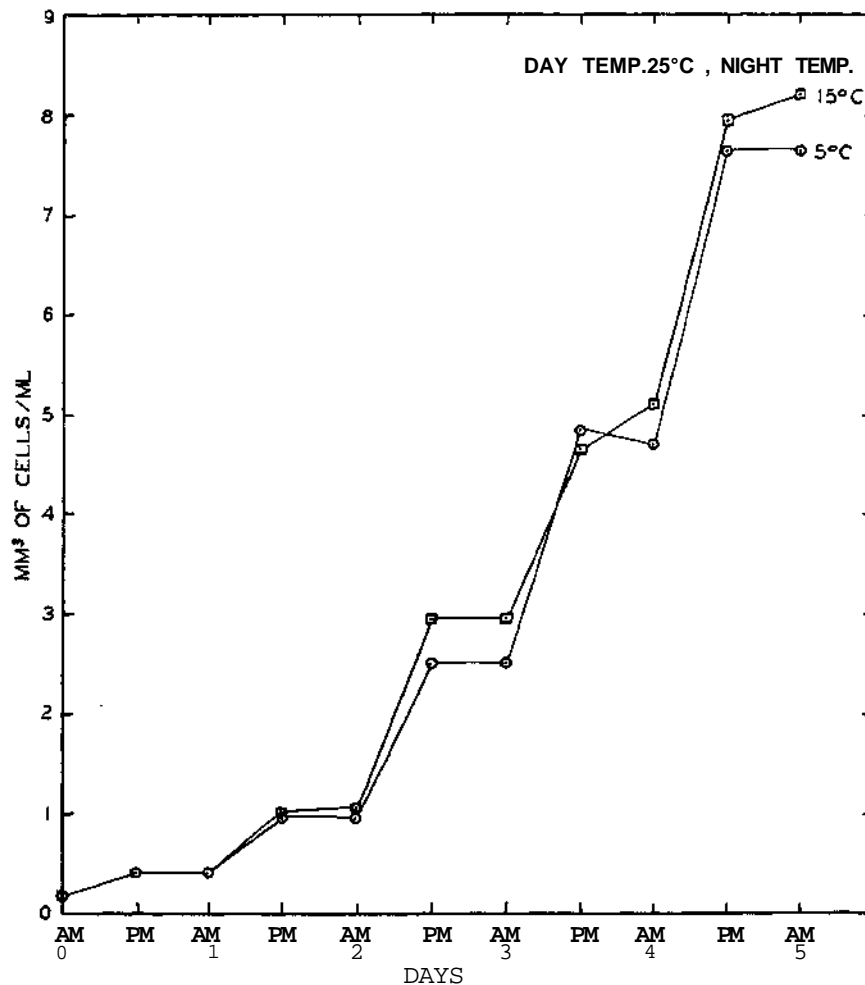
Fig. 18. Diurnal fluctuations in cell volume of Chlorella cultures grown at 25° C during the day and at 5 and 15° C during the night. Day, 9:00 a.m. to 9:00 p.m.; night, 9:00 p.m. to 9:00 ʼa.m.

therefore important in studies of diurnal fluctuations in cell division. Packed cell volumes and cell counts were determined after each light and dark period.

The night temperature was considered to be the factor responsible for diurnal fluctuations in cell division. During the day the cultures were grown at 25° C. The night temperatures were 5, 10, 15, 20, 15, and 30° C.

Very marked diurnal fluctuations in cell division and enlargement were found. Cell enlargement occurred during the day irrespective of night temperature, as shown in figures 17 and 18; it was during this time
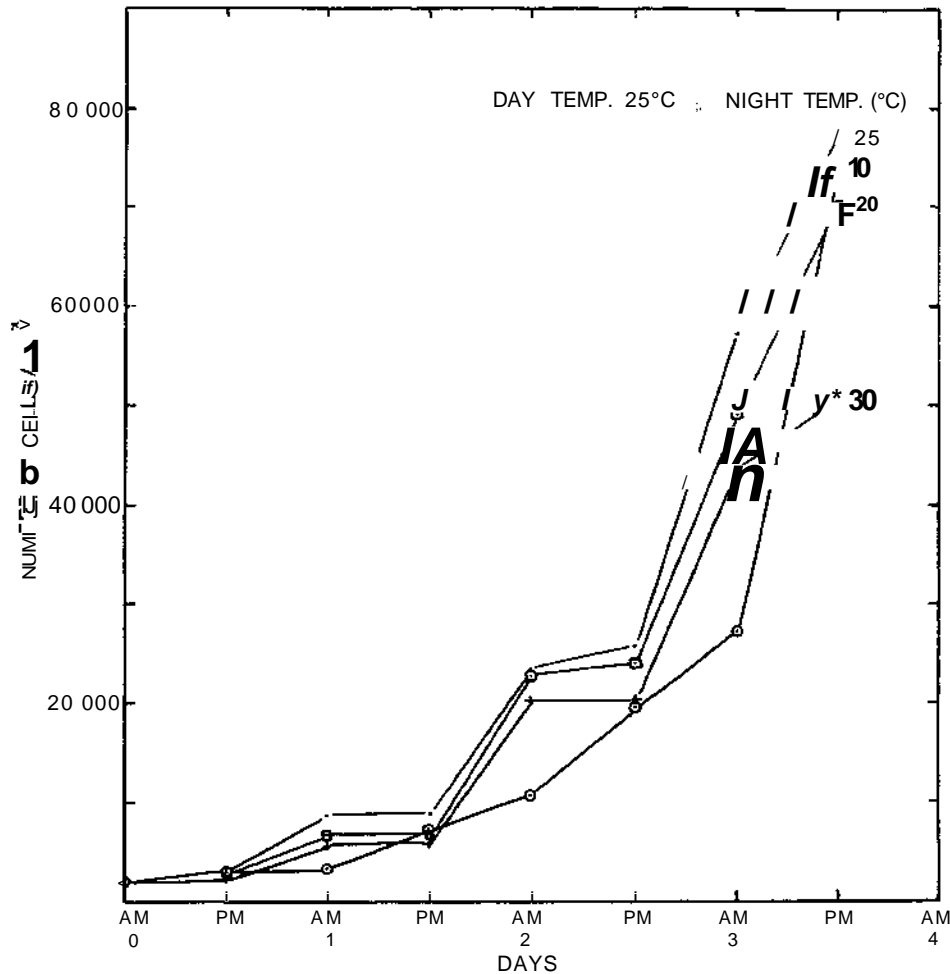
Fig. 19. Diurnal fluctuations in number of cells in Chlorella cultures grown at 26^0 C during the day and at 10, 20, 25, and 30° C during the nipht. Day, 9:00 a.m. to 9:0C p.m.; night, 9:1/0 p.m. to 9:00 a.m.

that the cells were actively photosynthesizing. Little or no further increase in size occurred at night.

Although night temperature did not influence the time of cell enlargement, it had profound influence on the time of cell division, as shown in figures 19 and 20. With night temperatures of 20, 25, and 30° C, cell division occurred chiefly at night. With a night temperature of 5° C, it occurred during the day. With night temperatures of 10 and **15°** C, it occurred during both the day and the night.

Cultures which had been given 20, 25, and **30°** C nights- contained in the morning small cells produced by cell division. On illumination, these
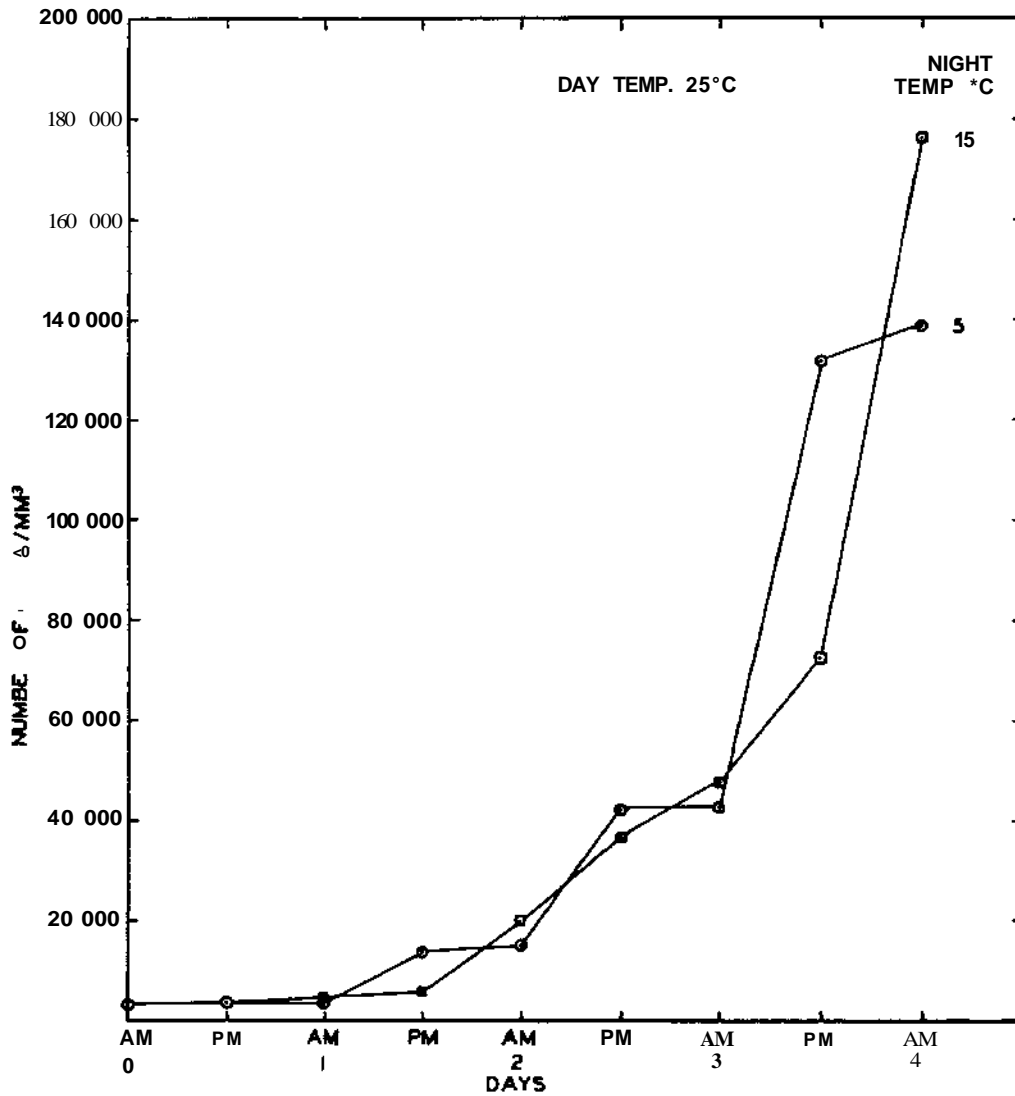
Fig. 20. Diurnal fluctuations in number of cells in Chloreila cultures grown at 25° C during
the day and at 5 and 15° C during the night. Day, 9:00 a.m. to 9:00 p.m.; night, 9:00 p.m. to
9:00 a.m.

small cells, now actively photosynthesizing, increased in size. By the end
of the day they were large enough to divide. When the cultures were kept
under favorable day and night conditions this process was repeatable.
During the first 4 days cell size increased, on an average, 2.2 times dur-
ing the day, whereas the number of cells increased 2.4 times during the
night, as shown in table 11.

Table 11

Diurnal fluctuations in cell size and volume in cultures grown at 25° C during the day
and at 20, 25, and 30° C during the night

Day	Night temp. (°C)	9:00 a.m.			9:00 p.m.			Av. cell size (no. of times increase during the day)	Cell number (no. of times increase during the night)
		Cell count (no. cells/mm^3)	Cell volume (mm^3 cells/ml)	Av. cell size (ml)	Cell count (no. cells/mm^3)	Cell volume (mm^3 cells/ml)	Av. cell size (ml)		
0......	20	2,040	0.1	4.9X10-11	2,850	0.35	1.2x10-1°	2.4	2.4
	25	2,040	0.1	4.9X10-11	3,200	0.4	1.2x10-1°	2.4	2.7
	30	2,040	0.1	4.9X10-H	2,350	0.35	1.5X10-1°	3.1	2.4
1......	20	6,850	0.35	5.1X10-H	7,000	0.95	1.4X10-1°	2.7	3.2
	25	8,650	0.25	2.9X10-U	8,950	1.05	1.2x10-1°	4.1	2.6
	30	5,600	0.25	4.5X10-11	5,700	C.8	1.4x10-1°	3.1	3.5
2......	20	22,700	1.0	4.4X10-H	24,000	2.05	8.5x10-11	1.9	2.0
	25	23,300	1.0	4.3X10-11	25,600	2.35	9.2x10-11	2.1	2.2
	30	20,100	0.8	4.0X10-H	20,200	1.95	9.6X10-11	2.4	....
3......	20	49,000	2.0	4.1X10-H	70,200	4.0	5.7X10-11	1.4	1.7
	25	57,200	2.3	4.0X10-H	77,200	4.0	5.2X10-U	1.3	1.7
4......	20	119,000	4.0	3.4X10-U	170,000	6.0	3.5x10-11	1.0	....
	25	134,200	4.0	3.0X10-H	180,000	5.9	3.3X10-H	1.1	....

The time of cell division could be restricted to the night hours, by controlling the night temperature, only so long as the cells received ample light for photosynthesis during the day. When the culture density increased to the point where not all of the cells in the culture were receiving enough light, then division began to occur during the day as well as at night. Under such conditions many of the cells were in effect living under night conditions during the day.

In summary, then, it is possible by varying the night temperature to obtain cell division only at night, only during the day, or during both day and night. Cell enlargement occurred chiefly during the day regardless of night temperature.

D

Harvesting by Settling

Cultivation of Chlorella in a Vertical Sedimentation Tube (J. H. C. Smith)

The industrial cultivation of unicellular algae requires a leasible and economical procedure for harvesting. One possible method is gravitational sedimentation. The practicality of this method has been tested both by long-term experiments on growing Chlorella under natural conditions and by quantitative determinations of rates of settling.

The apparatus used for the long-term culture experiments (fig. 21) consisted of a glass container—a "percolator" tube—67.5 cm long and
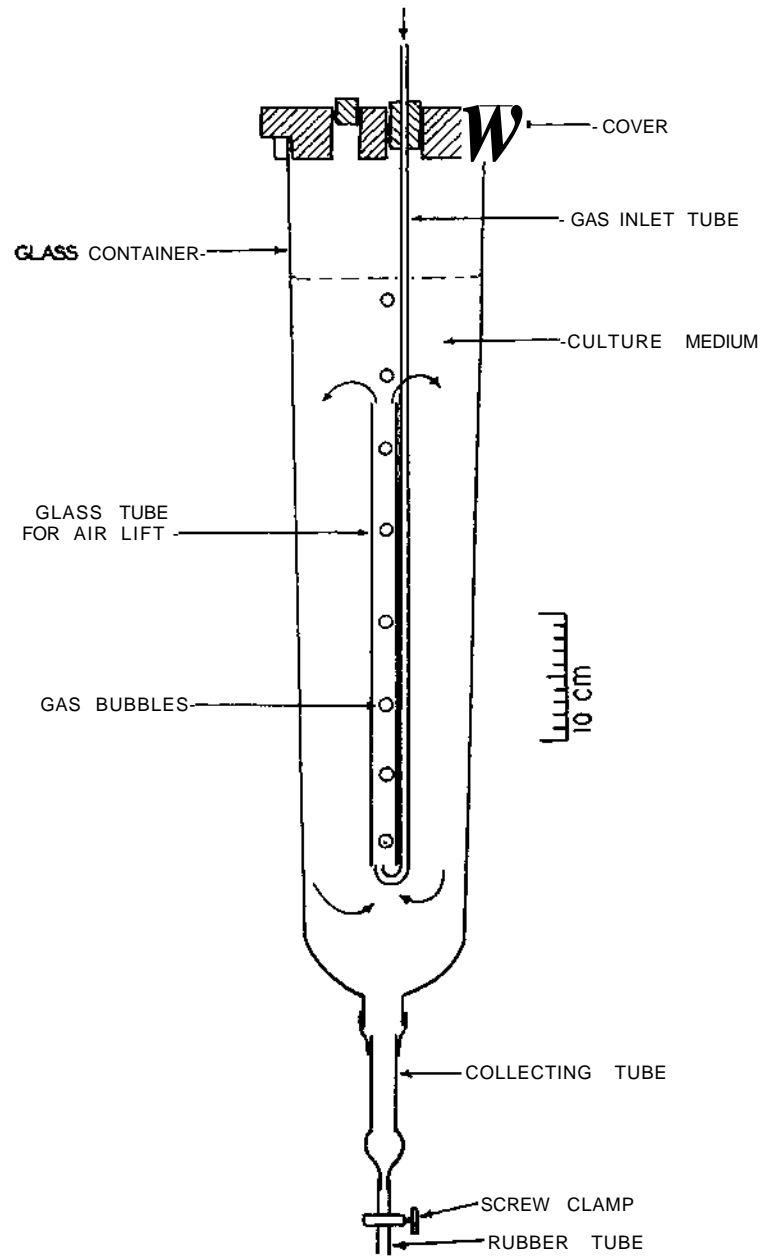
Fig. 21. Apparatus for cultivation of algae, arranged for continuous harvesting by gravitational sedimentation.

tapered from 15.5 cm diameter at the top to 12.5 *Cm.* at the bottorii. The outlet of the container was connected to a collecting tube 1.5 cm in diameter and 10 cm long. The end of this tube was closed with rubber tubing and a screw clamp. A gas-inlet tube of glass, inserted through the cover, passed down the center of the container to within about 10 cm of the bottom. This tube was bent upward and opened into a wider glass tube (2 cm diameter) which extended approximately from 10 cm above the bottom to 10 cm below the top of the culture medium within the container. In operation, air containing 5 per cent $CO2$ bubbled up through the wider tube and circulated the culture medium in the direction indicated by arrows in figure 21.

The culture medium was prepared from boiled tap water, 7.5 liters, and contained: $MgSO_4 \cdot 7 \wedge 0$, 37 g; KH_2PO_4, 18.5 g; $KNO3$, [19] & $FeSO_4 \cdot 7H2O$, 0.01 g. Carbon dioxide was supplied by bubbling air containing 5 per cent $CO2$ through the culture medium. The medium, after being placed in the culture apparatus, was inoculated with Chlorella cells.

The apparatus was placed outdoors and subjected to natural conditions. No controls of light or temperature were ever imposed, except on one hot day when the culture was shaded for a few hours.

During the growth of the culture some of the cells settled into the collecting tube at the bottom of the apparatus, and every morning the thick sludge thus collected was drawn off through the rubber tubing attached to the collecting tube. About 30 ml of cells and medium were withdrawn at each harvest. The harvested sludge was separated by centrifugation into two fractions, cells and liquid. The cells were weighed before and after being dried over calcium chloride at reduced pressure. The supernatant liquid was returned to the container with enough fresh medium to restore the culture solution to its initial volume. Nitric acid was added almost daily to the medium to maintain its pH relatively constant and to replenish its depleted nitrogen content. Occasionally traces of iron, manganese, copper salts, and other micronutrients (Arnon's A4 solution) were added to prevent development of mineral deficiencies.

The results of one experiment on Chlorella pyrenoidosa are shown graphically in figure 22. A summary of the results for three runs, two for C. pyrenoidosa and one for Chlorella Cornell no. 11, is given in table 12. (The original culture of the Chlorella Cornell no. 11 strain was obtained from Dr. David Appleman, of the University of California at Los Angeles.)

These experiments demonstrated that Chlorella could be cultivated for a long time (78 days) in the same culture medium without producing an inhibitor which seriously hindered its growth, and without irremediably depleting the supply of any necessary nutrient. They also demonstrated that the cells could be collected continuously in a highly concentrated suspension from which they could be conveniently harvested. In one run, the cell content of the harvested suspension was increased on the average 68-fold over that of the bulk of the culture solution. In individual harvests, this concentration factor was greatly exceeded. Chlorella Cornell no. 11 showed a greater concentration factor than did C. pyrcnoidpsa.
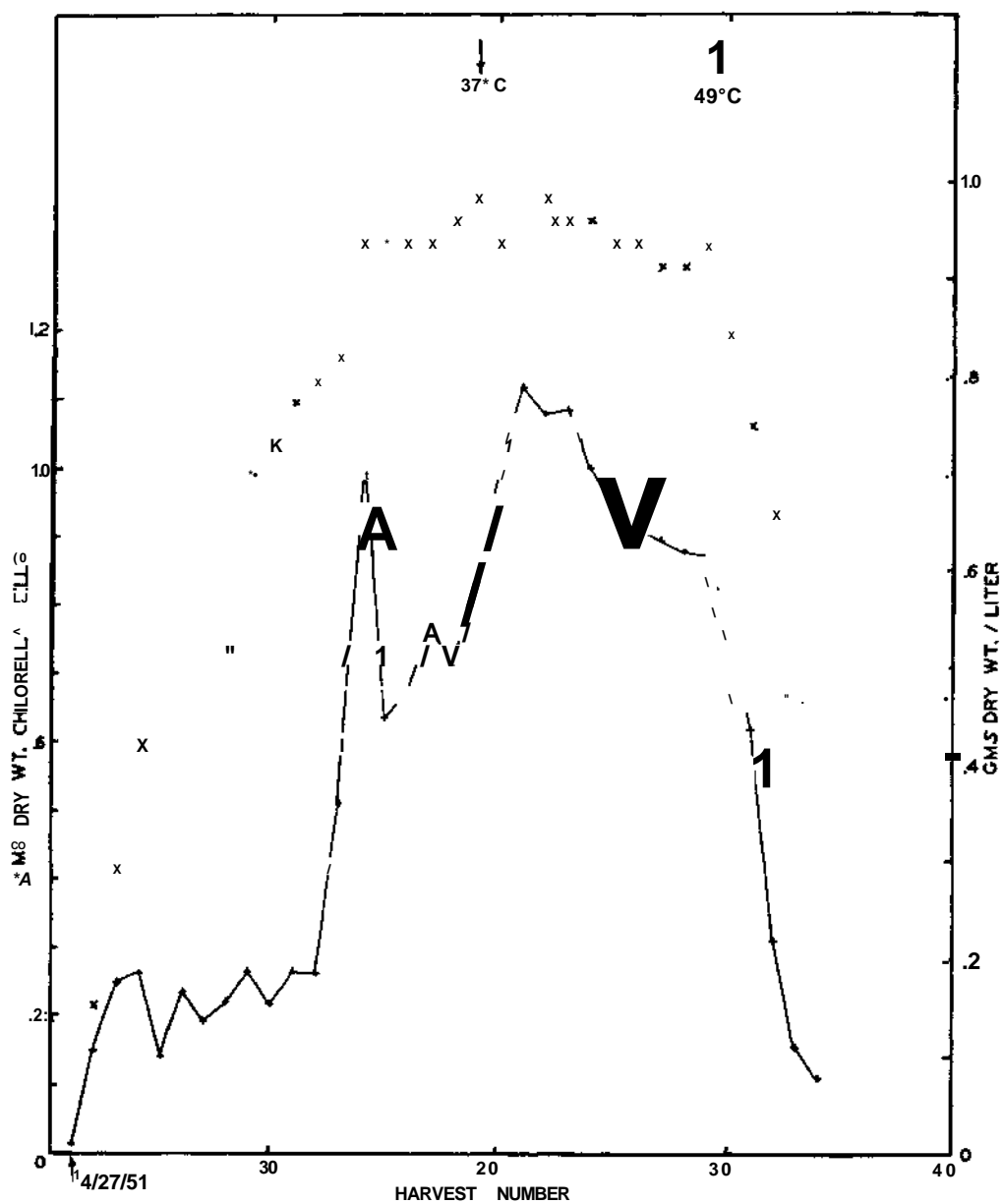
Fig. 22. Weight of CMorella pyrenoidosa cells harvested daily by gravitational sedimentation (solid line; left-hand ordinate) compared with content of cells in the culture solution (crosses; right-hand ordinate).

Table 12

Summary of experiments on cultivation of Chlorella in vertical tube to determine
feasibility of harvesting by gravitational sedimentation

	Chlorella pyrenoidosa	Chlorella pyrenoidosa	Chlorella Cornell no. 11
Dates of duration of experiment	Feb. 8 to Apr. 26, 1951	Apr. 26 to May 31, 1951	June 6 to Aug. 10, 1951 [a]
Total weight harvested (g):			
Wet weight	173.70	81.93	257.3
Dry weight	39.34	19.21	60.23
Average per cent dry weight of wet weight	22.65	23.44	23.41
Total number of harvests	78	33	56
Average weight per harvest (g):			
Wet weight	2.23	2.48	4.59
Dry weight	0.504	0.582	1.076
Average volume harvested (ml)	30.0	30.9	31.4
Average concentration of cells (g/1, dry wt,):			
In culture solution	C.736 [b]	0.759	C.501
In volume harvested	16.08	18.81	34.18
Concentration factor	21.9	24.8 [c]	68.2
Approximate cross-sectional area illuminated (m^2)	0.0687	0.0687	0.0687
Yield (g/m^2/day, dry wt.)	7.33	8.47 [c]	15.65
pH range	5.12-7.93	6.01-7.00	5.57-7.08
Temperature range observed (°C)	2-36	9-45	13-42

[a] The cells in this experiment were stored in the dark at about 4° C for 9 days, from June 16 to 25, 1951.

[b] In this experiment the cell density was not measured regularly. Only four determinations were made, and these were compared with the cell densities of the corresponding harvests to obtain the concentration factor.

[c] if only the best 16 consecutive harvests are taken for this run (cf. fig. 22), a concentration factor of 29.1 and a daily yield of 13.0 g/m^2 are obtained.

Temperatures as low as 2° C had no permanently deleterious effect on a culture, but a temperature of 45° C for a few hours caused an irretrievable loss of a C. pyrenoidosa culture (see fig. 22). 4 A temperature of 42° C did not kill a culture of Chlorella Cornell no. 11. It appears that the upper temperature limit tolerated by C. pyrenoidosa is something less than 45° C and by Chlorella Cornell no. 11 is something more than 42° C.

Addition of micronutrient elements to the cultures caused noticeable beneficial effects in some instances. When added to C. pyrenoidosa culture, Arnon's A4 micronutrient solution caused rapid increase in growth and production (see fig. 22, 12th day); when added to an unhealthy culture of Chlorella Cornell no. 11 it caused rapid greening and increased growth.

The storage of Chlorella Cornell no. 11 for 9 days at 4° C in the dark produced no harmful effects. The culture grew immediately upon being returned to natural outdoor conditions.

No precautions were taken to keep foreign organisms from the culture, and no serious trouble with infection was experienced.

[4] The culture of Chlorella pyrenoidosa in the pilot plant was heated to this .same temperature for periods of 5 minutes without serious harm (see chapter 17).--Ed.

On occasion, when the pH became too high, some difficulty with pre-cipitation of inorganic salts occurred. In a few harvests, the Chlorella cells were slightly contaminated with this precipitate.

From our experience with these cultures we can conclude that har-vesting by gravitational sedimentation is feasible.

Measurements of Sedimentation Rates of Chlorella (J. H. C. Smith)

The effects of various factors on the rate of settling of Chlorella pyre-noidosa have been studied quantitatively in order to estimate the practica-bility of settling as a means of harvesting.

The moving boundary method was used for measuring the rate of set-tling [293]. The cell suspension was introduced into glass-stoppered 10-ml measuring cylinders graduated in 0.2-ml units. The cylinders were placed against a quadrilled background. The boundary of the mass of Chlorella cells was sharp, and its position was readily determined by ob-serving the point on the cylinder scale at which the lines of the quadrille were obliterated. The downward movement of this boundary was timed and the rate of settling determined.

The settling is directly proportional to the time, and is also a function of the cell content of the medium, as is seen in figure 23. As reference to
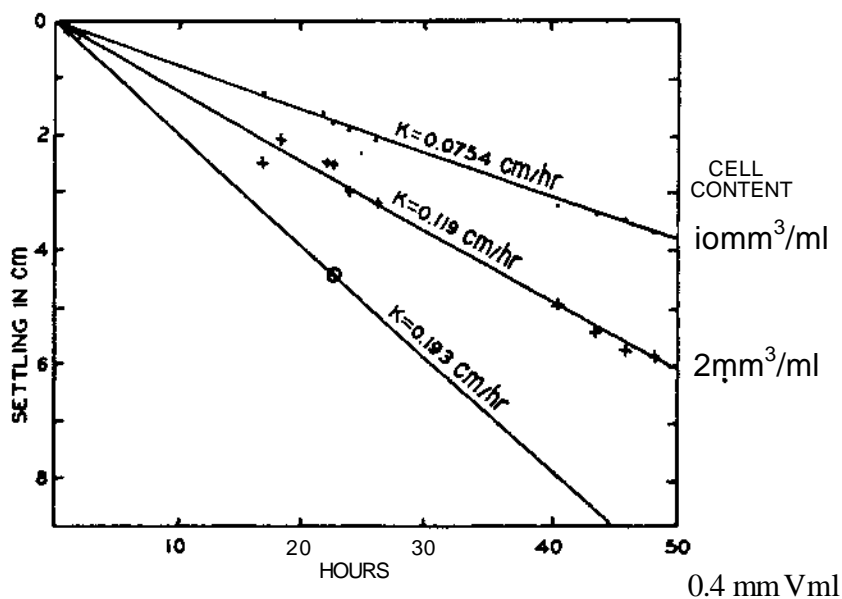
Fig. 23. Settling of Chlorella pyrenoidosa cells as a function of time: influence of the cell content of the culture medium. The cell concentration was determined by cenirifuging a known ¥olume of cell suspension in a graduated capillary centrifuge tube and is expressed in mm^/mi. For all practical purposes a cubic millimeter is equivalent to a milligram of fresh weight of cells* and these two terms have been used interchangeably.

table 13 shows, the rate of settling, k (cm/hr), was found to be related to the cell content, C (mg/ml), by the equation

$$K \; '' \; C^n \; '$$

where K and n are constants. From these data an approximate idea of the yield per day obtainable by settling can be estimated: the amount that will sediment in milligrams per square centimeter per hour equals kC.

Table 13

Applicability of the equation relating rate of settling with cell content, $kC^n = K$, when n = 0.292

C (mm3/ml)	$C^{0.292}$ (C)	$\frac{1}{k}$ k	$k/_n G.292$ $k(C)$	kC (mg/cm^/hr)	Daily yield[b] {g/nfi}
10.0 . . . . . . .1.96	0.0754	0.148		0.754	181
2.0 . . . . . . .1.225	0.119	0.146		0.238	57
0.4 . . . . . . .0.765	0.193	0.138		0.077	18.5

[a]The slopes of the straight lines in fig. 23.
*>Wetweight.

The daily yield in grams per square meter calculated from kC is given in the last column of table 13. (In these laboratory experiments on settling, all weight values are given in terms of wet weight.)

The rate of settling differs with different fractions of cells from the same lot. A batch of cells was separated into a "light" and a "heavy" fraction by differential centrifugation. After the two fractions had been recovered, they were resuspended in portions of the same culture solution at the same cell content. The rate of settling differed for the two lots, as the slopes of the lines in figure 24 show. Whether this differential sedimentation is dependent on cell size, or cell specific gravity, or both, remains to be determined.

In figure 25 a curve relating sedimentation rate to cell content is given.

A comparison of these results with those obtained with the large tube used for experiments on continuous harvesting by sedimentation, which has been described in section B of this chapter, is given here. The diameter of the large tube was 15 cm; its cross-sectional area was 177 cm^. Under certain conditions (cf. footnote c, table 12) the average concentration of cells in the culture medium was 4.04 *uim^/xnl* and the average daily yield harvested was 3.63 g wet weight. At this cell concentration, the settling rate is 0.4 mg/cm^2/hr (cf. fig. 25). In the tube, therefore, the amount settled should have been 1.7 g/day (i.e. [0.0004x177x24] g/day). This is roughly half the amount actually observed.

This discrepancy between the calculated and the observed values can perhaps be attributed to at least three causes: first, in the large-tube experiment the settling rate observed was that of the heavier cells, whereas in the free-settling experiments it was that of the lighter cells; second, in the continuous-harvesting experiment the circulation of the cells gave
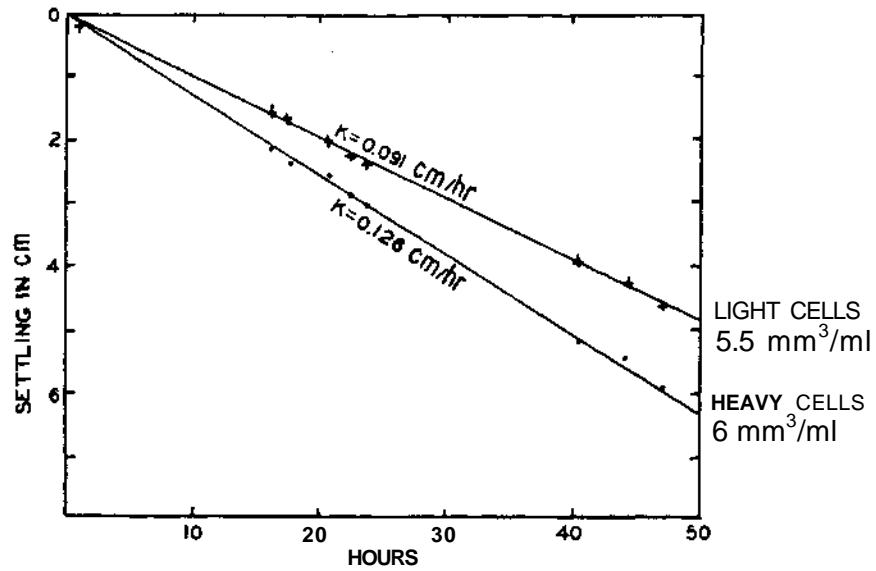
Fig. 24. Settling of Chlorella pyrenoidosa cells as a function of time: influence of specific properties of the cells.
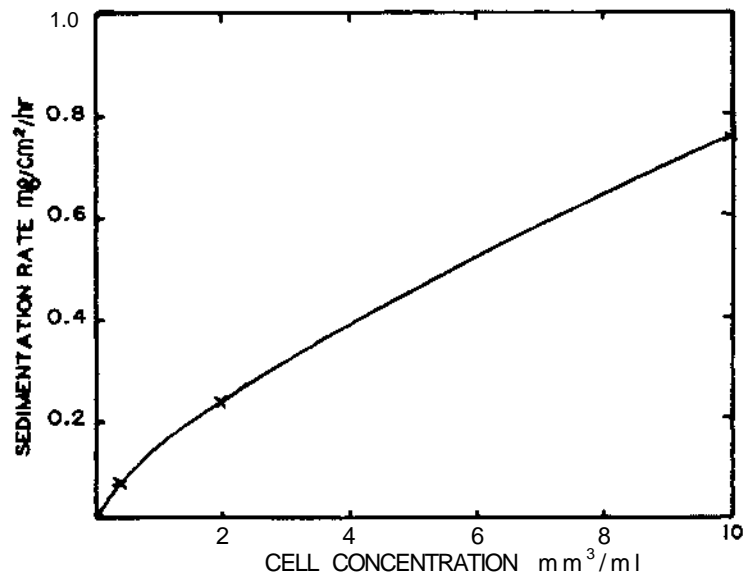
Fig. 25. Rate of sedimentation of Chlorella "pyrenoktoga cells as a function of cell concentration in the culture medium.

them a downward component of force which aided in their settling; and, third, the rate in the large tube may have been enhanced by maintaining a constant supply of cells in the main body of the culture solution.

We estimate that to obtain settling of a kilogram fresh weight per day, an area of 5.5 m2 would be-needed, given a cell content of 10 g/1 wet weight. This estimate is based on the free-settling experiments carried out in the laboratory. If the yield from the large-tube experiment is used as a basis of the estimate, approximately half this area would be required.

The Increase in Cell Density in the Settling Chamber of the Glass Tubing Culture Apparatus (E. A. Davis)

The plastic and glass tubing apparatus were designed to permit the harvesting of concentrated suspensions obtained by settling. To test the practicality of this method of harvesting a continuously growing culture, the following experiment was performed.

During the day the culture in the glass tubing culture apparatus was circulated and aerated with 5 per cent CO_2 in air. At night pumping was discontinued. The cells therefore settled in the tubing and in the separatory funnel used as a settling chamber. In the morning the slurry in the bottom of the settling chamber was drained off and the volume of culture was restored with fresh medium.

As the data in table 14 indicate, this method of harvesting a concentrated cell suspension obtained by settling was found to be highly success-

Table 14

Increase in cell density in settling chamber of glass tubing apparatus

Harvest	Vol. of culture harvested (ml)	Fresh wt. of harvest (g)	Cone, of cells in culture (g/1 fresh wt.)	Cone, of cells in harvest (g/1 fresh wt.)	Concentration factor
1	50	7.95	42.4	159.0	3.8
2	50	8.32	37.9	166.4	4.4
3	30	5.95	35.0	198.3	5.7
4	20	3.06	32.6	153.0	4.7
5	20	3.08	32.6	154.0	4.7
6	20	3.21	31.8	160.5	5.0
7	20	2.57	30.6	128.5	4.2
8	20	2.76	28.6	138.0	4.8
9	20	3.06	24.9	153.0	6.1
10	20	2.14	28.2	107.0	3.8
11	25	3.08	31.2	123.2	3.9
12	25	4.33	28.7	173.2	6.0
13	20	2.65	21.6	132.5	6.1
14	20	2.76	22.4	138.0	6.2
Average	26	3.92	**30.6**	148.9	5.0

fal. With an average of 30.6 g fresh weight of cells in the culture, the density of the harvests averaged 148.9 g/L This represents a fivefold concentration. Such a procedure would appear to have practical application in the mass culture of algae. In helping to reduce centrifugation costs.

E

Conclusions: Optimum Conditions for Growth of Chlorella

E. A. Davis and H. W. Milner

Sunlight has at least ten times the intensity that can be utilized by Chlorella for maximum growth. To obtain the highest possible yield of Chlorella, it is Important that as much as possible of the energy of sunlight be used for photosynthesis. This may be partially accomplished by means of culture turbulence, making use of the Intermittent-light effect, so that when one cell is shaded another cell receives the light. Increased yields presumably caused by turbulence have been demonstrated in laboratory experiments.

The daily yield of a Chlorella culture remains constant over a wide range of cell densities. It is of advantage in mass-culture work in a given system to maintain the highest cell density possible without decreasing the yield, since the quantity of culture to be handled during harvesting is thus lessened. It is more economical to use a shallow culture with high cell density than a deep culture with low cell density.

The yield of Chlorella is influenced by both day and night temperature. When different temperatures are maintained continuously, 25° C Is most favorable for *-apid growth. The yields at 25° continuously are exceeded by using a 25°[f] day and 15° night, with both laboratory and outdoor cultures. An even higher yield Is observed in outdoor cultures with a 30° day and 20° night.

The requirement for cooling outdoor cultures may be lessened by using a high-temperature strain of Chlorella that grows best at 39°, giving the same yield as ordinary Chlorella at 25°

Growth rates of Chlorella were not significantly different when the medium contained 0.56 to 4.43 per cent by volume CO_2- With low concentrations of carbon dioxide the gas must be supplied fast enough so that the culture medium is in equilibrium with the carbon dioxide concentration of the gas stream. With dense cultures and Inadequate means for keeping the medium in equilibrium with a low carbon dioxide concentration in the gas, higher percentages should be used. A mixture of 5 per cent CO_2 in air Is usually adequate. The yield of Chlorella Is not greatly affected if the supply of carbon dioxide Is cut off at night.

The maintenance of an adequate supply of fixed nitrogen in the medium Is mandatory for rapid growth of Chlorella In dense cultures. Urea as a nitrogen source has been found superior Iji several respects to the commonly used potassium nitrate. For an equivalent nitrogen concentration

urea gives greater yields; it can be used in higher initial concentration without harmful effect; and it causes smaller pH fluctuations in the medium during cell growth than is the case with potassium nitrate.

Another factor of great importance is the maintenance of a favorable concentration of available iron in the medium at all times. The chelating agent ethylenediamine tetraacetic acid (EDTA) has been used satisfactorily for this purpose. A medium having the advantages obtainable by the use of urea and also of chelated iron was found to be highly satisfactory for growing Chlorella. This medium contains:- urea, 1.05 g; $MgSO_4 \cdot VH^\wedge G$, 2.46 g; KH_2PO_4, !-[22] SJ ferric potassium EDTA stock solution, 1 mi; Arnon's A4 microelement solution, 1 ml; distilled water, 998 ml. The pH is adjusted to 6.0 with NaOH or HC1.

Experiments lasting as long as 78 days have demonstrated that Chlorella can be harvested continuously from the same medium if the supply of nutrient salts is replenished as needed.

Studies of cell division in relation to grams daily yield of Chlorella demonstrated that the two are virtually independent. That- is, weight for weight, large and small cells gain weight at the same rate.

Chapter 10

STCFFPRODUKTION DURCH GRttNALGEN UND DIATOMEEN IN MASSENKULTUR [1]

H. von Witsch und R. Harder

University of Göttingen,
Göttingen, Germany

Die Massenkultur autotropher Mikroorganismen wurde in den GSttinger Botanischen Anstalten in den Jahren in und nach dem Kriege auf die Möglichkeit untersucht und entwickelt, praktisch verwertbare Substanzmengen zu gewinnen. Vor allem die Fähigkeit mancher Algen zur Bildung und Speicherung erheblicher Fettmengen erschien uns von grösster Wichtigkeit. Aber auch Wirkstoffe und für die Ernährung wichtige Ergänzungsstoffe wurden beachtet.

A
Methode

Unsere ersten Untersuchungen wurden mit Diatomeen durchgeführt. Es stellte sich bald heraus, dass die erreichbaren Zelldichten bei pennaten Grunddiatomeen wesentlich h5her lagen als bei reinen Planktonformen. Da Gnmddiatomeen zum normalen Wachstum im allgemeinen ein festes Substrat ben5tigen> kultivierten wir sie nach mannigfachen Vorproben in Glaszylindern von 5 cm Durchmesser und 80 cm H5he, die, locker mit Glaswolle gefiillt, 1 Liter NShrlOsung enthielten. Die Rohre wurden von einem konstanten schwachen Luftstrom meist unter Zusatz von 0,5 % CO_2 durchstrQmt.

Brachte diese Kulturmethode auch sehr gutes Wachstum, so war doch die Glaswolle ein grosses Hindernis bei der Ernte. Wir suchten daher nach rein planktontisch kuitivierbaren Organismen mit gleicher oder noch besserer Massenentwicklung. Unter den pennaten Diatomeen fand v. Denffer [29] Nitzschia palea als eine sehr geeignete Form; unter den planktontischen Griinalgen ftihrten Versuche mit einer aus der Umgebung Göttingens stammenden Linie von Chlorella pyrenoidosa zum erstrebten Ziel (v. Witsch [177, 178]).

Als Kulturgef&sse.dienten meist Glasrohre von 3 cm Durchmesser und 25-30 cm H6he, die untcn trichterartig zu einer 1-mm weiten Diise ausgezofi'en waren. Ein schwacher Luftstrom (etwa 15 Liter/Stunde) wurde durch

[1] English summary appears at end of chapter,

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die Dlise gepresst Er verhinderte das Absetzen der Algen und sorgte fiir stete Durchmischung der NShrlösung. Auch hier warden der Luft meist 0,5 % CO2 zugesetzt. Sollten grossere Algenmengen herangezogen werden, so wurden derartige Rohre mit 6 cm Durchmesser und 150 cm H5he verwendet.

Ans&tze mit Flaschen als Kulturgef&sse fiihrten stets zu viel geringerer Zelldichte, da infolge zu grosser Schichtdicke sehr bald das Licht ins Minimum geriet. Neben Versuchen am Tageslicht (im Gewachshaus) wurden zur exakten Prlifung einzelner Wachstumsbedingungen und der physiologischen Eigenschaften unserer Versuchsobjekte auch zahlreiche Kulturen im Laboratorium bei elektrischem Licht durchgeftihrt. Als Lichtquelle diente meist eine wassergekiihlte 300-Wattlampe um welche die Kulturrohre gruppiert wurden. Je nach Abstand wurde eine Beleuchtungsstarke bis zu 10 000 Lux erreicht.

Neuerdings verwendete Aach [180] Kulturgefasse mit Beleuchtung von innen. In 5-16 cm weite Glaszylinder wurden Osram-Leuchtröhren eingebaut und die N5hrlösung direkt in den 1-20 Liter fassenden Zwischenraum zwischen Leuchtröhre und Glaszylinder geflillt. Hierdurch wurde eine optimale Ausntitzung der Lichtquelle gewahrleistet und die Aufstellung exakter Strahlungsbilanzen ermöglicht.

Fiir Nitzschia wurde folgende Nährlbsung verwendet (v. Denffer [29]): dest. Wasser 800 ml, Erddekokt 200 ml, $NaNO_3$ 0,2 g, Na_2HPO_4 0,04 g, Na_2SiO_3 0,1 g. Fiir Chlorella (v. Witsch [177]): dest. Wasser 1000 ml, $Ca(NO_3)_2$ 0,5 g, $MgSO_4 \cdot 7H_2O$ 0,1 g, KH_2PO_4 0,15 g, KNO3 0,15 g, Fe-Lactat 6 mg. Ein Zusatz von 10 ml Erddekokt erwies sich in vielen FSllen als giinstig. Vor allem ftir Ans&tze mit grossen Fliissigkeitsmengen wurde mit bestem Erfolg folgende einfachere N&hrlbsung angesetzt: Göttinger Leitungswasser (sehr hart) 1000 ml, $MgSO_4 - 7H_2O$ 0,1 g, KH_2PO_4 0,04 g, KNO3 ^{8,55} S> Fe-Lactat 6 mg, Erdlösung 10 ml.

B

Massenertrag

Mit den Glaswollekulturen erreichten wir mit dem damals leistungsfShigsten Diatomeenstamm in 10 Tagen einen Reinertrag bis zu 9 g Frischgewicht im Liter (Harder und v. Witsch [55]).

Die Ertr&ge bei unseren planktontisch kultivierbaren Formen stehen den genannten Werten nicht nach. Bei giinstigen Wachstumsbedingungen (Beleuchtungsstarke 10 000 Lux) konnte v. Denffer [29] bei Nitzschia palea ohne weiteres Kulturen mit $5,2 \cdot 10^9$ Zellen im Liter, entstrechend einem Trockengewicht von 0,98 g/L erzielen.

Chlorella **ergab** (v. Witsch [177]) unter ähnlichen Bedingungen bei Durchlüftung mit Luft + 0,4 % COo und 4000 Lux Beleuchtungsstarke in 28 Tagen eine Zelldichte von **1,6-foil** Zellen im Liter entsprechend einer Trockenmasse von 3,3 g/L. Durch ErhShung der Nahrstoffkonzentration auf den 3fachen N und doppelten P-Gehalt liess sich die Ernte auf $3,3 \cdot 10^{11}$ Zellen bezw. 5,3 g Trockenmasse steigern (siehe auch Fig. 2).

Geniigendes Alter der Kulturen vorausgesetzt bestand die Trocken-masse zu40-50% aus Fett (Stherlösliche Substanz).

Aach [180] erzielte mit Chlorella in den beleuchtungsmSssig gtinsti-geren 1-1,5 Liter fassenden Kulturgefässen Zelldichten von 4,5-5- IQH Zellen im Liter und einen maximalen täglichen Zuwachs von 0,34 g Trok-kenmasse.   10-Liter Kulturen lieferten einen Tageszuwachs bis zu 1,1 g. Der Lipoidgehalt Slterer Zellen konnte bis 70 % betragen. Hierbei ist allerdings zu beachten, dass Aach eine wesentlich verbesserte undgriind-lichere Extraktionsmethode anwandte, welche auch Lipoproteide angfiff. Es k5nnen daher seine Zahlen mit *den* Fettprozenten unserer früheren Arbeiten nicht direkt verglichen werden.

Von Scenedesmus liegen weniger auf reine Massenproduktion gerich-tete Versuche vor. In 18 Tage alten Kulturen stellte Gerdes [223] Zell-dichten von etwa 4- 1Gl0 und Trockengewichtsernten von 2,3 g im Liter fest.

C

Fremdinfektionen

In Übereinstimmung mit den Erfahrungen anderer Autoren traten bei unseren Kulturen trotz st&ndiger Durchliiftung mit nicht oder nur durch ein einfaches Wattefilter filtrierte Luft kaum Fremdinfektionen auf. Auch Sltere Kulturen blieben rein, abgesehen von einem leichten und vollkommen unschädlichen Bakteriengehalt, ausser wenn die Kulturbe-dingungen sehr unglinstig waren. Vor allem bei Kulturen am Tageslicht musste stSrkere direkte Besonnung vermieden werden, sonst wurden die Algen so stark geschädigt, dass besonders farblose Flagellaten die Kul-turen vernichteten.

D

Analyse der Algenlipoide

Wenn im Vorhergehenden von Fett gesprochen wurde, so wurde hier-unter stets die Gesamtheit aller mit Äther extrahierbarer Substanzen verstanden. Die n&here Analyse derselben wurde durch Kathen [237] bezw. Deuticke, Kathen und Harder [31] an drei Objekten durchgeftihrt und zwar dem GSttinger Chlorella pyrenoidosa-Stamm, an der von v. Denffer isolierten Nitzschia palea sowie einem ebenfalls in GSttingen isolierten Scenedesmus Klon.

Im wesentlichen zeigen die drei Algenlipoide eine dem Fett hetero-tropher Mikroorganismen (Nectaromyces Reukaufii, Nektarhefe, Rippel-Baldes [269]), und jenem aus dem Samen hOherer Pflanzen (z.B. Mohn51) sehr Shnliche Zusammensetzung. Die Analysenergebnisse Kathens [237] sind in Tabelle 1 wiedergegeben.

Die die Hauptmasse der Gesamtlipoide ausmachenden Triglyzeride bestehen vor allem aus FettsSuren mit durchschnittlich 18 C-Atomen.

Tabelle 1

Prozentuale Zusammensetzung von Algenlipoiden (nach Kathen [237])

Lipoide	Gesamtlipoide *(% der* Trockensubstanz) in		
	Chlorella pyrenoidosa	Nitzschia palea	Scene-desmus sp.
Farbstoffe	14,3	16,0	38,9
Phosphatide	0,9	0,5	0,7
Kohlenwasserstoffe und Wachse	0,5	1,4	3,9
Sterinester?	2,4	1,6	0,3
Freie Sterine	0,3	1,5	3,6
Triglyzeride	78,5	80,5	50,6
Freie FettsSuren			
	96,9	101,5	98.0

Nach den Verseifung-, Jod- und Rhodanzahlen kann man mit grosser Wahr-scheinlichkeit Triglyzeride folgender Zusammensetzung annehmen:

Bei Chlorella:

$$\text{Glycerin} \begin{matrix} ^\wedge & \text{StearinsSure} \\ — & \text{ÖlsSure} \\ {}^{"\wedge} & \text{LinolsSure} \end{matrix} \qquad \text{Glycerin} \begin{matrix} ^\wedge & \text{Linolsäure} \\ — & \text{LinolsSure} \\ {}^\wedge & \text{LinolsSure} \end{matrix}$$

Bei Nitzschia:

$$\text{Glycerin} \begin{matrix} ^\wedge & \text{StearinsSure} \\ — & \text{ÖlsSure} \\ {}^{"\wedge\wedge} & \text{LinolsSure} \end{matrix} \qquad \text{Glycerin} \begin{matrix} ^\wedge & \text{StearinsSure} \\ — & \text{LinolsSure} \\ {}^{"\wedge} & \text{Linolsäure} \end{matrix}$$

Bei Scenedesmus:

$$\text{Glycerin} \begin{matrix} ^\wedge & \text{StearinsSure} \\ — & \text{LinolsSure} \\ {}^{\wedge"\wedge} & \text{LinolensSure} \end{matrix}$$

E
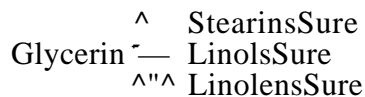
Waehstumsphasen

Die genauere Analyse des Wachstums unserer Objekte und des Einflus-ses einzelner Wachstumsfaktoren fiihrte zu Resultaten, die nicht nur f!ir die Massenkultur von Algen wichtig sind, sondern auch tiefere Einblicke in das Wachstum und die Vermehrung der untersuchten Organismen erlaubten.

Sowohl bei Chlorella (v. Witsch [177, 178]) wie bei Nitzschia (v. Denffer [29]) lassen die in den durchlflfteten Glasrohren heranwachsenden Kulturen stets zwei Waehstumsphasen erkennen, ¥on denen die erste durch rasche Zellvermehrung, die zweite durch Stoffspeicherung, also vor allem Fett-biidung charakterisiert ist, Diese beiden Entwicklungsabschnitte, die sich im wesentlichen mit den von Denk gepragten Begriffen Konkordanz- und Diskordanzstadium decken (nlheres siehe v. Witsch [178]), warden als Teilungs- und Speicherphase bezeichnet.

Nitzschia palea

Bei Nitzschia, deren vegetative Vermehrung durch Zweiteilung der Zellen erfolgt, ergab die laufende Bestimmung der Zelldichte einer Kultur ein anfänglich exponentiales Ansteigen der Individuenzahlen. 1st etwa die halbe endgliltige Besiedlungsdichte erreicht, zeigt die Zuwachskurve einen Wendepunkt, wird flacher und verlSuft schliesslich horizontal. Ungefähr nach Abschluss des exponentialen Wachstums werden in den Kulturen die ersten Anzeichen einer Verfettung mikroskopisch nachweisbar.

Die H8he der endgiiltig erreichbaren Besiedlungsdichte einer Nitzschia palea-Kultur ist ausschliesslich von der Konzentration der NShrstoffe ab-hSngig. Temperatur, Lichtintensität und Einsaatdichte bestimmen nur die Geschwindigkeit mit welcher der stationäre Endzustand erreicht wird. Fig. la zeigt das Resultat einer Versuchsreihe mit abgestufter Nähr-salzkonzentration, Fig. 1b einer solchen mit verschiedener Einsaatdichte.

Chlorella pyrenoidosa

Bei Chlorella liegen die VerhSltnisse nicht ganz so einfach. Auch hier hat die Wachstumskurve einer Durchltiftungskultur typische S-Form. Da aber die Zellvermehrung nicht durch einfache Zweiteilung erfolgt sondern je nach den Wachstumsbedingungen aus einer Mutterzelle 2 bis 8 entsprechend kleiner Tochterzellen entstehen, die erst allmählich zur noririalen Grösse heranwachsen, bietet die Feststellung der Teilungsfolge gewisse Schwierigkeiten. Ausserdem muss naturgem&ss zur Beurteilung des Massenertrages neben der Zelldichte auch die Zellgr5sse beriicksichtigt werden.

Teilungs- und Speicherphase lassen sich bei Chlorella auch an der durchschnittlichen Zellgr5sse erkennen. Nach Abschluss des Teilungs-wachstums nehmen die Zellen noch eine zeitlang an Grösse zu. Gleich-zeitig setzt die Ablagerung mikroskopisch nachweisbarer Fettmengen ein. Der mittlere Zelldurchmesser einer Slteren Kultur unseres Chlorella-Stairfmes betrSgt 4,8 M, kurz nach Abschluss der Teilungsphase jedoch $r^\wedge r$ 3,7^.

Auch unsere Versuche an Chlorella bestatigten die Erfahrung, dass Fettbildung einsetzt sobald in der N&hrl5sung Mangel an verwertbarem N auftritt. Fiir den Übergang vom Stadium der Zellteilung zur Phase des Zellwachstums liess sich keine einfache Beziehung zu einem einzelnen Element nachweisen. Die Dauer der Teilungsphase wurde nicht nur durch eine ErhShung der N-Konzentration sondern in viel stSrkerem Masse durch gleichzeitiges Hinaufsetzen des N und des PhosphorsSure-Gehaltes der Normall5sung verl^ngert. Da mit einer VerlSngerung der Teilungs-phase naturgemSss auch der Beginn des Wachstums und der Fettbildung hinausgeschoben wird, miissen gleichalte Kulturen mit verschieden hoher N- und P-Konzentration nicht nur verschieden hohe Zelldichten sondern auch verschiedene ZellgrSssen, MassenertrSge und Fettprozente aufweisen (v. Witsch [177]). In Fig. 2 1st ein solcher Versuch im Diagramm wiederge-geben.
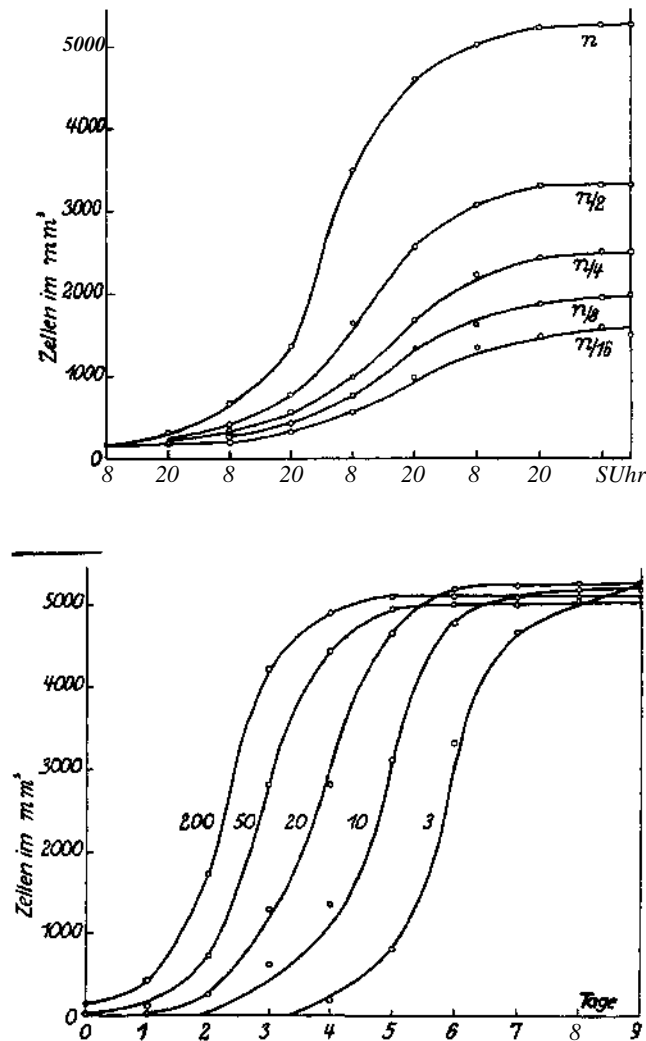
Fig. 1. Nitzschia palea. Planktonkultur. (a) Wachstum bei verschiedener NShrlSsungskon-zentration. n=Normallbsung, n/2-n/16=Verdiinnungsstufen. (b) Wachstum bei verschiedener Einsaatdichte (3-2QQ Zellen je mm*). (Nach v. Denffer [29].)

Growth curves for Nitzschia palea, showing in (a) that maximum cell count depends on concentration of culture medium; and in (b) that maximum cell count is independent of initial density of inoculum (shown by numbers on the curves), but that maximum is attained more quickly when initial density Is high. (After von Denffer [29].}

Sehr aufschlussreiche Untersuchungen liber die Energieausniitzung durch Chlorella führte neuerdings Aach [180] durch. Infolge der eingangs geschilderten Kulturmethode mit Innenbeleuchtung der Gefässe war er in der Lage die Verwertung der eingestrahlten Lichtmenge durch die Alge zu messen. In Kulturen solcher Zelldichte, dass die gesamte Strahlung der Lichtquelle absorbiert wurde, fand er bei Schwachlichtkulturen 6 % der eingestrahlten Energie in der organischen Substanz der gebildeten
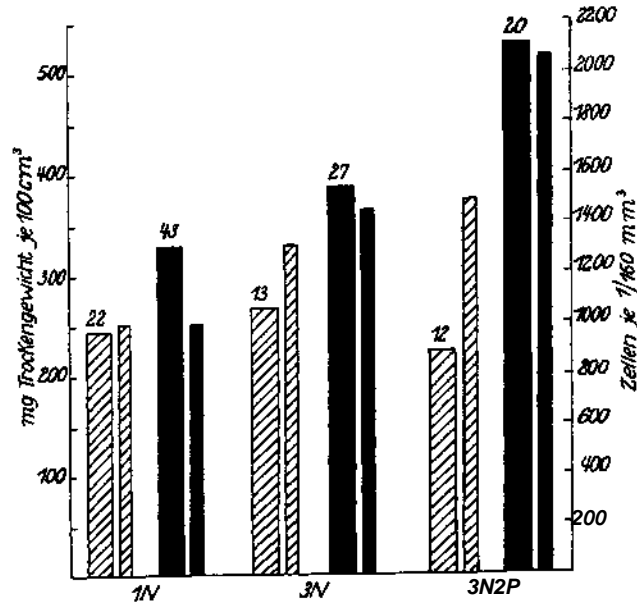
Fig. 2. Chlorella pyrenoidosa, Planktonkultur. Erntetrockengewichte (breite Säulen) und Zelldichte (schmale SSulen) in Mineralsalz-NormallSsung (IN), bei dreifachem Stickstoffgehalt (3N) sowie bei dreifachem Stickstoff- und doppeltem PhosphorsSuregehalt (3N 2P). Ernte nach 14 Tagen (schraffiert) und 28 Tagen (schwarze Säulen). Zahlen über den Trockengewichten: prozentualer Fettgehalt der Ernten. (Nach v. Witsch [177].)

Influence of concentration of nitrogen and phosphorus in a culture medium on yield of dry matter and cell count of Chlorella pyrenoidoga. Ordinate on left gives dry weight, corresponding to wide bars; number at top of each wide bar gives percentage of fat in dry matter. Ordinate on right gives cell count, corresponding to narrow bars. Numbers at base give relative concentrations of nitrogen and phosphorus in culture medium, the one at left being the usual solution with respect to both nitrogen and phosphorus. For each concentration, yield and cell count are given after 14 days (cross-hatched bars) and after 28 days (solid bars). (After von Witsch [177].)

Zellen festgelegt (Nutzeffekt), bei stSrkerer Beleuchtung 3 %. Die einzelnen Ergebnisse und Versuchsdaten sind in Tabelle 2 zusammengestellt. In diinneren nur einen Teil des eingestrahlten Licht absorbierenden Algensuspensionen (zirka 300 Zellen in 1/100 ml) war auch im Starklicht der Nutzeffekt auf die tatsSchlich absorbierte Energie berechnet, etwa 6 %, auf die eingestrahlte Gesamtleistung bezogen allerdings nur 2 %.

F

Das "physiologische Alter" und Wertigkeit als Impfmaterial

Eine Reihe von Versuchen wurde der Frage nach dem Einfliiss des "physiologischen Alters" des Impfmaterials gewidmet. Unter "physiologischem Alter" wird hierbei der Entwicklungszustand verstanden, in welchem sich die Zellen zur Zeit der Beòbachtung befinden. Vor allem interessierte ein Vergleich von Impfungen mit Zellen von in lebhaftem Wachstum begriffenen physiologisch jungen Kulturen mit der Verimpfung stark verfetteter schon lange in der Speicherphase stehender physiologisch alter Zellen. Hierbei darf das physiologische Alter keinesfalls mit dem Alter einer Kultur in Tagen verwechselt werden, denn je nach den Kulturbedingungen kSnnen die einzelnen Entwicklungsphasen natfirlich ganz verschieden schnell ablaufen (siehe Versuch Fig. 2). Diese Versuche gestatteten auch, die physiologische Bedeutung der Verfettung für die Algenzelle zu beurteilen. *Von* manchen Autoren wurde bekanntlich die Verfettung als

krankhafter Vorgang gewertet und dementsprechend von Degenerationsfett gesprochen (naheres hierUber siehe v. Denffer [29] und v. Witsch [178]).

Bei unseren Versuchen mit Massenkulturen in Durchliiftungsrohren war sowohl bei Nitzschia wie bei Chlorella physiologisch altes verfettetes Impfmaterial mindestens ebenso leistungsfähig wie junge in der Teilungs- phase stehende Zellen. Dasselbe fand auch Gerdes [223] für Scenedesmus. Stets ergab verfettetes Impfmaterial zum Mindesten in den ersten Tagen sogar raschere Zellvermehrung und ffihrte oft in kiirzerer Zeit zu gleich hoher Massenproduktion und Zelldichte als physiologisch junge nicht ver- fettete Zellen. Die mikroskopische Beobachtung lehrte, dass verfettetes Impfmaterial beim Wiedereinsetzen der Zellteilungen die gespeicherte Fettmenge glatt verbraucht und gerade durch diese Nährstoffreserven jungen fettarmen Zellen gegenüber im Vorteil ist. Bei Nitzschia, welche normalerweise nur am Licht zur Zellteilung schreitet, sind stark verfettete Zellen sogar in der Lage, sich auf Kosten ihres Speicherfettes auch im Dauerdunkel zu teilen. Wir haben also alien Grund, die Fettablagerung in der Algenzelle als reine Reservestoffspeicherung anzusehen, welche mit Degenerations- oder Krankheitserscheinungen nichts zu tun hat. Nur extrem verfettete Zellen aus iibermässig alten Kulturen zeigten eine leichte Verringerung ihrer Leistungsfähigkeit.

Eingehendere Untersuchungen iiber die stoffliche Zusammensetzung der Chlorella-Zelle in der Wachstums- und Speicherphase verdanken wir Aach [180]. Zunächst stellte er fest, dass die Teilungsfähigkeit bei Chlo- rella pyrenoidosa erlischt, wenn der N-Gehalt der Zelle unter $1,6 \cdot \overline{10\text{-}13}_g$ absinkt. Er fand ferner, dass N-Aufnahme und Eiweissgehalt und im Zusam- menhang damit auch Generationsdauer und Teilungsfrequenz in einer Kultur in hohem Masse von der absorbierten Strahlungsmenge abhängig sind (siehe Tabelle 2).

Tabelle 2

Chlorella pyrenoidosa: Wachstum und Strahhmgsausnutzung in Schwachlicht- und Starklichtkulturen1 (nach Aach [1.80])

	Strahlungsstärke, rel.	Strahlungsleistung, t (Watt)	4 Stdn,	Zn, rel.	Mittlere N c tr.	Zelle absorbierte Leistung; (Watt·10⁻¹²)	(Stdn)	Nutzeffekt der Ausstrahlten
Schwachlichtkulturen.............	1	**0,464**	103	222	3120	1,4	20,0	
	1	0,459	101	220	2790	1,5	**20,0** } ca 6	
Starklichtkulturen	6	2,98	336	113	5010	5,4		
	6	2,70	**305**	113	4400	5,6	7,7 } ca 3	

Auch Aach beobachtete bei seinen Analysen die grosse Speicherfähigkeit der Chlorella-Zelle vor allem auch flir N. Schon nach wenigen Tagen ist der NShrlBsung der gesamte Vorrat an assimilierbarem N durch die Algen- zellen entzogen und in diesen angereichert. Fiir Fe gilt wie schon frtiher erwShnt, dasgleiche. In einer Kultur gehen daher die Zellteilungen auch nach ErschBpfung des N-Vorrates der Lbsung solange weiter bis die N- Reserven der Zellen bis zum oben genannten Grenzwert abgesunken sind. Junge Zellen mit hoher Teilungsfrequenz (2 x täglich Teilung) ergaben bei der Analyse eineinhalb Mai soviel Eiweiss, sechs Mai soviel Fe und dop- pelt soviel Asche als ältere Zellen, die sich nur noch alle 20 Tage einmal teilten.

Nach Erlöschen der Zellteilungen wird nahezu die ganze absorbierte Strahlung zum Aufbau von Fett verwendet. Die starke Fettspeicherung (bis 70 %'cier Trockenmasse) geht jedoch nicht auf Kosten der übrigen organischen Substanz, die in schwach und stark verfetteten Zellen in etwa gleicher Menge gefunden wurde. Eine Ausnahme hiervon bilden nur die Farbstoffe, welche mit fortschreitender Verfettung zunehmend abgebaut werden. In allzu alten iiberstSndigen Kulturen nimmt allmählich auch die iibrige organische Substanz wieder ab.

G

Wirkstoffproduktion durch Algen

Aneurinbildung;

Testversuche, welche zuerst bei Chlorella pyrenoidosa durchgefiihrt wurden (v. Witsch [175, 178]), zeigten, dass Teilungs- und Speicherphase auch durch unterschiedlichen Gehalt an Aneurin (Vitamin Bi) charakteri- siert sind. Junge in lebhaftem Teilungswachstum befindliche Kulturen wiesen einen mehrfach höheren Gehalt an Vit. Bj auf als Sltere schon zur Speicherphase libergegangene. Ausgedehntere Untersuchungen wurden von Gerdes [223] durchgefiihrt. Sie wies an zahlreichen im Freiland gesam- melten Stisswasser- und Meeresalgen dieselben ZusammenhSnge zwischen Entwicklungszustand und Aneuringehalt nach und fai\d im allge- meinen Aneurinwerte, welche denen der höheren Pflanzen entsprechen.

Von besonderem Interesse sind ihre eingehenden Versuche tiber den Aneuringehalt von Scenedesmus. Dies wurden im Laboratorium unter genau definierten Bedingungen an Kulturen in Durchliiftungsrohren durch- gefiihrt. Laufende Vitaminteste zeigten besonders schön den engen Zusam- menhang zwischen Entwicklungszustand und Aneuringehalt einer Kultur. In Fig. 3 sind die Mittelwerte aus zwei derartigen Versuchen wiedergegeben. Die genauere Testung junger Kulturen ergab, dass im tSglichen Licht- Dunkel-Wechsel die Aneurinwerte stets in der Dunkelphase anstiegen, in der Lichtperiode wieder zurtickgingen. Diese Versuche wurden durch Serien mit verschiedenen Licht-Dunkel-Rhythmen sowie in Dauerlicht und Dauerdunkel erweitert und bestatigt. Ein direkter Zusammenhang mit dem
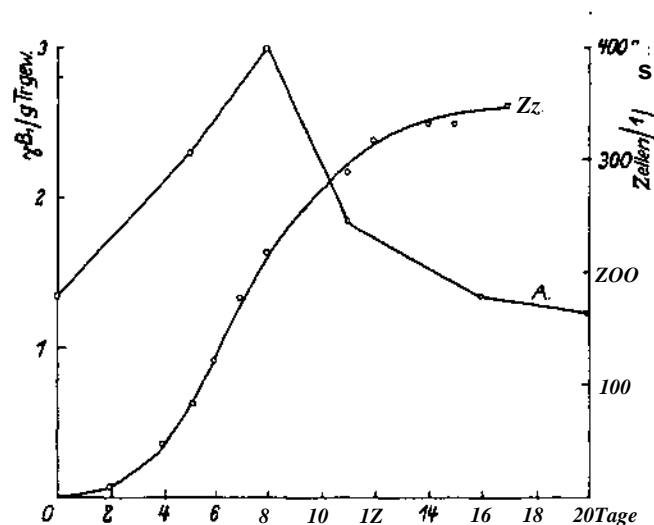
Fig. 3. _Scenedesmus bijugatus_. Planktonkultur. Zellenzahl (Zz) und Aneuringehalt (A) bei zunehmendem Kulturalter. (Nach Gerdes [223].)

Change in vitamin Bj content (curve A shows gamma per gram dry weight) of _Scenedesmus bijugatus_, contrasted with cell count (curve Zz) as culture increases in age. (After Gerdes [223].)

bei Scenedesmus normalerweise im Dunkeln ablaufenden Zellteilungen liess sich jedoch nicht nachweisen.

Hemmstoffe

Zum Schluss sei noch erwähnt, dass v. Denffer [30] bei _Nitzschia palea_ auch die Bildung von Hemmstoffen nachweisen konnte. Diese werden von den Zellen in das Kulturmedium abgegeben und verhindern nach Erreichen einer bestimmten Konzentration die weitere Zellvermehrung. In eindruckvollen Versuchen konnte er die Wirkung dieser Hemmstoffe vor Augen führen. Die erreichbare Zelldichte einer Kultur wurde nur dadurch, dass die normale NShrsalzmenge nicht auf einmal gegeben wurde sondern in 7 maligem Wechsel einer 1/7 normalen NShrlSsung, um 154 % gesteigert. Durch den mehrmaligen Fllissigkeitwechsel wurden die ausgeschiedenen Hemmstoffe stets wieder entfernt

Die zytologische Untersuchung bewies, dass die Hemmstoffe vor allem als Mitosegift wirken. WShrend die Assimilation und Fettbildung beim Erreichen des stationären Endzustandes ungehindert weiter gehen, werden alsbald gehSuft Zwillingsgruppen als Ausdruck des gehemmten Mitoseablaufs beobachtet. Die AuszShlung gefSrbter PrSparate von jungen Kulturen ergab etwa 90 % Ruhekerne und 10 % Teilungsstadien. PrSparate von alten Kulturen zeigten nur 40 % Ruhekerne und 60 % offenbar nicht mehr zu Ende geftihrte steckengebliebene Teilungsstadien. Der Anteil an

späten Telophasen, die als Zwillingsgruppen auch am lebenden Material leicht kenntlich sind, betrug im ersten fall 4, im zweiten jedoch 19 %.

English Summary by H. A. Spoehr

Production of Organic Material by Green Algae and Diatoms

Laboratory experiments on the mass culture of autotrophic micro-organisms were inaugurated in Gòttingen during the Second World War, particularly with a view to the production of fat. From preliminary experiments with various organisms it was concluded that the diatom Nitzschia palea and the green alga Chlorella pyrenoidosa were most promising for practical purposes of culture. These organisms were cultured in nutrient solutions in glass tubes, 3 cm x 25-30 cm and 6 cm X 150 cm, with an air stream enriched with about 0.5 per cent CO_2 at about 15 liters per hour, and illuminated with water-cooled 300-watt lamps, yielding up to 10,000 lux (1 lux = 0.0923 f.c).

Chlorella, cultured with air plus 0.4 per cent CO_2 and 4000 lux, gave a yield of 5.3 g dry weight per liter in 28 days. Scenedesmus, under presumably comparable conditions, yielded 2.3 g/L Little difficulty was encountered from infection, except when the cultures were illuminated with direct sunlight, in which case the algae were occasionally destroyed by colorless flagellates.

The lipid content of the cultured organisms ranged from 40 to 70 per cent. The composition of the fat was found to be very similar to that of fats from heterotrophic microorganisms and from the seeds of higher plants. The fat of Chlorella, Nitzschia, and Scenedesmus was found to be composed of the triglycerides of stearic, oleic, and linoleic acids (see table 1),

With Chlorella and Nitzschia, grown in the aerated cultures, two growth phases could be distinguished. The first of the phases is characterized by rapid cell division, and the second by storage of food material, primarily fat.

In Nitzschia cultures, cell counts at first show an exponential increase with time. At about the point where one-half the final population density is attained, the growth curve flattens out, and it finally becomes horizontal. At about the end of the exponential period the first signs of fat accumulation become microscopically detectable. The finally attainable population density of a Nitzschia culture is dependent upon the concentration ol the nutrients* Temperature, light intensity, and inoculum density determine only the rate at which the stationary state is attained (see fig, 1).

In Chiorella cultures the relations are more complex. Here also the growth curve has a typical **S-form.** A single Chlorella mother cell can produce 2 to 8 daughter cells, which only gradually attain normal size. In the estimation of total yield, not only cell density, but also cell size must be considered. Division and storage phases can? however, also be

distinguished in Chlorella cultures. After termination of division, the cells increase in size, and simultaneously deposition of fat is observable microscopically. Shortly after completion of the division phase the cells had a diameter of 3.7 microns; those of older cultures had a diameter of 4.8 microns.

The investigations confirm the observation that fat formation is initiated when there is low concentration of available nitrogen in the nutrient solution. The duration of the division phase was increased not only by increase of the nitrogen concentration, but even more by simultaneous increase of the available nitrogen and phosphate content of the nutrient solution (see fig. 2).

From the unpublished results of Aach it is concluded that when cultures are internally illuminated with weak light in such a way that all the light is absorbed, 6 per cent of the light energy is converted into organic material. With sixfold stronger illumination about 3 per cent is converted (see table 2).

The comparative value for inoculation purposes of cells of different "physiological age" was determined for Nitzschia and Chlorella. By "physiological age" is understood the stage of development, or growth phase, of the cells at the time when they are used for starting new cultures by inoculation. Old cells, in which considerable fat had accumulated, produced cultures which during the first few days grew more rapidly than those which were started with young, actively dividing cells. An inoculum of fat-containing cells produced cultures which attained a given cell density and amount of organic matter in a shorter time than cultures started with young, fat-free cells. Microscopic observation revealed that when fat-containing cells are placed in fresh nutrient medium, the fat is readily consumed, and that this appears to give these cells an advantage, as regards cell division, over the young, fat-free cells. Nitzschia, which normally divides only in the light, was found to divide also in the dark if the cells contained a considerable amount of fat.

The capacity of Chlorella cells to divide ceases when the nitrogen content of the cell sinks below $1.6 \times 10\text{-}13$ $g_\#$ Nitrogen absorption and protein content of a culture, as well as its frequency of division, are dependent upon the amount of absorbed radiant energy (see table 2). Young cells which divide twice daily contain 1.5 times as much protein, 6 times as much iron, and twice as much ash as old cells which divide only once every 20 days.

After cessation of cell division, almost all the absorbed radiation is used for the formation of fat. But this fat storage (up to 70 per cent of the dry weight) is not produced at the expense of the rest of the organic matter of the cells. The latter occurs in about the same amount in cells with both high and low fat content. An exception to this is found in the pigments, which decrease with increasing fat content.

Young, actively dividing cells contain more vitamin Bj than older, fat-storing cells. This is shown particularly in Scenedesmus (see fig. 3).

The formation of substances inhibitory to growth has been demonstrated with Nitzschia- The action is apparently due to the inhibition of mitosis.

Chapter 11

NONSTERILE LARGE-SCALE CULTURE OF CHLORELLA
IN GREENHOUSE AND OPEN AIR

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A

Introduction

The work carried out here was undertaken to evaluate the possibility of biological utilization of the huge quantities of carbon dioxide from waste gases available in the industrial district of the Ruhr. The work of Harder and von Witsch [54, 55] on diatoms (see the preceding chapter) was the first in Germany to show the feasibility of the large-scale culture of algae. Studies of the literature and further investigations indicated that Chlorella was a more suitable genus for this purpose. For a year and a half the authors have experimented to find out whether large-scale nonsterile cultures of algae can be grown under the local light and temperature conditions. The optimum conditions for such cultures as well as economic utilization of the algal product were also studied. The following report can give only a brief description of this work.

B

Experimental Plants and Culture Conditions

Experimental Plants

An experimental plant in the open air and a greenhouse with seven cement tanks and two of ceramic material were used. The experimental plant in the open air (fig. 1) included four culture trenches with a fall of 6 mm/m. These trenches were 9 m long, 70 cm wide, and 20 to 2^A cm deep at the low ends. They were rammed down in loam and were lined with plastic: two with Mipolam, a polyvinylchloride synthetic, and two with gray Oppanol, a polyisobutylene material. These plastics were ascertained to be nontoxic to Chlorella. The gas mixture for aeration was led into the cultures by iron tubes having many small holes, and protected by an inert coating. Each trench held 600 liters of culture when filled to a depth of 9 to 15 cm.

The greenhouse tanks, set up some years ago, hold 100 to 200 liters. They are 82 to 122 cm long, 82 to 66 cm wide, and 15 to 21 cm deep. The aeration system is similar to that described for the trenches.
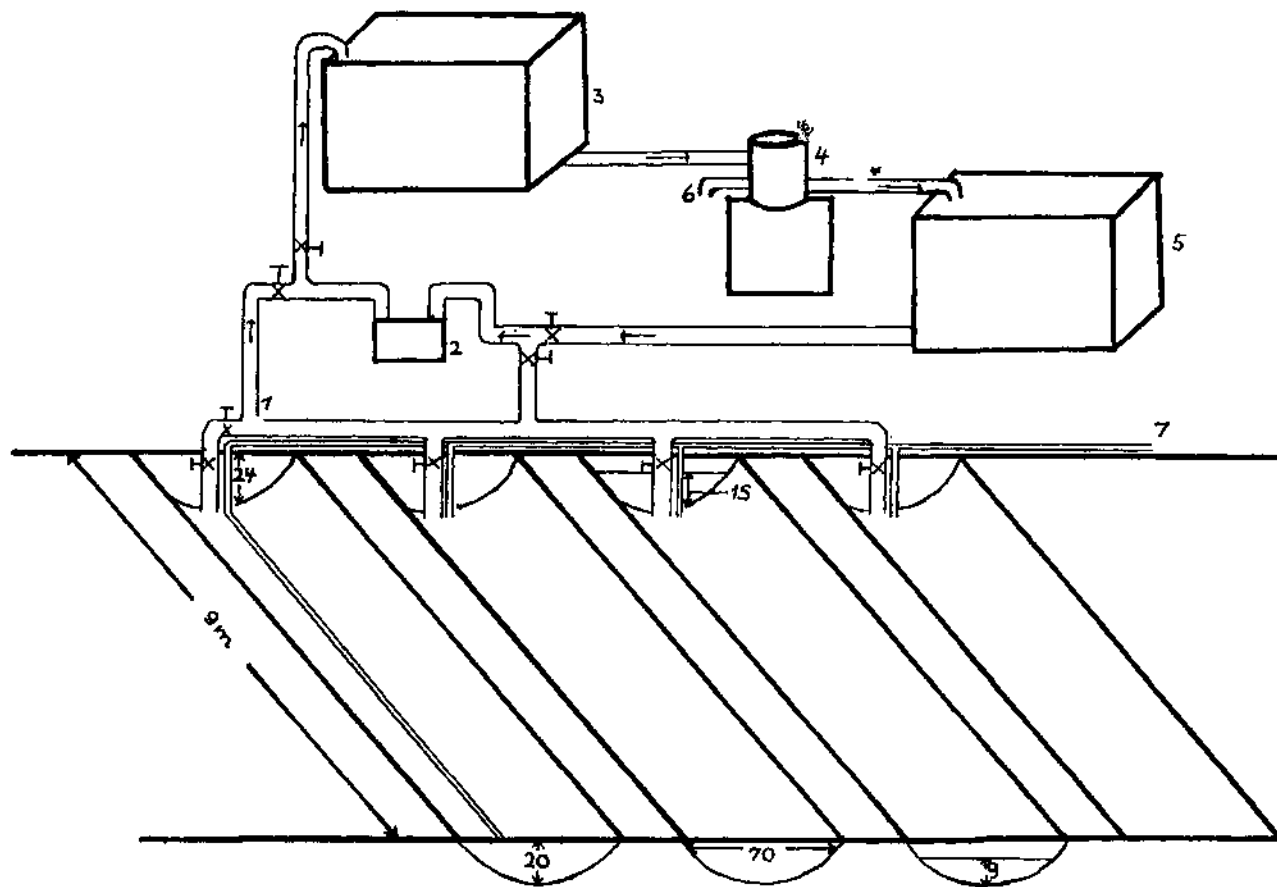
Fig. 1. Open-air plant. 1, pump pipeline; 2, pump; 3, collecting vessel for suspension; 4, centrifuge; 5, collecting vessel for filtrate; 6, centrifuged fresh substance; 7, gas pipeline.

None of the systems for mass culture described in this chapter is artificially illuminated or maintained at a constant temperature. The cultures in tanks in the greenhouse are, however, provided with a cooling device for use in the hot summer months.

Nutrient Medium

The nutrient medium used was a modified mineral salt solution, described by Kolkwitz, which yielded results similar to those from the media used by Pratt ([126], no. 25), Spoehr and Milner [151], and Myers [161], and other media used for Chlorella cultures. Its concentration was: 0.034 M KNO_3, 0.0062 M KH_2PO_4, 0.0022 M $MgSG_4 \cdot 7H2O$; total molality, 0.042. The pH of this solution when fresh was 6.1, and it increased during the growth of the culture to 7 to 8, depending on the rate of growth and the duration of the period of growth. The pH was measured daily with a Pusl "lonoscope," which uses a quinhydrone electrode.

The microelement solution also corresponded to the ones generally used. All the nutrient solutions for these large-scale cultures were made up in tap water, filtered through charcoal to remove chlorine.

Temperature

The summer temperature ranged from 18 to 24° C. A short-time increase to 30 or 35° on hot days, or a nightly cooling to 10 or 12° during the cooler late summer and autumn nights, did not seem materially to influence the growth. This finding agrees with those of Cook [22] and of Spoehr and co-workers [152].

Aeration

The flow of gas was 0.60 m^3/hr for open-air cultures and 0.55 m^3/hr for greenhouse cultures. This rate of gas flow was sufficient to maintain turbulence of the cultures. Although a gas mixture of 5 volume per cent of carbon dioxide is known to be more favorable, we were obliged by economic considerations to work with only 1 volume per cent of carbon dioxide, since pure carbon dioxide from gas cylinders had to be used. Purified waste gases containing carbon dioxide can be used for this purpose, as was shown experimentally. Adequate technical installations for supplying large-scale cultures with waste gas-air mixtures containing higher carbon dioxide concentrations are under construction.

Strain of Chlorella

Preliminary experiments showed that not all species or strains of Chlorella are suitable for large-scale cultures. Further experiments on the physiological characteristics of the more-promising species or strains were made. In agreement with Winokur [172], we found considerable dif-

ferences in rate of division and in yields of organic matter in relation to cell size. A strain of <u>Chlorella pyrenoidosa</u> was chosen which excelled in ability to remain in suspension and in its rate of growth. This strain was used to inoculate all the large-scale cultures.

Light Conditions

The curves in figure 2 show the cell counts and the course of growth of large-scale Chlorella cultures which were exposed to sunlight for 10 to 12 hours daily. The culture period was seven weeks. For comparison, a growth curve for cultures in glass tubes (diameter, 5.5 cm) is shown.

As was to be expected, there are considerable differences between the absolute figures of the several growth curves. Owing principally to the different conditions of illumination, the initial phase and the logarithmic period of growth end at different points in the three types of culture container. The horizontal trenches and tanks are unfavorable for the absorption of oblique illumination. The depth of the suspension primarily determines the light conditions in a culture. The figures in table 1 show that the rate of division, population density, and yield of organic matter are inversely related to the depth. Also the smaller yields in the greenhouse tanks are chiefly caused by the greater depth of the cultures. In the summer of 1951, which was almost entirely cloudy, Chlorella cultures 30 cm deep in open-air tanks had to be stopped after a short time. The trench cultures, on the contrary, being not nearly so deep, were in operation throughout the summer. This shows that the best prospects are for growing cultures in thin layers. Myers [161] obtained similar results.

Although the yield was ten times as great, culture in glass tubes was, for the present, out of the question for economic reasons. So also was operation with sterile cultures, which, in the case of large-scale systems containing several hundred liters, is carried out only with difficulty, and at a considerable increase in operating expense. The "open-air trenches described above, therefore, seem to be the most practical plant, since the depth of the suspensions is relatively small.

C

Harvesting of the Open-Air Cultures

Cultures were harvested according to "the theory of the optimum catch" [66]. The most effective continuous harvesting of Chlorella cultures is done at *the* cell concentration at which the highest growth takes place. Only then can the richest harvest be obtained in the long run. This cell concentration depends on different factors, mainly the daily duration and intensity of illumination. The growth curve for Chlorella cultures presented in figure 3 was obtained under the optimum local light conditions, that is, a number of summer days with about 10 hours of sunshine. The curve shows that,
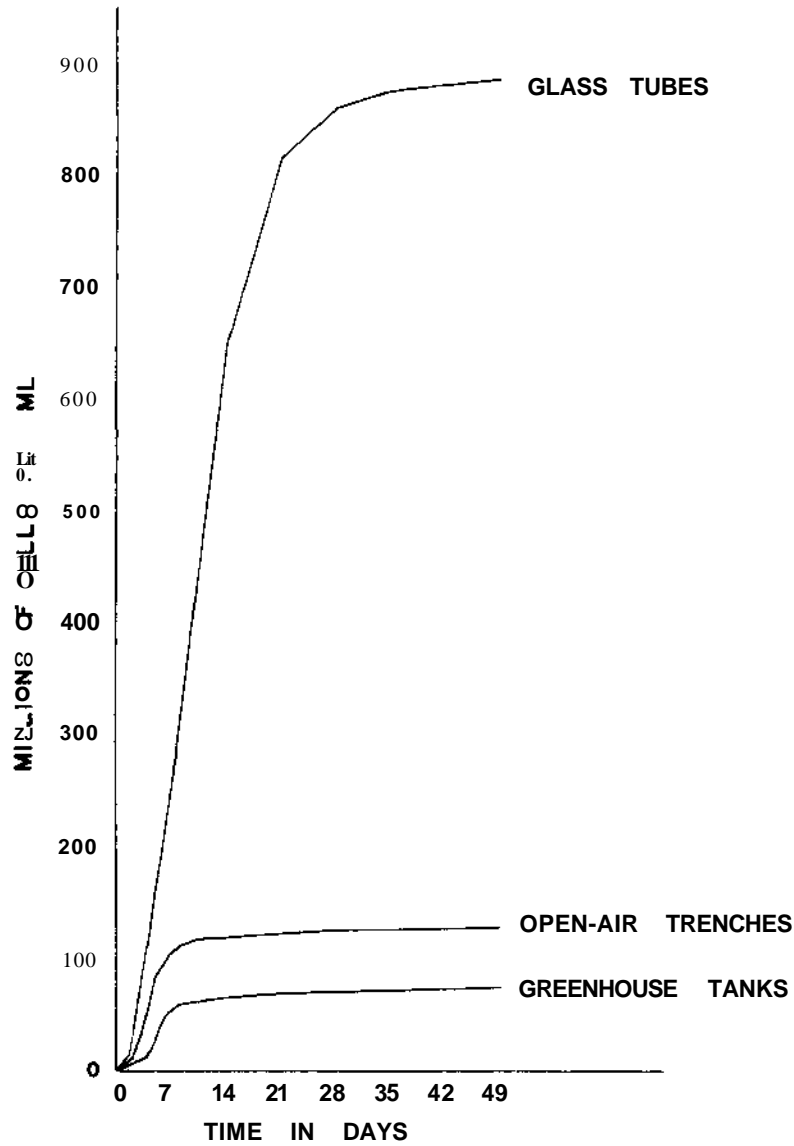
Fig. 2. Growth curves of Chlorella cultures in glass tubes, open-air trenches, and green-house tanks.

between $8x10^6$ and 64x106 cells per milliliter, the cell count doubles within 24 hours. Since greater yields can be expected at a higher population density, half the culture was centrifuged as soon as the cell count was within the range of $40X10^6$ to $64x10^6$ cells per milliliter, at which cell density, according to Pratt [131], inhibitory substances had not yet accumulated. The culture removed was replaced by cell-free centrifugate or, if necessary,

Table 1

Influence of depth on Chlorella cultures

	Tube enures	Cultures in open-air trenches	Cultures in greenhouse tanks
Depth of suspension (cm)	5.5	9-15	20
Maximum cell concentration (no. cells/ml)	886X10⁶	126X10⁶	72X106
Yield of dry substance (g/1)	3.07	0.82	0.47
Rate of division[a] per day for following ranges of concentration (million cells/ml):			
1-10	1	1	0.72
10-40	1.73	1	0.81
40-70	1.34	0.67	0.18

[a] Calculation of the rate of division was made by the formula $k(t-t^{\wedge}) = \ln x/x^{\wedge}$, where $x =$ final cell concentration, $x\pm$ = initial cell concentration in the period $(t-tj)$, $k/\ln 2$ = rate of division.
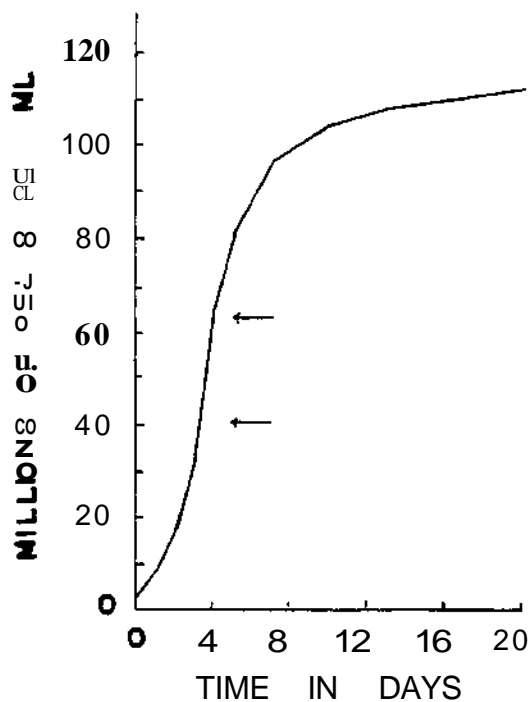
Fig. 3. Growth curve of Chlorella cultures in open-air trenches. The arrows mark the range of concentration within which the culture was harvested.

by fresh medium. Doubling of the number of cells within 24 hours with about 10 hours of sunshine daily was observed repeatedly. If optimum light conditions and cell concentration could be maintained, it should be possible to harvest daily; but a continuing culture process in which half the culture would be harvested each 24 hours within the stated cell concentrations has not yet been achieved.

Figure 4 shows the course of two Chlorella cultures in which continuous culture was tried during 10 days with about 10 hours of sunshine daily. The arrows mark the days and the cell concentrations at which the cultures were centrifuged. The curves show that the harvesting must meet the requirements imposed by growth in order to maintain the population density between the desired limits. If half the culture was centrifuged on two successive days, centrifugation had to be omitted on the third day in order to maintain the population density.

Thus there was a decided rhythm in the growth pattern. Before harvesting, a culture population doubled in 18 to 20 hours. After a harvest, 30 to 35 hours were required for doubling the cell count, and after harvesting on two successive days, an-even longer time was required for the population to double. After a day's rest from harvesting, the cycle was repeated. This rhythm of growth cannot have been induced by varying daylight conditions, because intensity of radiation was practically the same on each of the 10 days.

The growth rate of algae is greatly influenced by the considerable variation in intensity of sun or sky radiation. This is shown by one of the curves
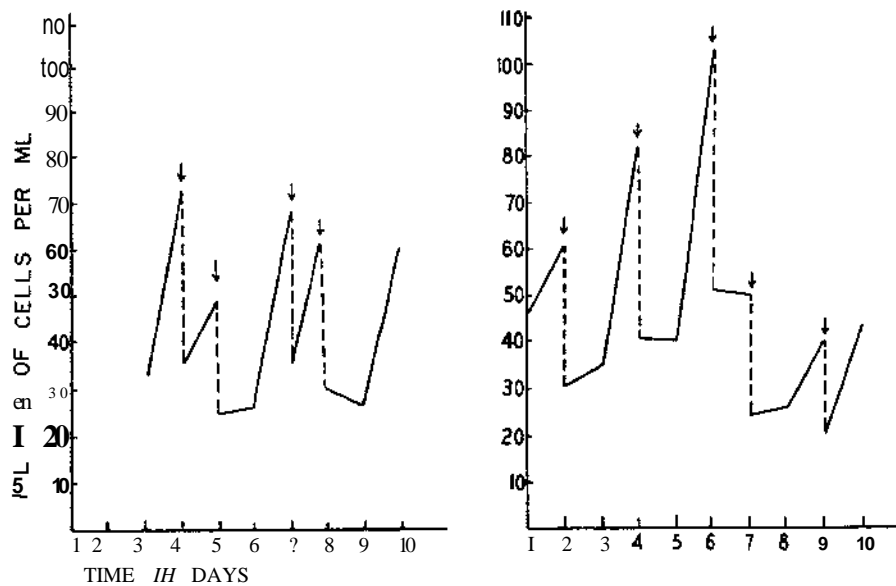
Fig. 4. Growth of two Chlorella cultures in open air. The arrows mark the day and cell concentration at which cultures were centrifugecl to hall concentration,
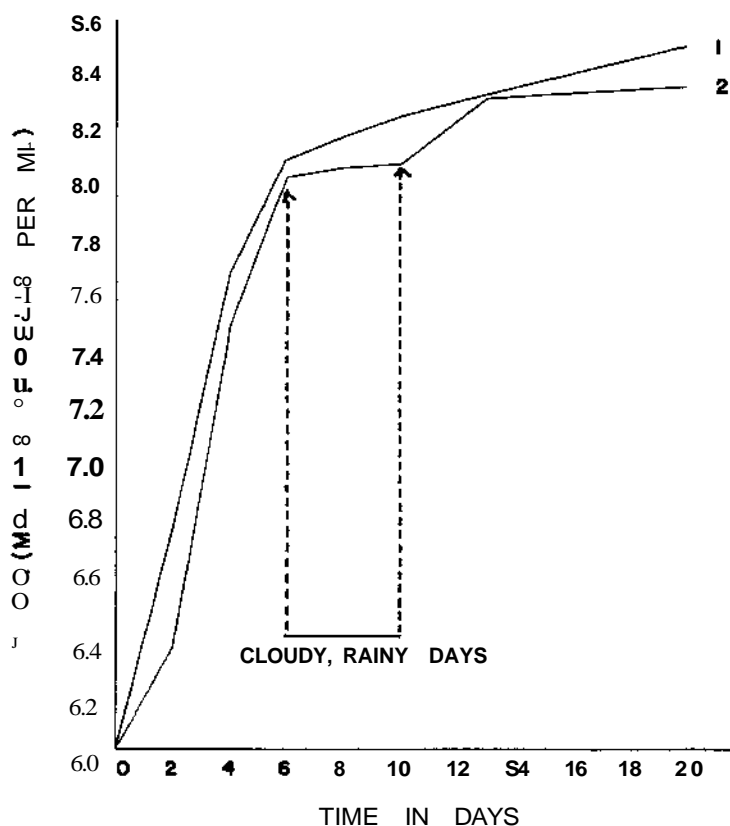
Fig. 5. Growth curve of two Chlorella cultures in 300-mm'Erlenmeyer flasks. No. 1 under artificial illumination by 25-watt Osram tubes, type HNI; duration of illumination, 16 hours; distance from cultures, 5 cm. No. 2 under natural illumination in a greenhouse; duration of daylight, about 16 hours.

in figure 5. Two cultures were illuminated 16 hours daily, no. 1 with electric light, no. 2 with natural light. The second curve shows clearly the effect of cloudy days. In both cultures the nutrient medium, temperature, gas mixture, volume, and inoculum were the same.

D
Contamination

Foreign Algae

A problem not easily solved in nonsterile, open-air, large-scale algal cultures is prevention of contamination by foreign algae and protozoa. Blue-green algae were repeatedly observed in 1950: Chroococcus minutua principally in the spring and Pseudanabaena catenata in the summer. As several species of blue-green algae seem to require calcium as a macro-

nutrient, whereas Chlorella requires this element in traces only, calcium salts were not used in the nutrient media in 1951. In contrast with the previous year, blue-green algae were not observed in the summer of 1951. Green algae, Chlamydomonas and Scenedesmus, appeared only occasionally. The cell count of these foreign algae was always less than 5 per cent that of Chlorella.

<u>Protozoa</u>

In 1950 protozoa were hardly ever observed, whereas in the summer of 1951 the cultures were heavily infected by zooflagellates, ciliates, and amoebae. Because of their rapid growth, the zooflagellates caused most of the difficulty. In 2 to 4 days after infection, cultures became useless because of the contamination. The extent of contamination was clearly different in nonsterile cultures grown in tubes, in the open air, and in the greenhouse. Infection was heaviest in the greenhouse cultures, very trifling in rapidly growing cultures in tubes. Again it should be pointed out that the culture conditions were better in the tubes, especially because the thinner layers of the suspensions, as compared with those in the greenhouse tanks, provided better illumination. A parallel observation was that protozoa were hardly perceived in the sunny summer of 1950, whereas heavy infection occurred in the cloudy summer of 1951. These briefly outlined observations lead us to believe that Chlorella cultures are more readily attacked by protozoa, the more unfavorable the conditions become for growth of the alga.

Attempts to exterminate the protozoa by different means were practically without success. Use of "E 605 forte" 1 in low concentrations was partially successful; within 24 hours some cultures were freed of protozoa. This treatment, however, failed in some cases and caused a retardation of algal growth, the duration of the effect depending on the rate of evaporation of the E 605 forte.

Late in the summer of 1951 it was observed that the alga Scenedesmus had a greater resistance to protozoa than did Chlorella. These observations were proved correct by experiments with mixed cultures of Scenedesmus with Chlorella and flagellates or ciliates. Following this, Scenedesmus cultures were set up in the greenhouse tanks and have been in operation for three months. During this time practically no protozoa have appeared in these cultures, in contrast with the Chlorella cultures set up at the same time under the same conditions. Whether this result will lead to a final solution and whether Scenedesmus is adapted to such cultures cannot be said at present.

[1] "E 605 forte" is a dimethylfluorophasphate produced by the I. G. Farben as an insecticide. It causes an irreversible inactivation of animal nerve fibers.

E

Yields from Greenhouse and Open-Air Cultures

The preceding sections clearly show that many difficulties may arise with large-scale Chlorella cultures under the conditions described. These difficulties interfered with the original intention to investigate the economic utilization of nonsterile large-scale cultures. The figures given in table 2

Table 2

Yields of substance for Chlorella cultures in greenhouse (G) and open air (O)
(All figures based on dry weights)

		May	June	July	Aug.	Sept.	Oct.
Yield of dry substance (g/1/day)[a]	G	0.016	0.020	0.039	0.018	0.018	0.011
	O			0.055	0.040	0.029	0.030
Yield of dry substance (g/1)[a]	G	0.15	0.17	0.17	0.15	0.15	0.07
	O			0.27	0.21	0.24	0.22
Yield of energy (%)[b]	G	0.46	0.50	0.98	0.37	0.47	0.38
	O			0.48	0.41	0.45	0.52
Ash(%)	G	9.88	13.01	11.67	14.52	9.00	12.53
	O			18.07	12.92	13.47	13.62
Lipides without pigments (%)	G	2.49	2.52	4.41	1.98	1.77	2.22
	O			2.50	1.73	1.65	1.39
Chlorophyll (%)	G	5.30	4.97	4.12	4.45	4.30	4.25
	C			3.66	4.13	4.37	3.90
Protein (%)	G	48.20	48.20	49.20	48.70	52.70	**49.30**
	O			31.21	51.10	51.50	52.60

[a] The yield is the average value for a number of different cultures grown for varying numbers of days during any one month. The average culture time varied from 4 to 9 days.
[b] Yield of energy is referred to total radiation of all wave lengths received from sun and sky as measured with a Moll thermopile.

are for yields which were obtained under such difficulties in the summer of 1951 and are not to be considered normal yields. The figures show average daily yields calculated from the monthly yields. It is supposed that many factors disturbing the course of the cultures can be removed in the course of further development.

Yield of Organic Matter

In July the yield in dry weight of organic matter attained a maximum for both greenhouse cultures and open-air cultures, a result which corresponded to local light conditions. This maximum is more pronounced for greenhouse cultures than for cultures in the open air. The higher yields *in* open air are caused by the greater quantity of sunlight received, and by the higher ratio of illuminated surface to volume.

Utilization of Energy

The sun and sky radiation falling on a horizontal surface was compared with the energy content of the algal substance produced, as determined by heat of combustion. The figures in table 2 represent the percentage of the incident light energy recovered as heat by burning the dry plant substance. The heat of combustion checks within 1 per cent with the energy content calculated from analysis of the substance. The yields of energy from both greenhouse and open-air cultures were calculated on the basis of the sun and sky radiation in the open air.

Yields of Lipides, Chlorophyll, and Protein

The yields of lipides shown in table 2 were determined by exhaustive extraction of the dry algae with petroleum ether. The chlorophyll content was ascertained from the methanol extract of the fresh algae by saponifying the chlorophyll to potassium chlorophyllide, which was determined colorimetrically. Yields of protein were calculated from the nitrogen content of the dried algal cells.

This chapter describes the first steps toward operating large-scale Chlorella cultures in the open air under local conditions. Whether these conditions are suitable and to what extent the several deficiencies **can** be removed will be learned only by further investigation.

Chapter 12

THE ACCUMULATION OF LIPIDES BY ALGAE

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Many algae are known to accumulate lipides, but quantitative information about the extent to which different forms are able to do this is scanty. This chapter gives a preliminary account of work which aims at comparing the lipide-producing capacities of algae representing different groups. Such an investigation seems particularly desirable in view of the possibility that algae may be of economic value as producers of fat [54, 116, 206].

Direct comparison of the lipide production of different species is difficult. The lipide content of an alga may vary considerably during growth and according to culture conditions [55, 151, 177], and thus, if valid comparisons are to be made between different species, the material used should be grown under similar conditions and should represent equivalent stages of growth. The specialized growth requirements of many algae, however, make this ideal impossible to attain, since standard culture conditions favorable for one species may not suffice for another. Hence it seems that comparison between different forms can be only indirect, and that for each species lipide content should be determined in samples taken at different stages of growth.

Accordingly, in this investigation each species has been grown in the medium found to be most suitable for it, but culture conditions have otherwise been kept as far as possible the same. A sample for analysis has usually been taken at the end of tjie exponential growth phase, and others at different times subsequently. Since nitrogen nutrition has been found particularly important in determining the lipide content of Chlorella [151, 177], determinations of nitrogen content of the algal material have been made as well as determinations of lipide.

The following algae have been studied so far, each in pure bacteria-free culture: Chlorophyta, Chlorella vulgaris Beijerinck (Hopkins and Wann); Chrysophyta, Tribonema aequale Pascher (Pringsheim), Monodus subterraneus Petersen (Lewin); Cyanophyta, Anabaena cylindrica Lemmermann (Fogg), Oscillatoria sp. (Allen). The name of the. isolator is given in each case in parentheses.

The following culture media have been used: (1) solution C2 of Spoehr and Milner [151] with ferric citrate as a source of iron; (2) solution no. 10 of Chu [19] at double concentration; (3) solution no. 10 of Chu [19] with the concentrations of all constituents except nitrate doubled; (4) a solution without combined nitrogen [221]; (5) a modification of a solution recommended by Allen (personal communication) for the culture of Oscillatoria: $MgSO_4$- $7H_2O$, 0.25 g; KNO_3, 0.20 g; $Ca(NO_3)_2$, 0.025 g; KH_2PO_4, 1.00 g; Na_2CO_3, 1.50 g; NH4CI, 0.050 g; FeCl3, 1.25 mg; water, 1 liter; traces of Mn, B, Zn, Cu, and Mo.

Drechsel bottles fitted with sintered glass distributors and containing 400 ml of medium have been used as culture vessels, the cultures being maintained at 25.0° C in a mean light intensity of 650 foot-candles from an incandescent-filament lamp, and aerated at the rate of 10 liters per hour per culture with air containing 0.5 per cent CO_2. Suitable precautions have been taken to keep cultures uncontaminated, results from occasional contaminated cultures being discarded.

For the determination of lipide, the method of Belia [189], which is based on that described by Lemeland [244], has generally been used. This procedure involves preliminary extraction with ethanol followed by saponification, and results in the separation of "fatty acids" and an "unsaponifiable fraction."[5] For complete recovery of lipide it has been found necessary to saponify the residue as well as the extract. The possibility of omitting the preliminary extraction and carrying out the saponification directly on the material has been examined. The results obtained in this way with Chlorella and Anabaena are distinctly low as compared with those obtained with preliminary extraction, but in the case of Monodus greater proportions of lipide were obtained by direct saponification, and the results given below for this species were obtained by this method. Lipides have been dried to constant weight at 50° C in an atmosphere of oxygen-free nitrogen. Determinations of total nitrogen have been made by a conventional micro-Kjeidahl method using a selenate mixture as catalyst in the digestion.

The principal results obtained are summarized in table 1. Considerable differences in fatty acid content of the different species have been found, and the results confirm the generally held view based on qualitative observations that fat accumulation is characteristic of the Chrysophyta but not of the Cyanophyta. The content of unsaponifiable lipoid is more uniform. The somewhat lower values found for the two blue-green algae may perhaps result from the fact that members of this group lack sterols [207], which generally form a high proportion of the unsaponifiable fraction in microorganisms [239]. There appears to be no correlation between the amounts of the fatty acid and the unsaponifiable fractions either in any particular alga or when mean values for the different species are considered.

In Chlorella vulgaris and in the two representatives of the Chrysophyta there was an increase in fatty acid content as the cultures aged, and in Tribonema more fatty acids accumulated in the material grown in the

Table 1
Analysis of various algae grown in pure culture

Species and special culture conditions	Age of culture (days)	Total dry wt. (mg/1 medium)	N (% dry wt.)	Fatty acids (% dry wt.)	Unsaponifiable lipoid (% dry wt.)
Chlorella vulgaris	11	755	4.34	20.3	2.2
Medium 1 (2.25 m.-equiv. N/l); aeration with 2.5% CO2 in air	18	J1210 \1003	2.42 3.30	25.9 21.3	2.1 2.2
	25	1380	2.67	24.6	1.8
	32	1340	1.89	25.9	1.8
Tribonema aequale		J 417 \ 255	2.85 2.95	10.0 9.5	1.3 2.7
Medium 2 (1.0 m.-equiv. N/l) ,		I 597 \ 387	1.74 2.62	14.8 8.2	1.1 2.1
	[25				
Medium 3 (0-5 m.-equiv. N/l).........	f 12	f 450 \ 172	1.56 3.36	10.8 16.1	1.7 5.0
	I25	523	0.97	20.6	1.6
Monodus subterraneus	f 14.5	333	2.94	18.3	2.4
Medium 2 (1.0 m.-equiv. N/l).........	21	675	1.28	31.1	1.6
	I 27.5	f 510 X 758	1.45 1.22	21.8 34.4	1.2 1.7
Anabaena cylindrica	11	f 504 X 682	8.65 9.00	4.9 4.9	1.0 1.6
Medium 4 (no combined N supplied)...	23.5	J2100 J2182	7.76 7.14	4.7 4.4	1.6 1.3
	30	2190	6.95	4.0	0.8
Medium 4 without Mo, stagnant culture.............................	21	350	3.04	5.0	2.4
	34	680	3.88	4.5	1.7
Oscillatoria sp. Medium 5 (3."3 m.-equiv. N/l); aeration with 2.5% CO2 in air.........	21	142	7.42	5.5	0.5
	24	222	7.00	6.2	1.5
	36	f 699 l 608	7.38 5.65	4.4 4.4	0.7 1.2

medium containing a lower amount of nitrate. Such results are in conformity with those obtained with Chlorella pyrenoidosa [151]. In contrast, the proportions of fatty acids in the two blue-green algae were much the same in all samples examined. Even in material of Anabaena which was starved of nitrogen as a result of deficiency of molybdenum, an element necessary for the fixation of free nitrogen, which this organism is able to accomplish [221], the fatty acid content was not increased.

Such differences in accumulation of fatty acids seem best considered in relation to nitrogen content. In this consideration it will be assumed that the total amount of combined nitrogen in the cells is the factor of preponderant importance in determining lipide accumulation, an assump-

tion in accord with the available experimental evidence but almost certainly an oversimplification of the situation. Under conditions favorable for rapid growth, the products of photosynthesis in a simple alga are used chiefly for the synthesis of protoplasm, and the cells have a high nitrogen content but little reserve material [99, 117]. When growth is retarded, the primary products of photosynthesis may be diverted to the production of reserve materials, carbohydrate or lipide being formed presumably according to the relative activities of the enzyme systems concerned.

The amount of nitrogen available to the alga would appear to determine the amount of lipide accumulated in two ways. First, by limiting growth, deficiency of nitrogen causes photosynthetic activity to be directed to the synthesis of reserve materials, including lipide. Secondly, the ratio of lipide to carbohydrate formation may be altered as the nitrogen content of the alga changes. This might be brought about by changes in the relative amounts of components of the enzyme systems concerned. Other explanations are, of course, possible, but this supposition does not seem unreasonable in view of the finding that different nitrogen fractions in micro-organisms are affected to different extents by nitrogen starvation [290].

This second effect may be studied if the proportion of fatty acids in the total reserve material is considered to be a function of the nitrogen content of the organism. In figure 1 the fatty acid contents of the algae studied have been expressed as a percentage of the dry material other than protein (protein being taken as amount of nitrogen x 6.25). This procedure is not altogether satisfactory, since only total dry weight, including inorganic constituents, has been determined; but the error introduced does not appear to be important, since essentially similar results were obtained when the amounts of fatty acids were expressed as percentages of amount of fatty acids plus hydrolyzable polysaccharide. For comparison, some results for Chlorella pyrenoidosa [91] have been expressed in the same way and included in the diagram.

It will be seen that, compared at corresponding nitrogen contents, the algae studied differ considerably in their capacity to produce fatty acids, the two blue-green algae producing least and the two species of Chlorella producing most. The effect of variation in nitrogen content is also markedly different in the different species. In Chlorella vulgaris, at least within certain limits, the proportion of fatty acids produced changes little as the nitrogen content of the cells varies. In Monodus and Chlorella pyrenoidosa and probably in Tribonema a decrease in nitrogen content produces a considerable alteration in favor of fatty acid synthesis, whereas in Anabaena and possibly in Cscillatoria there is a less marked trend in the opposite direction. It thus appears that nitrogen deficiency affects the ratio of fatty acid to carbohydrate production to different extents in different algae, and the results for the two species of Chlorella suggest that such differences may exist *not* only between groups, but between closely related forms.

Since in Chlorella pyrenoidosa the lipide content has been found to vary between extremely wide limits according to the conditions under
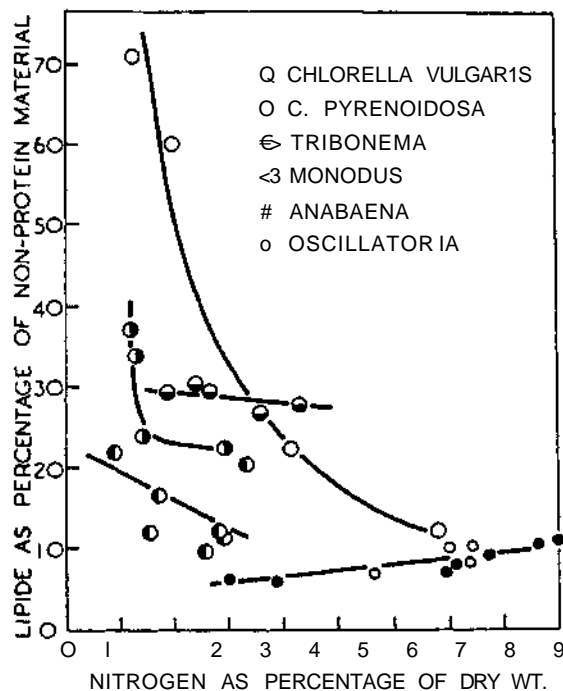
Fig. 1. Effect of variation in nitrogen content on the amount of lipide (fatty acids only), expressed as a percentage of dry material other than protein, produced by various algae.

which the alga is grown [151], and since various species of Chlorophyceae and a diatom have been found to contain approximately equal amounts of lipide after growth under similar conditions [68], it seems that the accumulation of lipide which is regarded as characteristic of certain algae may perhaps be dependent more on the environmental conditions under which such species habitually grow than on genetically determined peculiarities of metabolism (see, for example, [99]). The results reported in this chapter indicate that, nevertheless, there are considerable inherent differences in the capacity of algae to accumulate lipide.

Chapter 13

EXPERIMENTS WITH CHLORELLA AT JEALGTT'S HILL[1]

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In 1949 experiments were started at Jealott's Hill to investigate the possibilities of culturing unicellular algae as a source of food. The growth and composition of these organisms are influenced by many factors, most of which are interrelated. Determination of the optimum cultural conditions would involve considerable time and effort. Cur approach has therefore *been* somewhat arbitrary, the aims being to devise a technique which would give reasonably high yields of a product with a tolerably constant composition, and to determine the composition of the product and its value as a food [47].

This work has been conducted solely with Chlorella vulgaris var. viridis, but it is recognized that this may not be the most suitable alga.

Although no attempt was made to keep the cultures sterile, little difficulty was caused by contaminating organisms. Frequently ciliates were present, but, although they engulfed a considerable number of cells, they did not appear to reduce the yield.

A
Methods Ufed for Culturing Chlorella vulgaris

Culture Vessels

Chlorella vulgaris has been cultured in cylinders, in a battery of large tubes, and in large aspirator bottles in the laboratory, and outdoors in a tall Perspex[2] tank (table 1).

Medium

At the start of these investigations the following nutrient solution [213] was used: KNO_3, [2]-[538] & $MgSO_4 \cdot 7H_2G$, 4.93 g; KH2PC4, 2.45 g; sodium citrate,[3] 0.00294 g; $FeSO_4 \cdot 7H_2O$, 0.00278 g; distilled water, 1 liter. To this

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Table 1

Dimensions of vessels in which Chlorella vulgaris var. viridis was cultured

Vessels	Dimensions	Volume of medium in vessel
Cylinders	15 in. long, 1.75 in. in diameter	250 ml
Tubes	4.5 ft. long, 2.75 in. in diameter	4 1
Aspirator bottles	18 in. high, 10.5 in. in diameter	Varied
Perspex tank	4.5 ft. high, 1.5 ft. long, 4 in. broad	60 1

solution were added minor elements [160] in these concentrations, expressed as parts per million: Zn, 0.07; Si, 0.06; Al, 0.09; I, 0.01; B, 0.05; Cu, 0.002; Ni, 0.01; Na, 0.12; Li, 0.005; As, 0.01; Co, 0.01; Mn, 0.44; Mo, 0.02. Later, we found that when nitrate nitrogen was replaced by ammoniacal nitrogen rather higher yields were obtained, and that when ammonium nitrate was used the organism preferentially absorbed the ammonium ion. Accordingly, in all subsequent experiments the basal ingredients were: $(NH_4)_2HPO_4$, 1.652 g; KH_2PO_4, 0.749 g; K_2SO_4, 3.267 g; $MgSO_4 \cdot 7H_2O$, 4.93 g; sodium citrate, 0.00294 g; $FeSO_4 \cdot 7H_2O$, 0.00278 g per liter.

From the second day after inoculation, the pH of the medium, initially adjusted to 6.0, was readjusted twice daily and never allowed to fall below 5.0. In the earlier experiments potassium hydroxide was added, but later ammonium hydroxide was used.

Inoculum

There are four distinct stages in the growth cycle of Chlorella vulgaris var. viridis: a lag phase, an exponential phase, an approximately linear phase when the growth rate is less than in the exponential phase, and finally a stationary phase [47]. The yield was considerably increased by eliminating the initial lag phase in growth (table 2). This was done by

Table 2

Effect of age of inoculum on the growth rate of cultures of Chlorella vuigaris var. viridis

(The inoculum was grown in the same medium, under light of the same intensity, received the same amount of carbon dioxide, etc., as was used iii the culture vessels to which it was transferred.)

Stage of growth of inoculum	Age of inoculum (days)	Size of inoculum (mg/1 dry matter)	Concentration of cells in subculture after 2 days (mg/l dry matter)
Exponential	2	5	M0
Linear	3	5	230

inoculating with cells that were in the exponential growth phase. The size of the inoculum used in all our cultures was equivalent to 5 mg dry matter per liter.

Temperature

The optimum temperature for this strain of <u>Chlorella vulgaris</u> was found to be about 25° C. At 20 or 30° growth was very poor. In all the experiments described here, the temperature was maintained at 25±1° C.

Carbon Dioxide Supply

In the early work a 5 per cent CO_2 and 95 per cent air mixture was supplied to the cultures at the rate of 20 ml/l/min. This was not enough to prevent sedimentation of the cells. Later experiments showed that the gas flow could be reduced to 6.25 ml/l/min without altering the growth rate or the "crude protein*' or carbon content of the product.

To *get* an idea of the amount of carbon dioxide utilized, this same gas mixture was bubbled through cultures of <u>Chlorella vulgaris</u> in vertical tubes (see table 1), and the gas escaping from the tops of the tubes was collected and analyzed.. The results are summarized in table 3.

Table 3

Apparent utilization of carbon dioxide by <u>Chlorella vulgaris</u> var. <u>vlridis</u>

Amt. of gas mixture supplied to cultures (ml/l/min)	Apparent utilization of CO_2 (% of amt. added) incubation period (hr)														
	1	5	23	24	28	48	49	52	53	72	96	120	124	125	144
7.5	70	....	62	....	68	....	90	....	97	93	72	87	....	81	80
6.25	80	38	....	65	75	100	....	100	....	99	98	75	75	....	72

It appears that during the first few hours after inoculation of the cultures (5 mg/l of dry matter), a large amount of carbon dioxide dissolved in the medium. During the second and third days, when the organism was growing exponentially, practically all the carbon dioxide supplied was utilized. After the third day, when the organism grew more slowly, the rate of utilization of carbon dioxide declined slightly.

In the experiment where the gas mixture was supplied at 6.25 ml/l/min, 1.9 *g* of dry matter were harvested from each liter of culture solution after incubation for 6 days. Since it may be assumed that the culture solution was saturated with carbon dioxide at the end of the experiment, and since dry Chlorella cells contain about 47 per cent carbon, it appears that approximately 85 per cent of the carbon dioxide supplied was utilized, or, more correctly, only 15 per cent escaped to the atmosphere.

Recently 20 per cent CO_2 in air has been used instead of 5 per cent, the same amount of CO_2 being used in both cases. The growth rates of the alga were identical.

Light

It would seem that any large-scale algal culture unit would, for economic reasons, have to depend on daylight. In our tests in the laboratory, where other factors were being studied, it was of course necessary to use a constant source of light. Daylight fluorescent tubes were used.

The results of certain investigations suggest that high light intensities may be harmful [100]. In our experiments outdoors, it appeared that light intensities of 12,000 f.c. were not inimical to growth in agitated cultures.

Effect of Dark Periods

We compared the growth of Chlorella vulgaris in continuous light with that when the cultures were illuminated for different periods every day. ^
In all cases the light intensity was 1300 f.c. The results are given in table 4.

It appears that keeping the cultures in the dark for periods up to 16 hours every day had no deleterious effect. During the periods of darkness the organism ceased to grow, but it started growing exponentially once the light was switched on. Also it is interesting to note that the yield per liter per unit of light was increased. The nitrogen content of the cells was not affected by this treatment.

Energy Yield

The heat of combustion of dried Chlorella is about 550C cal/g. Rough calculations suggest that the "efficiency of utilization" of light in tubes 2.75 inches in diameter directly illuminated on one side at 1300 f.c, **
and giving a yield of 0.44 g/1 dry matter per day (about 15.3 g/m2/day), is about 20 per cent. 6

Depth of Culture

Shallow ponds or troughs are likely to be the least costly type of culture vessel, and it is important to know the optimum depth of culture in relation to capital and operating costs. (It is, of course, realized that optimum depth may vary with li^ht intensity.) A test was run in large aspirator bottles, illuminated from below only. The intensity of light available was rather low, viz. 1000 f.c. The results are given in table 5.

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^Similar experiments by Tamiya and co-workers are described in chapter V.—Jbd.

** Length of tube occupied by 1 liter = 10.3 inches. Area of tube illuminated was assumed to be *rl.

6 This efficiency of utilization is the same as the maximum reported by Wa&sink and co-workers in chapter 5.--Ed.

In this single experiment it appears that the yield of dry matter per liter was inversely proportional to the depth of solution exposed to light, therefore the yield of dry matter per unit area was not affected by the depth of the solution within these limits.

Table 4

Effect of intermittent light on growth of Chlorella vulgaris var. viridis

No. hours in light each day	No. hours in darkness each day	Incubation period (days)	Total illumination (days)	Yield of dry matter (g)	
				Per liter when harvested	Per 24 hrs illumination
24	0	5.8	5.8	1.7	0.29
16	8	6.8	4.8	1.7	0.35
24	0	6.0	6.0	1.7	0.28
12	12	12.0	6.0	1.9	0.32
24	0	6.0	6.0	1.5	0.25
8	16	13.0	4.3	1.5	0.35

Agitation

We found that bubbling the carbon dioxide-air mixture through the cultures helped to keep the cells in suspension, but alone it was not sufficient. A blast of air was therefore blown through the cultures in the cylinders and tubes for about 30 seconds at intervals of 30 minutes, and the culture in the Perspex tank was continuously stirred by blowing air through a ceramic filter in the bottom of the tank.

This agitation resulted in marked frothing. Several defoaming agents were tried, and of these Silicone DC200 (1 drop per liter) proved remarkably effective. It is innocuous to the organism. It is reasonable to expect that it would be more difficult to prevent sedimentation in shallow, horizontal troughs than in tall vessels.

Table 5

Effect of depth of culture on yield and nitrogen content of Chlorella vulgaris var. viridis

Depth of culture solution (in.)	Volume of culture solution (liters)	Concentration of cells (rag/1 dry matter)		N (%)	"Crude protein"[a] (%)
		Initial	After 8 days		
4	5	5	800	7.7	48.1
12	16	5	190	8,1	50.8

[a] "Crude protein" ˢ 6.25 x nitrogen content.

B

Yield of Dry Matter

In the early experiments the cultures were harvested when they were 6 days old, and the average yield of dry matter from cultures grown in the tall tubes was 1.9 g/1 in 6 days. Later a semicontinuous process was used, the aim being to maintain a fairly dense population growing exponentially in a solution whose nutrient content was kept nearly constant.

From the tall tubes (continuously illuminated at 1300 f.c.) a portion, usually three-quarters, of the liquid was run off each day. The cells were centrifuged out and the liquor returned with the necessary amount of extra nutrients.

The procedure with the Perspex tank (outdoors, illumination by sunlight) was similar except that the culture was harvested every second day. Production per liter of culture solution was lower than in the tall tubes because illumination was for not more than 16 hours a day and was sometimes poor, and the depth of culture was greater. The liquid in the tank had to be cooled (by water through a tubular glass coil) on very sunny days.

In these trials, the average daily yield of dry matter was 0.44 g/1 in the tall tubes and 0.3 g/1 in the tank.

C

Composition of Dry Matter

The average percentages of certain components found in Chlorella cells, harvested from cultures which had been growing for 6 days in the tall tubes, are given in table 6. The determinations were carried out on freeze-dried material, but all the results are expressed on a dry-weight basis.

Table 6

Composition of Chlorella cells

Component	Percentage	Component	Percentage
Moisture	5	Phosphorus	1.1
Nitrogen....*	8	Potassium	1.5
"Crude protein"	50	Magnesium........*	0.5
"True protein"	45.5	Iron	0.04
Ether extract	3.0	Sulfur	1.1
Ash	7.4		

Preliminary examination of products obtained by the semicontinuous process, where the nutrient status of the medium was kept constant, and of products from the batch process showed that the percentage of inorganic constituents (notably phosphorus and potassium) in Chiorella- cells was influenced by the composition of the nutrient solution.

The amount of "fat" extracted by ether alone was between 1 and 5 per cent, but ether extraction after dilute acid hydrolysis or straight methanol extraction removed about 20 to 25 per cent of the material. (Cf. [91].)

Preliminary results suggest that freeze-dried Chlorella cells contain more than 1200 ppm of beta carotene. It was estimated by the A.O.A.C. extraction procedure (alcoholic potassium hydroxide). Extraction of pigments from this type of material appears to be far from complete, for the results were found to be only roughly reproducible. They are probably considerably below the true figures.

D

Nutritional Value

Dr. Kathleen M. Henry, of the National Institute for Research in Dairying, Shinfield, Reading, Berkshire, fed our freeze-dried Chlorella to young rats. The diet fed contained 17 per cent Chlorella, which at this level had no ill effects on the animals. For comparative purposes, dried skim milk, dried brewers' yeast, and peanut meal were also included in the feeding trial. Groups of six rats were fed diets containing 8 per cent "crude protein/* and gains in weight in four weeks were recorded. These were expressed in terms of the protein efficiency ratio (grams gain in weight per gram protein intake), as listed in table 7.

Table 7

Protein efficiency ratio of Chlorella
and other sources of protein

Protein source	Efficiency ratio
Chlorella	1.84 ±0.11
Dried skim milk	2.83 ± 0.25
Dried brewers* yeast	1.69 10.10
Peanut meal	1.34^0.09

E

General Observations

It seems clear from our preliminary estimates that Chlorella would be too costly to produce solely for use as a food, and that economical production is likely to depend upon the possibility of using one or more of its constituents as a basic material in chemical manufacture, any residue being disposed of to best advantage.

It appears that the main characteristics of a plant for the mass culture of algae are:

(1) The process must use sunlight, as artificial light is likely to be too costly. The plant should be located in an area where the light intensity is sufficient to enable growth to occur for the maximum number of days per year.

(2) Clearly, for most economical production, the yield per unit area per day must be as high as possible.

(3) The culture vessels should preferably take the form of enclosed shallow tubes or troughs laid horizontally, as vertical vessels would be much more costly.

(4) Means of avoiding sedimentation of algal cells will have to be devised.

(5) Means of keeping the temperature close to the optimum for the particular organism used will have to be provided. The cost of this item may be reduced by locating the plant in an area with a mild climate or by selecting species or strains which will make optimal growth at reasonably high temperatures, e.g. 30-35° C. [7]

(6) Culture conditions may possibly vary according to the type of product (e.g., high protein, high fat, etc.) required.

(7) For some purposes it may not be necessary to dry the product. If it has to be dried, then the effect of method and temperature of drying on the properties of the product will have to be determined.

7 See the end of section B of chapter 4 for an account of a preliminary search for such a strain of Chlorella.--Ed.

Chapter 14

CULTIVATION OF COMPLEXES OF ALGAE WITH OTHER FRESH-WATER MICROORGANISMS IN THE TROPICS

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The industrial interest in the large-scale cultivation of microalgae which has been manifest in the United States and elsewhere since the publication of the work of Spoehr and his co-workers with the versatile <u>Chiorella pyrenoidosa</u> may justify the publication of work carried on for many years here in Venezuela on the cultivation of highly heterogeneous complexes of fresh-water microorganisms. The following narration of this work will reflect more the insistent struggle to develop a basically sound idea than the presentation of precise biochemical data. It is hoped, however, that the significance of these observations on the use of microalgal cultures in human nutrition will offset the comparative grossness of our methods and data.

A

The Maracaibo Lake Project

Need for Carotene

Our work at Cabo Blanco with fresh-water microorganisms is a sequel to the Maracaibo Lake Project; which dates back to 1932, and which contemplated the large-scale use of the phytoplankton of Lake Maracaibo for the extraction of vitamins, notably carotene, and for processing to obtain proteins. There was in the Scandinavian countries in the early thirties considerable interest in getting carotene at low cost for use as an additive product in margarine and for the winter feeding of dairy cows. Since it was known that many leaves equaled or excelled carrots as a source of pro-vitamin A, it was thought that some very abundant tropical foliage might be used as a raw material. Jorgensen undertook to examine a number of such materials in Venezuela in 1932 to get a rough idea of their relative carotene content. The method consisted simply in comparing the intensity of the orange-yellow tinctures obtainable from the dried material by extracting with gasoline and subsequently shaking out chlorophyll with an aqueous solution of caustic soda.

These crude tests were made along the shores of Lake Maracaibo, which often, especially in morning calms, shows large areas covered with very dense phytoplankton colonies held in suspension close to the surface. The idea occurred to Jorgensen that this practically unlimited supply of chlorophyllic material could be obtained as a concentrate by large-scale filtration of the water, and could then be reduced to a thick fluid, if a continuous process of centrifugation could be evolved. It could next be reduced to a powder on a vacuum drum drier and extracted with a suitable solvent to obtain carotene-rich lipides.

Collection from the Lake

Cn several trips along the shores of the lake, most often in dugout canoes, microalgal surface colonies on the becalmed waters were scooped up with a calabash dipper and filtered through a couple of old felt hats. The quantitative yield at the best was 1320 mg (dry weight) per liter of agua espesa. This term, which means literally "thick water," is used by the native fishermen to describe those parts of the lake water containing myriads of pinhead-sized colonies of microscopic algae within an inch or two of the surface. The "thick water" is dispersed somewhat by the trade wind that springs up after ten o'clock in the morning, and then the yield of algae is less. One collection from the agitated "thick water" contained about 400 mg/1, but 150 to 200 mg/1 was the usual yield.

When the dried material was extracted with topping-plant gasoline, solutions of lipides were obtained with an orange color up to 6 times as intense as the best from leaves. The microalgal chlorophylls did not go into this solvent, a fact which gave the material an advantage over foliage, from which chlorophyll was always obtained with the carotenoid complex. A method of isolating carotene and carotenoids was developed, through the discovery that the whole complex could be adsorbed on lead sulfide, leaving the colorless lipides in the solvent. Typical rhombic crystals of carotene were later obtained abundantly from similar natural microalgal material. The common yellow xanthophylls as well as red analogues from the Myxophyceae were also recognized and obtained in separate solutions.

To utilize the defatted material, it was planned to develop a continuous process of separating proteins from carbohydrates by passing a gas through a mixed solution of both and then passing the froth into petroleum ether or hexane over water. The proteins would remain colloidally attached in the petroleum ether layer, while the carbohydrates would go into the water. Accompanied as they would be by a small amount of protamin, the carbohydrates could be utilized in a fermentation industry or as culture medium for yeasts or fungi.

Government Concession

In the year 1933 the Venezuelan Government granted Jorgensen patents of broad scope for the preparation of phytoplankton for industrial purposes

and for the extraction of carotene from algae. A twenty-year exclusive concession for the industrial use of the phytoplankton on the southern half of Lake Maracaibo was signed between Jorgensen and associates and the Government in 1936, and it was ratified by the national Congress in 1938. Under this concession a tax was to have been paid only on the weight of material harvested from the lake and not on subsequent increase by cultivation. Therefore it was planned to multiply natural harvests of phytoplankton from Lake Maracaibo by pumping them into basins of fertilized water of proper pH and to use carbon dioxide from flue gases as a source of carbon. The cost of obtaining a tax-free yield in this way was to be compared with the cost of obtaining the same quantity of material by filtering a larger quantity of lake water, and paying tax on the whole amount.

At first the project was received with skepticism and even with ridicule in Europe and the United States. It was compared to getting gold out of sea water. Almost all biochemists considered fish-liver oils cheaper and better sources of vitamin A than the pro-vitamin carotene. Recent research at the time had indicated that vitamin A from animal sources was superior to the pro-vitamin in both human and animal nutrition, as it went more readily into hepatic storage.

A certain interest in the project had been shown by an American pharmaceutical manufacturing company as early as 1934, but by 1940 neither that concern nor any other was sufficiently interested in the nutritional or therapeutic possibilities of phytoplankton to want to engage in their development. In that year the chemical department of a large food industry expressed the view that the project was sound scientifically but without commercial interest. The concession was canceled by the Venezuelan Government in 1941, because the proposed industry had not been started within the two years stipulated, with an additional year of grace.

B

The Cultures at Cabo Blanco

The Maracaibo Lake Project had received considerable publicity in Venezuela and there was perhaps a certain perplexity in government circles as to why foreign and domestic capital had not come forward to develop it. In July 1941 Jorgensen received permission to maintain at the Cabo Blanco Leprosarium, at the expense of the Ministry of Health and Social Welfare, a fair-sized experimental culture of fresh-water microorganisms, and to test their nutritional value, in co-operation with Dr. Jacinto **Convit,** in cases of low general health among the patients. The nutritional use of the cultivated algal **"soups"** would at the same time permit observations as to the course of the disease in cases **that** were aggravated or stationary in spite of treatment with chaulmoogra oil.

It was decided to carry on the cultures in such a way as to simulate as nearly as possible the natural occurrence of fresh-water phytoplankton, which directly or indirectly sustains an almost endless variety of animal

life. It was thought also that the variety of nutritional factors would increase with the number of species.

Method of Culture

As culture vessels we used bowls made locally of unglazed baked red clay. They measured 40 cm in diameter at the top and 20 cm in depth, and their capacity was about 15 liters. Each bowl was placed on a cement pillar 1 m in height. Evaporation from-the porous sides kept the water at a temperature of 28° C, even though the bowls were exposed to direct tropical sunlight.

The water used was the municipal supply of the town of Maiquetia. It was slightly alkaline and calcareous and at the time was untreated for the destruction of microorganisms. It contained a variety of phyto- and zoo-plankton species. Microalgal bottom sediments from an open cement water tank were used as seed material, but algae from local mudholes were later obtained and cultivated to provide additional species for which our culture medium was favorable. Chroococcaceae and Oöcystaceae were frequently obtained from lichens by placing these in filtered earth decoctions to which a clarified broth obtained by boiling microalgal "soups" had been added. The aerobic fungus would proliferate at the surface, and the algal ex-helot, having been released from its captivity in the fungal hyphae, would multiply at the bottom or in suspension. The algae thus obtained, if capable of rapid multiplication in our medium, would be incorporated in the complex of the general cultures.

During the first two years we used solutions from a commercial 6-1U-8 fertilizer to maintain in the bowls a nutrient concentration about 1 per cent above the natural content. We have since, however, used various other types. Any natural water to which any commercial fertilizer has been added will sustain some sort of microalgal complex. (We are at present successfully using a commercial preparation known as "Plantabbs" together with ammonium acetate to produce very dense cultures of a complex rich in lipides.)

The fertilized water gave a slightly acid reaction. Liberal additions of clarified decoctions from well manured garden soil or barnyard topsoil were made to each culture vessel twice a week. The cultures were stirred twice a day with a dipper to keep the most phototropic species from shutting off the light from the species at the lower levels. The water lost by evaporation was replaced daily.

Microorganisms Present

The typical complex consisted of bottom growths of Myxophyceae, mostly Chroococcaceae and matted aggregates of slender filaments of Homocystineae with infiltered cells of spherical green algae. Oöcystaceae such as Chlorelia, Oöcystis, and Ankistrodesmus, and some Scenedesmaceae occurred densely in suspension. On the surface we generally had very heavy

proliferations of a strongly phototropic green euglenoid without a flagellum, but with a red eyespot close to the anterior tip. It moved by gyrating the anterior part of its exceedingly elastic body. It would encyst readily, forming a dark green motionless sphere. Its "chloroplasts" seemed to be Chlorella cells harbored in its cytoplasm.

Ciliates such as Colpidium and Vorticella and at times Paramecium were usually abundant among the algae. Rotifers were rare, but micro-crustaceans, such as the copepod Cyclops and several species of Cladocera, would be present from time to time, the former at the bottom and the latter close to the surface. On the sides of the bowls the larvae of a species of Chironomus would build their shelter tubes from algae caught in their silk-spin. The intense red color of these "blood wigglers," being due to hemo-globin, might indicate the presence of hematopoietic factors in the micro-algae on which they lived.

We called the entire material "plankton" to facilitate reference and by reason of its similarity to the natural complex of fresh-water microorganic life commonly known by that name.

A fair allowance for error must be made as regards our identification of algae. There is as yet no biologist in Venezuela with special training in microalgal taxonomy whom we could consult. It is quite possible that some of the microorganisms called by us Myxophyceae belong to groups difficult to classify because of characteristics that place them as transition forms between bacteria and algae.

Harvesting

For the purpose of harvesting, we took advantage of the natural descent of the cells to the bottom during the night. In the early morning, before the upward phototropic movement had started, we siphoned off from 80 to 90 per cent of the water and brushed the precipitated material from the sides of the bowls into the bottom. In this way, we obtained from each bowl every 9 to 15 days an average of 1.5 liters of thick "soup" containing about 2.5 per cent dry weight of microorganisms. The water siphoned off was refer-tilized and returned to the bowls after each harvest.

The net amount of cellular material in the daily harvest was estimated by drying 20 ml of the "soup" and weighing the residue. By the end of 1943, we had 200 culture bowls in use with a daily average of about 3000 liters of water. We harvested during that year a total of 8060 liters of "soup" con-taining an estimated minimum of 204 kg of microorganisms. One bowl, each harvest from which was dried on a framed glass plate 0.5 xn2 in area, gave a total of 1368 g in one year.

The cultures were from time to time invaded by Cladocera, and in such cases the water was clear after harvesting and had to be reinoculated, but ordinarily no reinoculation was necessary. We maintained emergency seed cultures, however, in several extra bowls. The water lost by evapo-ration in these was always replaced with fertilized earth decoctions, and they were given carbon dioxide in the form of ordinary soda water. It was

observed that stale soda water excelled the gaseous product as a stimulant for reproduction, perhaps because of the greater ability of the water to retain carbonic acid. The "soups" harvested from these bowls at monthly intervals were of extraordinary volume and thickness. Harvests of 4 liters of "soup" with 45 g dry weight of microorganisms to the liter were commonly obtained. This gave us a rough idea of what might be produced in large culture basins of appropriate construction with the use of earth decoctions, a suitable fertilizer, and carbon dioxide.

Administration to Leprous Patients

During the years from the beginning of 1942 to the end of 1946 these plankton "soups" were used as accessory food for leprous patients. The harvested "soup" was boiled for about 20 minutes and a little salt was added to improve the flavor. The patients drank it willingly. The taste, which was not unpleasant, varied with the species complex.

The patients before receiving the "soup" were in poor general health and in an advanced stage of lepromatous leprosy. They were of all ages: 37 were from 8 to 20 years, 26 from 20 to 40 years, and 17 from 40 to 70 years. The daily dosage of plankton "soup" was 400 ml for the children and 600 ml for the adults. The length of treatment for an individual varied from one to three years.

Observation of the effect of the administration of the plankton "soup" naturally was interfered with by the well known spontaneous variation in the condition of leprous patients. Certainly no ill effects resulted from it. In a majority of the cases there was a marked improvement in energy, in weight, and in general health, all of which seemed to result from the plankton "soup" rather than from any other factor in the treatment of the patients. It was not possible to arrange a control group of patients that would match those fed the plankton "soup." The control had to be the record of the patients prior to treatment and the general experience here that lepromatous leprosy does not improve spontaneously in children.

In some cases of severe anemia and emaciation we employed a concentrate, obtained by boiling the ordinary "soup" down to about one-third of its volume to contain about 7 per cent dry weight of microorganisms. An outstanding case was that of a 33-year-old woman in this leprosarium, whose weight increased from 41 to 57 kg during one year when she received 500 ml of the concentrate daily.

Current Research

Here at Cabo Blanco we are now endeavoring to produce large quantities of concentrates of a certain fraction of microalgal lipides which seems greatly to accelerate the leprous reaction and the passage of active lepromatous lesions to cicatricial forms* Recent observations during the experimental treatment of a previously untreated case of very severe lepromatous leprosy in a 12-year-old girl point toward the possibility that the active

microfactor involved is a strongly hypophasic red carotenoid from the Myxophyceae. The production of this pigment by these microalgae is greatly increased when they utilize ammonium acetate as a source of carbon.

The pigment will be tried preferentially in a group of nonlepers who have been proved by a negative Mitsuda test to be naturally susceptible to the disease. Should this test change to positive after administration of the carotenoid during a certain period, it might be possible to prevent the spread of leprosy in countries where it is endemic, by giving to susceptible persons a natural resistance factor which they are, perhaps hereditarily, unable to produce in their skin tissues from common carotenoid precursors.

Chapter 15

EXPERIMENTS ON CULTURE OF ALGAE IN ISRAEL[1]

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A

Introduction

It is intended to describe briefly the work carried out on the culture of algae in Israel during the period March to December 1951, as well as the application of these results in the setting up of a small-scale pilot plant which will permit assessment of the method developed for mass production of algae under the prevailing economic conditions in Israel. The reason why this work is being undertaken here may be briefly recalled.

Economic Considerations

We have in Israel a situation of very limited areas of arable land, a restricted supply of water for irrigation, and a rapidly expanding population. These factors combine to create a severe shortage of domestically produced food, and, as a result, there is a high expenditure for the import of food to maintain a minimum standard of nutrition. Cattle feed also must be imported, because of the shortage of green fodder.

Climatic conditions include a year-round availability of sunlight (an average of 9 hours per day) and equable temperature conditions. The necessity of producing high yields per unit area by the efficient utilization of sunlight, resulting in freedom from dependence on large tracts of land, made the idea of mass culture of algae extremely attractive. Although the production cost of Chlorella must bear some relation to that of other domestically grown foodstuffs, the significant point in experiments here is that any local production will result in a saving of foreign currency even if the domestic price as such may be slightly higher than the world market. Therefore, a comparison with other products such as is given by

Cook in the Stanford Research Institute report [279J, for example, is not a decisive factor here.

For these reasons, it was decided to investigate the simplest possible method of mass culture of algae. The particular points on which to concentrate were as follows: the most uncomplicated set-up for the culturing of algae (again taking into account the saving of foreign currency in obtaining materials for the building of large-scale production plants), and the factor of re-use of the culture solution (resultant saving of water).

B

Chlorella Culture

The work was started on the alga Chlorella pyrenoidosa after a very cursory examination of C. vulgaris and of species of Scenedesmus and Stichococcus had shown that they all had much lower growth rates than the former and, therefore, were unsuitable.

Laboratory Set-up

Experiments to date have been conducted as follows: The algae were grown in large Erlenmeyer flasks (6 liters) containing 3 liters of culture solution. These flasks were placed on a south balcony so that they received, generally speaking, direct sunlight on about a third of their total surface (on their sides, not their tops) during the hours in which there was sunshine. The external area of the cultures which could theoretically receive light was 460 cm^. The area on the top of the cone which the cultures constituted was 175 cm^. As the light was from the side and illumination on a conical vessel is uneven, it is estimated that some 250 cm2 received direct sunlight. The variation of growth witfi the amount of sunlight was not investigated. For purposes of continuous stirring, an air stream was passed through these flasks at a rate of 300 liters of air per liter of medium per day. Carbon dioxide was provided at the rate of about 5 per cent of the air stream. This was not measured accurately, as the amount was in any case well above the requirement and served to keep the solutions charged with carbon dioxide.

It was found that the carbon dioxide (from cylinders filled here), if unwashed, was extremely toxic to the algae because of the presence of sulfur dioxide and hydrogen sulfide, which resulted in the death of the cultures in 1 to 1| days. The gas had to be washed by passing through alkaline permanganate. (This may be an important factor in large-scale work.) About 20 liters of solution were used in each run of the experiments.

Solutions

The culture solution finally used was as follows: 3.0 g (NH^SO^ 2.5 g KH2PO$_{4?}$ and 1 g MgSO$_4$ • 7H$_2$O per liter, made up in Jerusalem tap

water. (The latter contains a high percentage of chloride, 80 ppm, and 1 ppm free chlorine, besides having a high calcium carbonate content, about 65 ppm.,) To this were added iron and manganese at the rate of 2 drops per liter of saturated solutions of $FeSO_4$ and $MnC^$. The addition of other trace elements did not affect the growth rate.

Contamination

Apart from the sterilizing of the culture vessels, no precautions were taken against contamination, and the air stream was unfiltered and as heavily infected as is normal in Jerusalem. Over a period of more than half a year, contamination of the cultures was very rare and did not affect the growth rate. Contamination, when it occurred, was of two kinds: bacterial and jprotozoal. In no case was there contamination by foreign algae (with a single exception, when a culture became heavily infected by Chlamydomonas; but after a week the Chlorella had entirely supplanted the foreign alga).

Yields

By the means described, cell concentrations of 80,000 to 100,000 cells per cubic millimeter were obtained, corresponding to an average yield of O.7 to 1.0 g/1 in periods of 7 days to 3 weeks. (Yield is defined as the weight of Chlorella dried at 85° C, per unit volume of 1 liter.) No attempt was made to raise the population density above $1X1Q5$ cells/mm^. (This density corresponded to 1.0 g/1 dry weight.) The length of the growth period depended on the initial size of the inoculum, prevailing temperature and light conditions, and other variable factors. Temperature was a particularly important factor. On "khamsin" days, when the temperature of the cultures rose to 40° C or more at midday, growth was inhibited not only on that day but on a number of subsequent days, but the cultures were not killed.

Under these general conditions, the yield (i.e. increase in dry weight per day) was found to have a maximum of 0.4 g/l/day, with the average throughout these experiments falling between 0.1 and 0.2 g/l/day. The latter rate could be repeated more or less indefinitely, and occurred when the cell concentration was at about 40,000 cells per cubic millimeter. For the assumed area of illumination of 250 cm2 per flask containing 3 liters of solution, a yield of 0.1 g/l/day corresponds to 12 g/mVd&y.

Summarizing the above, it can be stated that if a light utilization can be achieved that is three times as high as the present, either by continuous Illumination or by exposing the entire culture instead of only one-third of its surface to the light, under these extremely simple conditions a yield of O.5 g/l/day can be expected. This presupposes a continuous harvesting system which will maintain the cell number at the above-mentioned optimum.

No continuous culture experiments were carried out except to verify the finding that harvesting at the 0.7 g/1 stage to half the concentration led to

continued growth at the optimum rate, without toxicity in the medium and
without its exhaustion. No drop in pH was observed, presumably because
of the buffer action of the phosphate. No signs of trace-element deficiency
were detected.

It is known that workers in the United States, e.g. Myers and co-workers
[161] and those working with the Carnegie Institution of Washington, have
obtained much higher daily and total yields. [2]   What is to be stressed about
the results obtained here to date is that they were obtained under the sim-
plest possible conditions without attempts to increase the surface-volume
ratio, which is a crucial factor in determining rates. No attempt was made
to control climatic or septic conditions.

Large Tanks

The results described have led to the design of larger-scale tanks with
the primary aim of producing under simple conditions amounts of algae
sufficient for feeding experiments. These tanks are to be an intermediate
step in expanding the size of culture units.

The tanks to be used are 135 cm long and 35 cm wide, with the maximum
depth of liquid 25 cm. Illumination for these tanks is to be overhead sunlight
in a greenhouse, supplemented by overhead artificial illumination at the rate
of 300 watts to each tank. Circulation of the water will be effected by a
mechanical stirrer, designed to suck water from one half of the tank and
move it into the other half over a partial partition. This will at the same
time aerate the water and ensure dispersal of the carbon dioxide to be
introduced into the stirrer. In these tanks it is hoped to have a steady pro-
duction at the rate of 0.5 g/l/day from some 500 liters of culture solution.

C

Projected Pilot Plant

A pilot plant is to be constructed, taking into account the above observa-
tions as well as the results of the workers of the Carnegie Institution.

Evaluation

Calculation and economic evaluation of these workers' results have
indicated to us that shallow-layer plants are not a practical solution of the
problem for Israel. Climatic factors in this country are such that shallow
layers either on land or on a water surface would undergo temperature in-
creases which would be inhibitory to growth if not lethal to the algae.

Also, a shallow-layer plant would largely do away with any saving in
area* For large-scale production in an industrial process the ratio of
surface to production would be quite unfavorable. From preliminary

[2] For example, see chapter 9.--Ed.

figures 3 for the yield from the 1951 Chlorella pilot plant at Arthur D. Little, Inc., we calculated that for the production of 30,000 tons yearly (a starting figure for Israel) the area required would be 6 km2 (2.3 square miles), which is not a workable unit for Israel.

Deep-Culture Plant

We therefore envisage a pilot plant having the following general requirements. The plant must be constructed on dry land. It must have a minimum depth of 1 m. The system is to be septic. The introduction of carbon dioxide into such a plant must have optimum efficiency, i.e. high dispersal, because of the relatively high cost of carbon dioxide.

Having the tank as deep as 1 m serves two purposes. In the first place, it makes it possible to utilize high-intensity sunlight more efficiently by lighting in depth, as described in the next subsection. In the second place, the large ratio of volume to surface area will decrease the heating of the culture by infrared solar radiation. It is hoped that this effect will be large enough so that artificial cooling will be unnecessary.

In view of relatively low electricity costs here, it may be permissible to use additional illumination during the night, but sunlight must be used as the chief source of energy. This must be done either by introducing it into the deeper parts of the tanks or by bringing the algae into constant contact with the light. The latter may be feasible if Warburg's observation (see [197]) stands, that the light-dark alternation is not a matter of fractions of seconds, but rather of minutes. In that case mechanical stirring may provide the answer to the problem.

Pilot-Plant Set-up

A double tank of 2 m^ capacity with an area of 2 m2 is being built. Stirring is to be effected by two perforated plates of 1 m^ each, one for each tank. These plates are alternately lifted 1 m high, and while one descends through the liquid to the floor of the tank it lifts up the other one by a pulley arrangement, so that the electric motor which moves the pulley has only to furnish power to take care of friction*losses.

The lighting in depth will be provided by vertically positioned tubes, spaced in alternate rows of 5 and 4 tubes each, all in all 5 rows or 23 tubes per square meter. At first these tubes will contain fluorescent bulbs of 40 watts each. The tank as such will be covered on top by a transparent plate for the double purpose of preventing escape of carbon dioxide and of collecting light.

Later an attempt will be made to reflect sunlight into the depth of the tank by using collector lenses, produced by suitable impressions in the transparent cover plate. The tubes will then not contain the fluorescent bulbs, but slender silver-papered cones with their base at the bottom of

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[3] The yield used (12 $g/m^2/day$) is somewhat higher than the final values presented in chapter 17. —Ed.

the tube and their highest point at the level of the liquid in the tank. The light bundle coming from the collector lens of the cover plate will be reflected into the liquid over the whole length of the tube. Such tubes of 9 cm diameter will provide, together with the top surface of the liquid, a light-exposed area of 7 m^ in each tank.

A collecting-lens surface of 18 cm diameter (254 cm2 area) over each tube will distribute the incident light over a vertical tube surface of 2700 cm2. If the optics of the system are made such that the light reflected laterally is of uniform intensity from the top to the bottom of the tube, this arrangement will result in a diminution of light intensity in the ratio of 1 to 11.

Under these conditions the light intensity in the liquid at the surface of the vertical tubes will be less than 400 foot-candles as long as the sunlight incident on the collector lens is less than 4400 foot-candles. This condition will be true for a large fraction of the daylight hours in Israel. Then none of the light will be wasted by being absorbed by algae at an intensity higher than the saturation intensity. 4

The distance between tubes, furthermore, is great enough so that the mutual shading of the cells in a moderately dense culture will allow for complete absorption of light at higher intensities. There will be some loss of efficiency, to be sure, because the cells closest to the vertical tubes will absorb the light at an intensity higher than 400 foot-candles; but this loss wili take place only during the shorter fraction of the day when the light intensity is higher than 4400 foot-candles, and the loss will not be nearly so great" as it would be if the same amount of sunlight were shining directly on the 1 m2 of the top surface of the tank.

Assuming a low average yield of 14.3 g per square meter of exposed area, each square meter of tank area should produce about 100 g (7x14.3) during the 12 hours of daylight. During the absence of daylight, additional light will be provided by sources of artificial light on top of the cover plate, and the yield will be increased correspondingly.

One tank is being built of stainless steel for experimental purposes, and simultaneously a concrete prototype will be set up. This concrete model will have a plastic lining consisting of a sprayed polyvinyl coating.

The tanks will be tried under local conditions, and an accurate cost evaluation will be made.

Laboratory Experiments

In addition to the above large-scale experiments, it is intended to pursue laDoratory work already started on the effect of various growth substances on the algae. The purpose of this work is to determine whether the growth rate can be increased, and, simultaneously, in what concentrations a selective herbicide like 2,4-D can be used to prevent secondary algal

* The value of 400 foot-candles for the saturation intensity in sunlight is based on the discussion by Myers in section B of chapter 4.—Ed.

miections. As far as the latter is concerned, preliminary studies show that Chlorella is fairly tolerant to 2,4-D, so that such treatment may be an important factor. Lastly, it is hoped to study a large diploid Chlorella, obtained by camphor treatment of the algae by Pearsall (unpublished data), which is said to have a division rate identical with that of normal Chlorella and may, therefore, improve the yield per unit time.

Chapter 16

KINETICS OF GROWTH OF CHLQRELLA, WITH SPECIAL REFERENCE TO ITS DEPENDENCE ON QUANTITY OF AVAILABLE LIGHT AND ON TEMPERATURE[1]

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A

Introduction

The rate of growth of algae is dependent on four main factors: (1) quantity of available light, (2) temperature, (3) composition of nutrient medium, and (4) availability of carbon dioxide. In algal mass culture outdoors, factors (3) and (4) may be controlled relatively easily, whereas factors (1) and (2) are subject, to a great extent if not entirely, to natural variations occurring both diurnally and seasonally. In order to obtain quantitative information as to the possible yield of algae in mass culture, it is, therefore, important to scrutinize quantitatively the dependence of algal growth on the quantity of available light and on temperature. It was with this purpose that the following kinetic studies were carried out

B

Methods

The experimental organism was Chlorella ellipsoidea, and the culture medium used in most of the experiments was of the following composition, per liter: KNO_3, 5.0 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $KH2PO4$, 1.25 g; $FeSO4 \cdot 7H2O$, 0.003 g; Arnon's A4 and B7 solutions [183], 1 ml each. The pH was 5.3-5.4. (Microelements, in ppm: Fe, 0.6; B and Mn, 0.5 each; Zn, 0.05; Cu, 0.02; Mo, Co, Ni, Cr, V, W, and Ti, 0.01 each.)

In the mass culture which was run outdoors, a medium containing urea as nitrogen source, ethylenediamine tetraacetic acid (EDTA) as chelating agent, and Hutner's mixture of microelements (as cited by Myers et al. [104]) was used. This medium contained, per liter: urea, 3.0 g; MgSO4 • $7H_2O$, 2.5 g; KH_2PO_4, 1.25 g; $FeSO_4$ • $7H_2O$, 0.003 g; EDTA (di-sodium salt), 0.037 g; Hutner's solution [104], 1 ml. The pH was 5.2. (Microelements, in ppm: Ca, 30; B, 20; Zn, 20; Fe, 10.6; Mn, Mo, and Cu, 4 each; Co, 1.)

These two media are called in the present chapter "nitrate-A" and **"urea-EH"** medium, respectively.
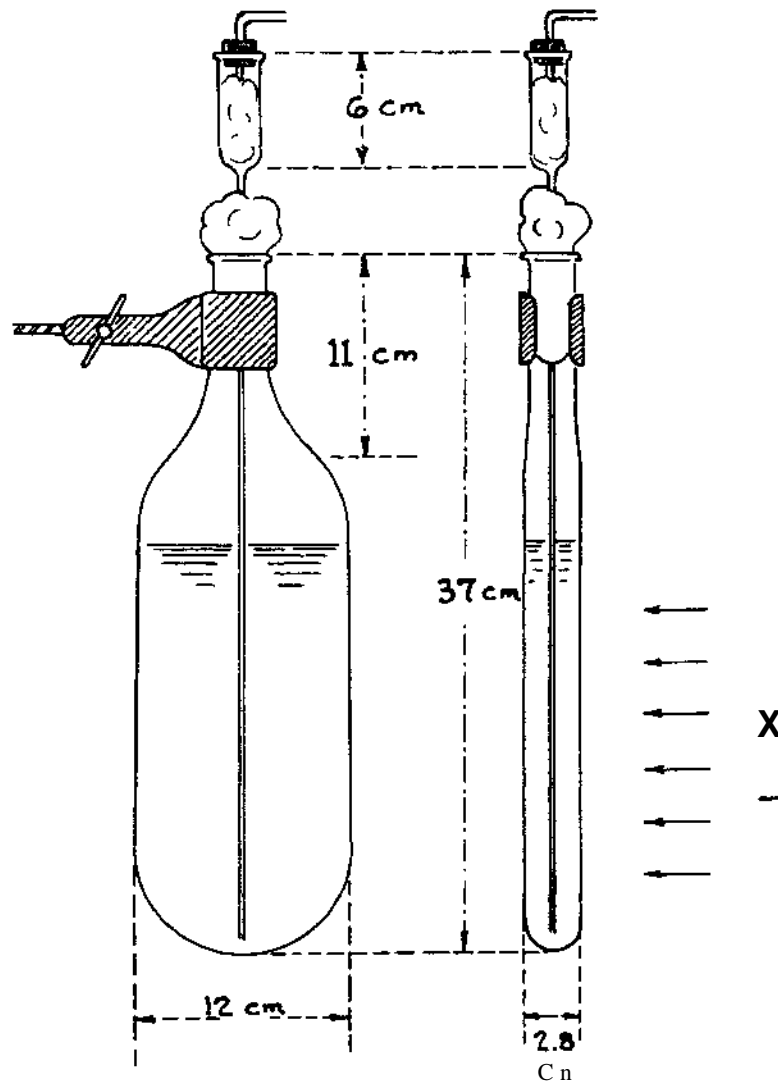
Fig. 1. Front and side views of the culture flask

In all experiments except the outdoor mass culture, the culture chamber illustrated in figure 1 was used. It is an oblong, flat flask with a uniform inner thickness of 2.8 cm and a total capacity of 600 ml, and has an opening with a cotton plug carrying a long glass tube, the lower end of which almost reaches the bottom of the flask. The upper end of this tube is widened and filled with cotton to serve as an air filter. In the upper opening of the tube is fitted a rubber stopper carrying a glass tube which serves to conduct the carbon dioxide-enriched air into the culture medium.

The flask was filled with 500 ml of culture medium and autoclaved. After seeding with algae, the main body of the flask was immersed in the water of a thermostat having glass walls, and from outside the thermostat the culture was illuminated in a direction perpendicular to the flat surface of the flask. The algal suspension was constantly aerated with air containing 5 per cent CO_2 at a rate of 200 to 300 ml per minute, which also served to homogenize the suspension during the experiment; at this rate of aeration the agitation of culture medium has no significance in bringing about growth acceleration by causing intermittent illumination of cells. Before entering the culture flask, the carbon dioxide-enriched air was brought to the same water vapor pressure as the culture medium by being scrubbed through gas washing bottles containing a solution having the same composition and temperature as the culture medium.

Illumination was furnished by a projector lamp or reflector flood lamp placed outside the thermostat. These lamps were operated at 70 to 85 volts, a voltage stabilizer and variable transformer being used. Light intensity was checked and regulated two or three times a day, by use of a photometer having a submersible photoreceiver. The light intensity at the surface of the culture flasks ranged from 140 to 50,000 lux (1 lux = 0.0929 f.c). The temperatures studied were 25°, 15°, and 7° C.

The growth of algae was followed by measuring the packed volume of cells after centrifuging. Except when otherwise stated, the culture medium was replaced every day so as to keep the composition of the nutrient solution constant throughout the experiment. For this purpose the whole cell suspension was centrifuged, the solution was discarded, and the cells were resuspended in a measured amount of fresh solution such that the concentration of cells was equal to that in the previous suspension.

Outdoor culture was conducted by means of apparatus which is illustrated schematically in figure 2. The culture, 40 liters in total volume, was circulated in a series of glass tubes 3 cm in diameter, connected with rubber tubing and submerged in water in a trough, and exposed to sunlight. The total length of the illuminated portion of the tubes was 33.3 m. The trough water served to prevent overheating of the culture on sunny summer days. The culture was circulated in the system by a motor-driven pump at a linear velocity of 15 to 30 cm per second; and by means of a gas-exchange tower (placed indoors) it was aerated with carbon dioxide-enriched air supplied at a rate of 1.0 to 1.5 liters per minute. The pump and the glass tubing were connected by iron tubes, and the inner surface of the pump was partly of iron and partly of Babbitt metal, both of which were found to be

INLET FOR NEW MEDIUM

5%CO$_2$
IN AIR

GAS OUTLET ←

SUNLIGHT

COOLINGWATER

t

PUMP

WOTO/?

SAMPLING
OUTLET
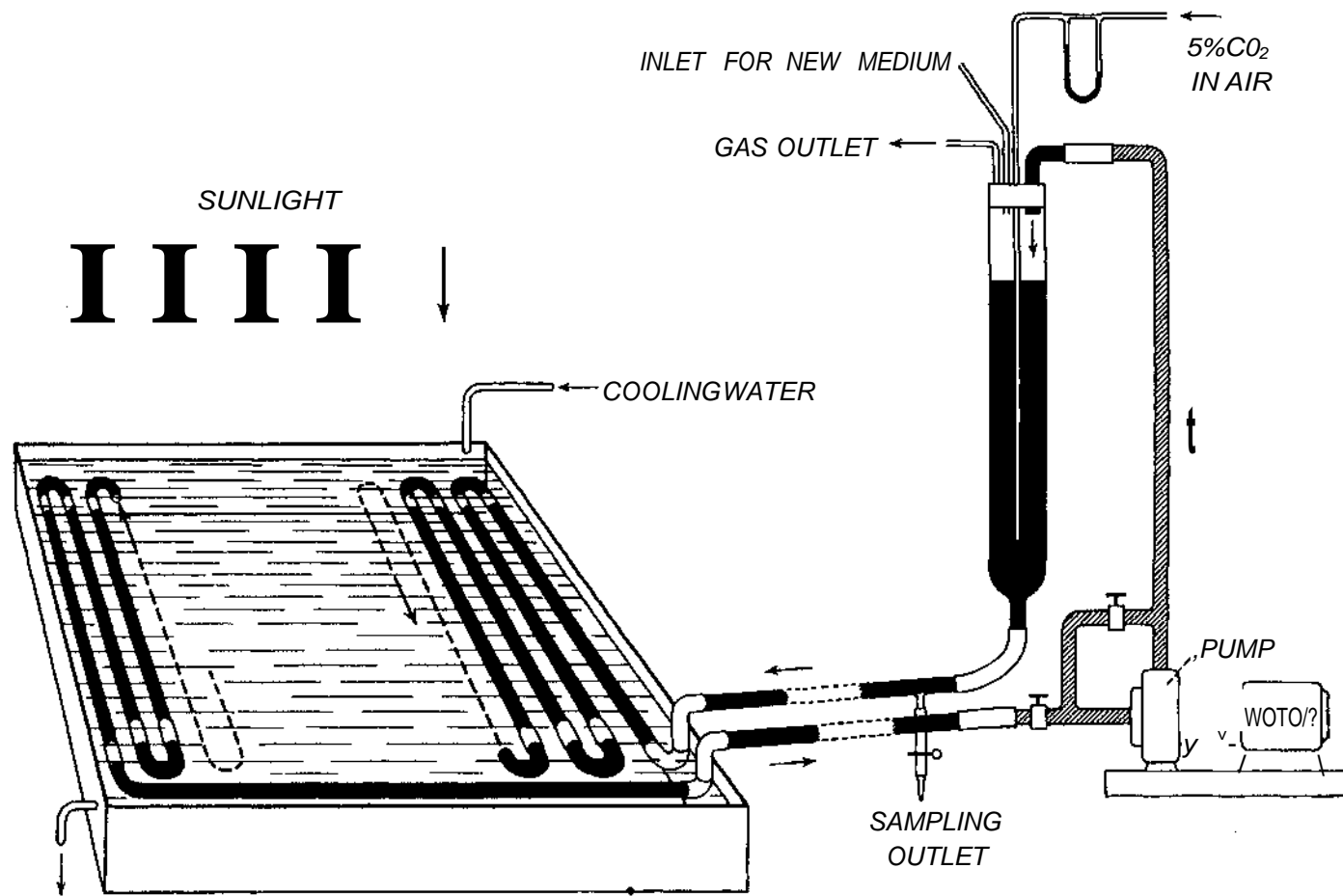
Fig. 2. Schematized representation of the outdoor culture apparatus

noninjurious to algae, at least when the culture medium contained a chelating agent such as EDTA. About 10 liters of culture was taken out every day or every other day and replaced with fresh medium so as to accomplish the turnover of medium in about 7 to 10 days.

The quantity of sunlight was measured with a Mazda "Integrating Luxmeter/' which recorded the daily quantity of solar energy (in the visible region) in terms of kilolux-hour. The photoreceiver of the luxmeter was placed in the open air, with its opening in a horizontal position, beside the water trough of the culture system. The meter was calibrated occasionally, using an incandescent lamp of standard intensity. The actual light intensity at the submerged position of the glass tubes (about 3 cm below the surface of the water in the trough) was 64 per cent of that measured in open air with the luxmeter.

C

Results

Exponential and Linear Phases of Growth

When a culture of algae is started with a sufficiently small inoculum so that only a small part of the incident light is absorbed, and the algae are allowed to grow under constant incident light and constant temperature in a culture medium that is also kept unchanged ("chemostated"), growth generally proceeds in two distinct phases. Typical examples of the phenomenon are given in figures 3 and 4. The two figures show the same experimental results, which were obtained with cultures (in "nitrate-A" medium) provided with different light intensities, 800, 2000, and 5000 lux (temperature, 25° C). In figure 3, the course of growth is illustrated by plotting the packed cell volume per liter (V) and its daily increase ($\Delta V/\Delta t$) against culture days, whereas in figure 4 the logarithms (Briggsian) of V and their daily increase are plotted against culture days.

It is obvious that for each culture $\Delta \log V/\Delta t$ is constant at earlier stages, whereas $\Delta V/\Delta t$ becomes constant at later stages, and that both these constant values are dependent on light intensity. In the following we shall call the earlier growth phase, in which $\Delta \log V/\Delta t$ is constant, the "exponential phase," and the later growth phase, in which $\Delta V/\Delta t$ becomes constant, the "linear phase." When the inoculum consists of cell samples taken from old cultures, the culture very often shows the so-called "lag phase" before entering the exponential phase. This phenomenon does not occur when the inoculum consists of active cells taken from fresh cultures. In all experiments presented here, the appearance of a lag phase was excluded by use of active cells.

In figure 5 are shown the linear relations of logV and time in the exponential phase, and those of V and time in the linear phase, at higher light intensities, 10,000, 25,000, and 50,000 lux (temperature, 25° C). Worthy of notice is the fact that at these higher light intensities the value $\Delta \log V/\Delta t$
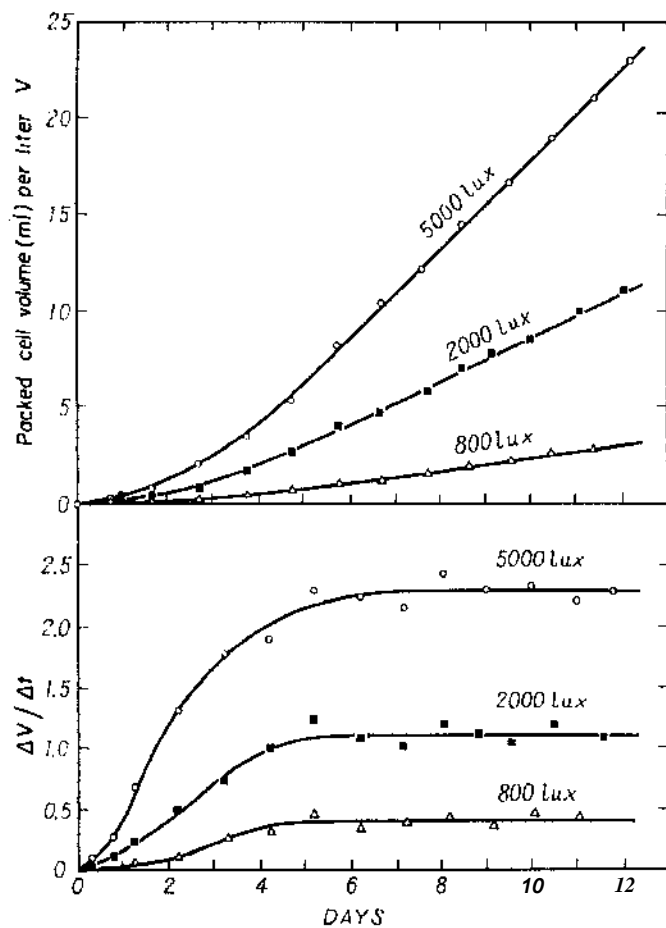
Fig. 3. Increase of cell concentration (V) and change in growth rate (AV/At) during the course of culture at three different light intensities, 800, 2000, and 5000 lux. Temperature, 25° C.
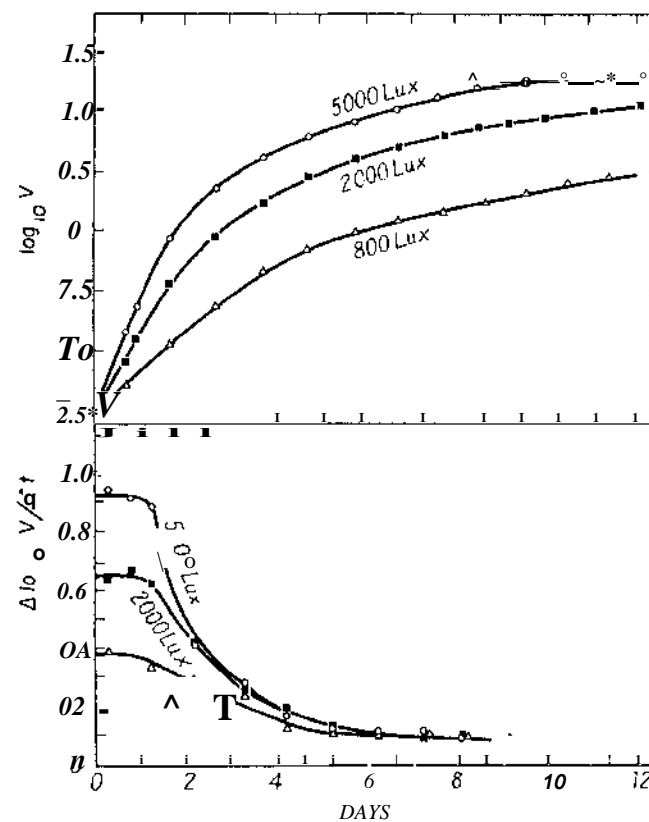
Fig, 4. The same data as in figure 3, presented in terms of logarithms of cell concentration ($\log_{10} V$) and their daily increase ($\Delta \log_{10} V / \Delta t$).
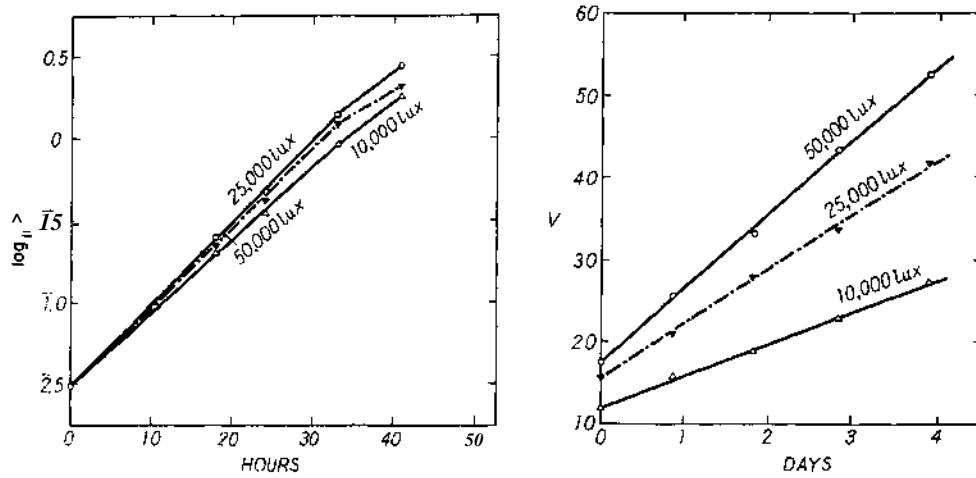
Fig. 5. Growth curves obtained at 10,000, 25,000, and 50,000 lux. Courses of growth at low population densities are presented on a semilogarithmic scale; those at high population densities are plotted on a linear scale. Temperature, 25° C.

during the exponential phase was almost independent of light intensity, whereas AV/At in the linear phase was found to be still largely dependent on light intensity.

Dependence on light intensity of AlogV/At at lower population densities and of AV/At at higher population densities is shown in figure 6. It will be seen that the value of AlogV/At soon becomes "saturated" at higher light
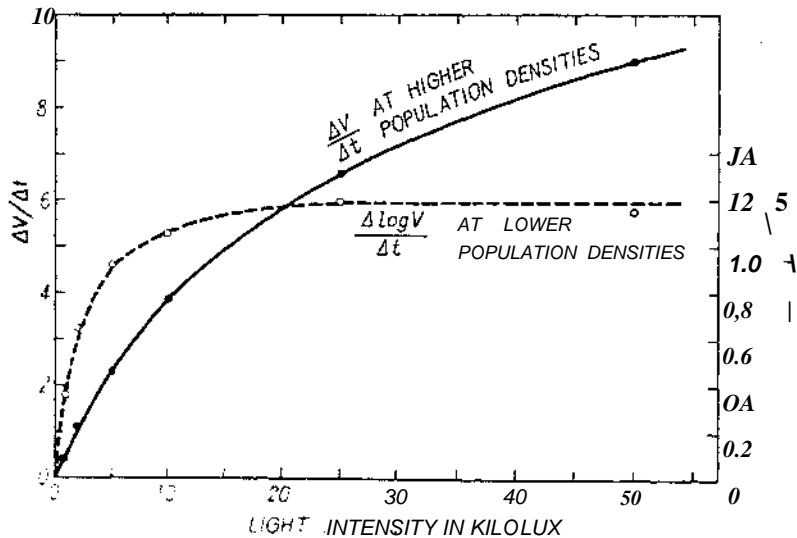
Fig. 8. Dependence of exponential and linear rates of growth on the intensity of incident light. Temperature, 25* C.

intensities, whereas no such tendency is found in the value of AV/At ob-
served at higher population densities.

   The curves presented in figures 3 and 4 show that the transition from
the exponential phase to the linear phase occurred at different population
densities according to the light intensities applied, and indeed that the
higher the light intensity, the higher was the population density at which
the exponential phase ceased and the linear phase set in. Approximate
values of these critical population densities are given In table 1. All

<u>Table 1</u>

Population densities corresponding to the exponential and the linear growth
phases in the 2.8-cm culture chamber at 25° C

Light intensity (lux)	Approximate population density (packed cell volume in ml/1)	
	at which exponential phase ended	at which linear phase began
800	.0.1	1.0.
2,000	0.2	2.5
5,000	0.5	6.0
10,000 25,000 50,000	>1.0	10.0

these values are referred to the particular conditions of our experiment,
that is, a culture-solution thickness of 2.8 cm, and average cell diameters
of 3 to 5.5 microns. The critical population density is expected to become
lower in proportion to Increase In the thickness of the culture solution.

Effect of Temperature on Exponential Growth Rate at Different Light Intensities

   The evidence set forth in the preceding section indicates that the tran-
sition from the exponential phase to the linear phase of growth is caused
by the change in optical density of the cell suspension during the course
of growth. Conceivably, the exponential phase of growth represents the
stage during which practically all individual cells in the culture receive
the incident light directly and do not cause appreciable decrease of avail-
able light by shading each other. Both by experiments and by the statisti-
cal considerations presented in the Appendix to this chapter, it was ascer-
tained that, under the conditions of our experiments (thickness of culture
solution 2.8 cm, diameter of cells 3 to 5.5 microns), the light-diminishing
effect of mutual shading of cells may practically be neglected when the
population density of the cell suspension is less than 0.1 ml packed cell
volume per liter.

   With the cell concentration kept always below 0.1 ml packed cell volume
per liter by diluting the culture frequently with fresh medium, the effect of
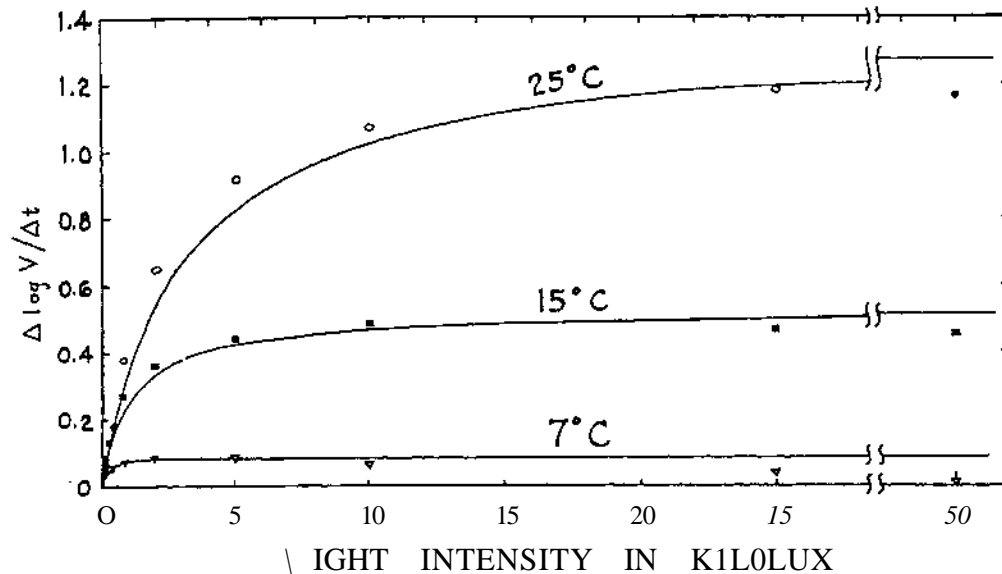
Fig. 7. The exponential growth rate as a function of light intensity at different temperatures. The curves correspond to the values calculated by equation (1). Values of $A log_{10} V/At$ are averages of several measurements.

temperature on the exponential growth rate at different light intensities was investigated (culture medium, "nitrate-A")- The results obtained are summarized in figure 7. The following facts emerge from these results:

(1) At all temperatures studied, the growth rate increases linearly with light intensity (I) at lower light intensities, but with further increase in light intensity, the increase in rate gradually slows down till the curves become flat and the growth rate becomes practically independent of the intensity. The functional relations observed may most simply, though somewhat approximately, be expressed by the formula for a rectangular hyperbola, as follows:

$$\frac{A log V}{\Delta t} = \frac{<^* k_G I}{k_G + \alpha I}$$

where k(j is the light-saturated value of AlogV/At, and *a* is the initial slope of AlogV/At as a function of I, namely:

$$oc = \left[\frac{d(A log V/At)}{dl}\right]_{I \longrightarrow 0}.$$

The curves shown in the figure are those calculated by formula (1) with the values of kQ and a given in table 2. Noteworthy is the fact that, whereas kQ changes remarkably with temperature, *oc* is decidedly independent of temperature.

Table 2

Values of constants used to calculate curves in figure 7

Temperature (°C)	(I/day)	(1/day-kilolux)
25"	1.34	0.45
15°	0.52	0.45
7°	0.092	0.45

(2) At all temperatures studied, but especially at lower temperatures, it was observed that growth was depressed at extremely high light intensities. At 7° C, growth was retarded even when the light intensity was about 5000 lux, and at 50,000 lux the cells completely ceased to grow within a few days and became entirely colorless. The tendency to color fading of cells was also observed, though to a minor extent, at higher temperatures. In all cases, cell suspensions grown under weaker light were dark green; with increase of light intensity, the color changed to light green and then to brownish pale green, and finally disappeared. The occurrence of such fading is also dependent on temperature; for example, the light intensity under which light green cells were formed increased with temperature as follows: 2000 lux at 7°, 25,000 lux at 15°, and 50,000 lux at 25°.

Effect of Thickness of Culture Solution on the Linear Growth Rate

At low population densities the growth rate is exponential and is independent of the thickness of the culture solution; but at high densities the growth rate is linear and is profoundly affected by the thickness of the solution. In an experiment using several culture chambers with different inner thicknesses, the linear growth rates shown by sufficiently dense cultures (more than 20 ml cells per liter) were compared (culture medium, "nitrate-A"; illumination, 25,000 lux; temperature, 25° C). The results obtained are given in table 3.

Table 3

Dependence of growth rate on thickness of culture

Thickness of culture (cm)	Linear growth rate AV/At (ml/(l)(day))
0.70	23.0
2.0	8.7
2.8	6.6
6.0	2.9

In figure 8 the values of AV/At are plotted against the reciprocals of the thickness of the culture. It may be seen that the growth rate is almost exactly inversely proportional to the thickness (D) of the culture.
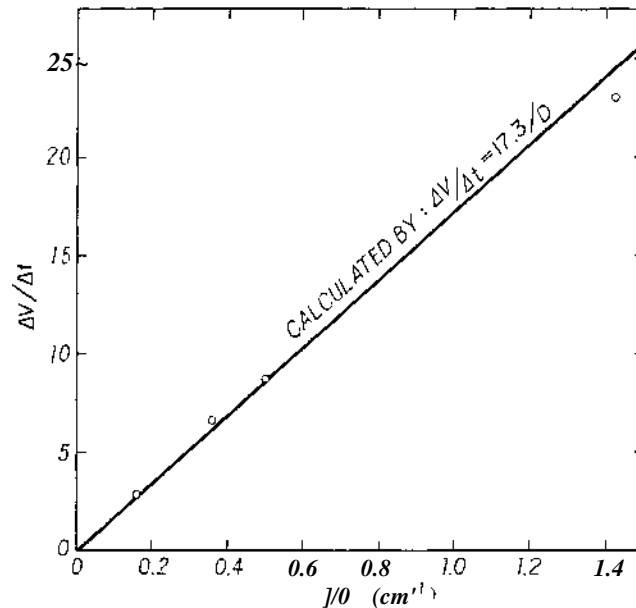
Fig. 8. Inverse proportionality between linear growth rate and thickness of culture solution.
The curve corresponds to the proportionality factor 17.3 ((cm)(ml)/(l)(day)).

If the quantity of algae contained in the whole culture is denoted by $, and the area of illuminated surface of the culture by A, the concentration (V) of algae in the culture is <J>/AD; therefore, AV/At = A$/ADAt The fact that A$/AAt (= DAV/At) was constant means that the rate of increase of algae per unit area of illuminated surface was the same in all cultures with different thicknesses. In the experiments described above, the rate of growth per unit area was 17.3 ml packed cell volume per square meter per day.

Significance of EDTA as a ChelatJng Agent and Comparison of Nitrate and Urea as Nitrogen Sources

Whereas in all the experiments described above "nitrate-A" solution was used as the nutrient medium, in the outdoor culture to be described later the medium used was '*urea-EH/' which differed from the former in containing urea instead of nitrate as nitrogen source, and EDTA as chelating agent together with considerably higher concentrations of various microelements. To make clear the significance of these differences and our reasons for preferring the latter medium for mass culture, it seems pertinent at this point to present in brief the data demonstrating the essential features of these two media.

Comparative experiments were conducted using, besides **nitrate-**A" and "urea-EH" media, the following culture solutions: "urea-A/[1] a

medium which contained urea instead of the nitrate in "nitrate~A"; and "nitrate-EH," a medium which contained nitrate instead of the urea in "urea-EH."

In one experiment, we left the cultures to grow in each medium without replacing the medium during the whole course of the experiment. (Illumination, 25,000 lux; temperature, 25° C.) The courses of growth of algae in such "standing" cultures are illustrated in figure 9. Owing to the very large "inoculum size" used in these experiments (initial population density, 12.4 ml cells per liter), the algae showed the linear growth phase from the beginning. As may be seen, the growth curves in both "A" cultures showed a linear ascent only during the first 2 days (with the formation of 11 to 14 ml cells per liter), after which the growth rate suddenly decreased and tapered off more and more with time. By contrast, the curves of "EH" cultures continued to ascend linearly for 8 to 11 days (with the formation of about 60 to 70 ml cells per liter), after which their ascent slowed down only gradually. The net increase in algal quantity attained at the end of the experiment was about 2.8 times as large in "EH" cultures as in "A" cultures.

Normal cells of our experimental alga when provided with adequate nutrient substances contain, per dry weight of cells, 5.2 to 9.5 per cent nitrogen and 1.2 to 1.8 per cent phosphorus. In view of the nitrogen and phosphorus content of the culture medium used, the early decline of the growth rate in the "A" cultures is obviously due to a deficiency in some of the microelements. (See the right-hand outside ordinate figures in figure 9. All media contained sufficient amounts of magnesium, potassium, and sulfate.) Experiments not reproduced here showed that the growth curve of "A" cultures, which had flattened down at later culture stages, suddenly ascended afresh when iron and Arnon's A4 and B7 mixtures were added to the medium.

The probable cause of the decline of growth rate in the "nitrate-EH" culture (with 5.0 g/1 KNO3) was a shortage of nitrogen. 2 The growth retardation which occurred in the "urea-EH" culture (with 3.0 g/1 urea) is to be accounted for by the shortage, not only of nitrogen, but also of phosphorus and possibly also of some microelement, which may have set in simultaneously.

In the experiment shown in figure 10, the four culture media were renewed every other day in order to exclude the occurrence of any deficiency phenomena. (Illumination, 25,000 lux; temperature, 25° C.) As was

2 Analysis of cells on the 17th culture clay gave the following results:
   Cells harvested from "nitrate-EH" medium: N_3 3.40 per cent; P, G.79 per cent.
   Cells harvested from "nitrate-A" medium: N, 5.85 per cent; P, 1.48 per cent.
   Whereas the cells grown in "nitrate-A" medium--in which the deficiency in microelements was the main cause of growth retardation--showed normal levels of nitrogen and phosphorus content, the cells harvested from "nitrate-EH" contained notably smaller amounts not only of nitrogen, but also of phosphorus. The low phosphorus content accompanied by the low nitrogen content is worth noticing here, because at the time of harvesting there was still an appreciable amount of phosphorus left in the culture medium. Cn the assumption of total consumption of the nitrogen in the medium, the nitrogen content of the cells harvested from the "nitrate-EH" culture was calculated to be 3.6 per cent, a value which is very close to the observed value 3.4 per cent.
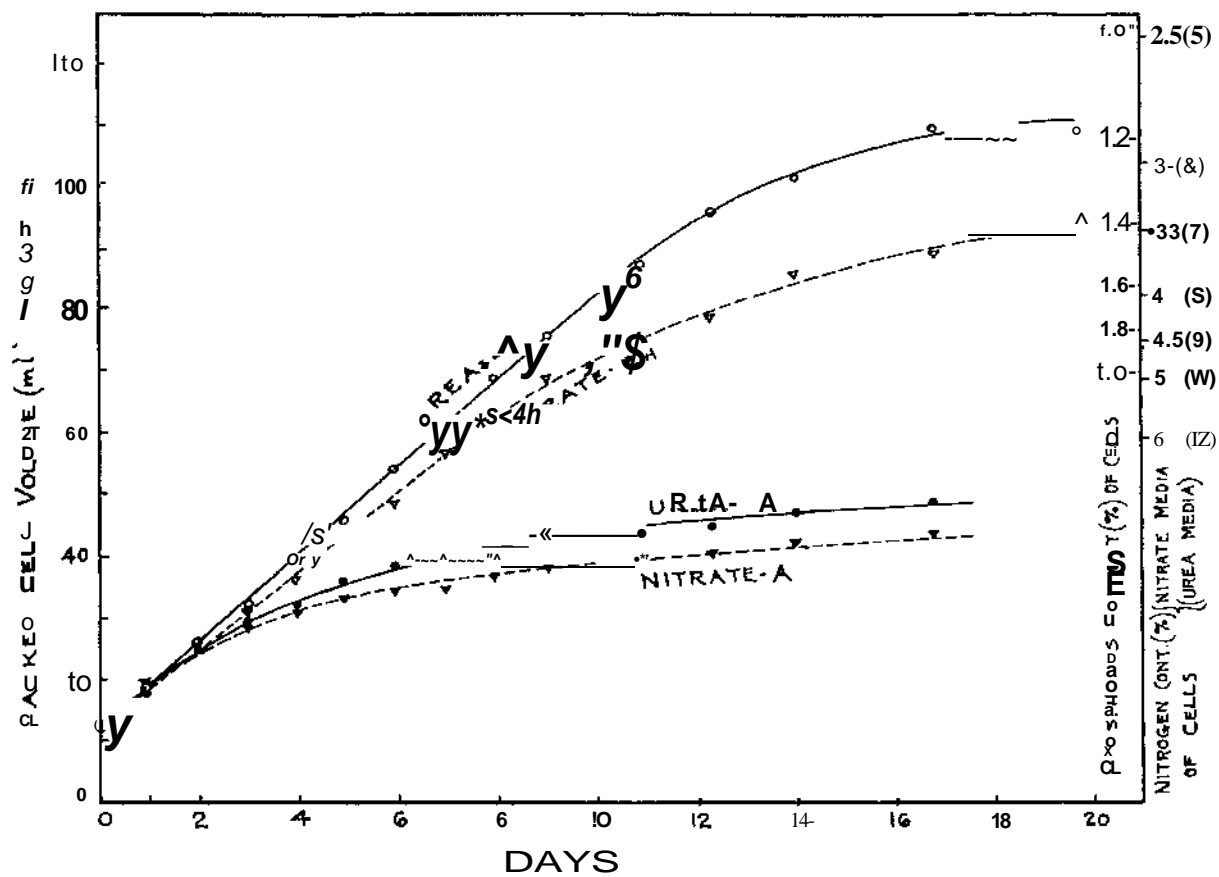
Fig. 9. Course of growth (in linear phase of growth) in "standing cultures" with and without EDTA plus Hutner's mixture of microelements. Light intensity, 25,000 lux; temperature, 25° C. Right-hand outside ordinate figures indicate the phosphorus and nitrogen content (per cent dry weight) which algal cells would be expected to attain if all the phosphorus and nitrogen in the culture media were used to build cell material at the respective concentrations.
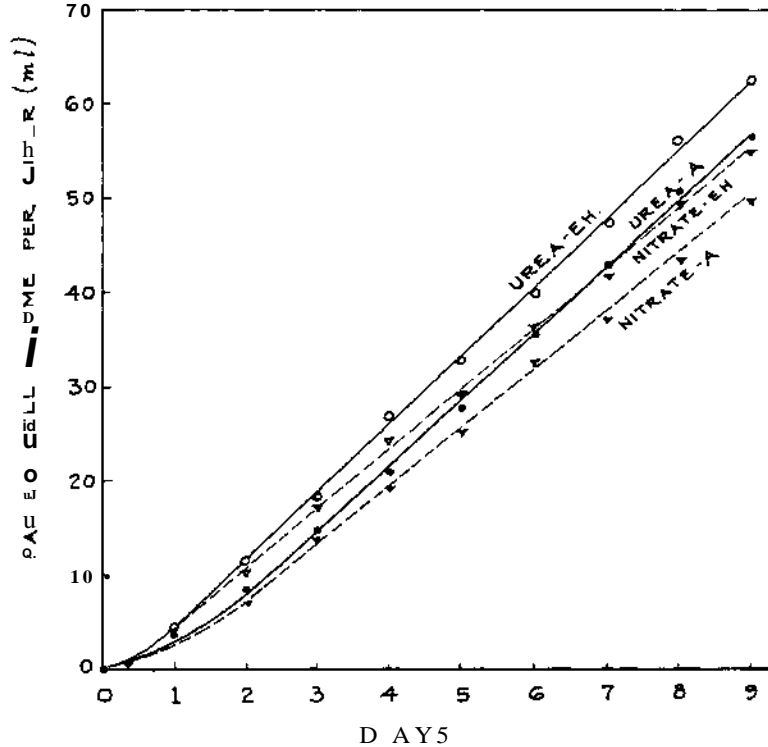
Fig. 10. Course of growth in "chemostatetf" cultures. The cells were resuspended in fresh culture medium every other day. The two culture media marked "EH" contained EDTA and high concentrations of micronutrients. Light intensity, 25,000 lux; temperature, 25° C.

expected, the growth curves in all these "chemostated" cultures showed continuous linear ascent which lasted practically indefinitely. Worthy of note is the fact that the growth rates were independent of the presence of EDTA but dependent on the sort of nitrogen source used.

We learn from these results: (1) that the use of EDTA in combination with a high concentration of microelements has the same effect as the frequent replacement of culture medium in ordinary cultures; this means thai the efficacy of EDTA in promoting the growth of microorganisms lies not in direct action on the organisms, but in its capacity for providing a large reservoir of microelements by forming complexes with elements which in a free state act poisonously in such high concentrations (cf. Hutner et al. [61]); (2) that urea is superior to nitrate as a nitrogen source, causing, on the average, 10 to 15 per cent faster growth of algae than does nitrate. Concerning the other merits of urea as the nitrogen source for Chlorella culture, see Davis et al.? chapter 9 in this monograph, pages 122 ff.

The desiderata for a nutrient medium to be used in mass culture of algae may be (1) that it support rapid growth lasting for a long period

without requiring frequent replacement or replenishment of its components, and (2) that its composition be well balanced in the sense that the proportions of the various components are the same as their proportions in algal cells; in other words, that all components of the medium become exhausted simultaneously. It is for these reasons that we have preferred, out of a number of solutions tested, the "urea-EH[?>] medium, with the composition given above (section B), as the nutrient solution for the mass culture of our experimental alga. 3

Yield of Algae in an Outdoor Culture as a Function of Available Light

The outdoor culture using the equipment shown in figure 2 was run from December 1951 to September 1952, during which period the culture temperature ranged between 1° and 28° C. The data cited here are those obtained during the period from late spring to midsummer 1952, the culture temperature (measured in the trough water) ranging between 17° and 28° C and the daily light quantity between 24 and 228 kilolux-hour. In figure 11 the observed values of daily increase in cell population are plotted against the light quantity per day. The values recorded here are only those observed at a population density higher than 7 ml packed cell volume per liter; that is, in the range of population density showing the linear phase of growth.

As may be seen, the daily yield is distinctly dependent on available quantity of light, and, though the points in the figure are rather scattered, their plot is, by and large, similar to that of linear growth rate against

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[3] Besides EDTA, a large number of chelating agents were tested for their applicability to algal culture. Good results, comparable to those from EDTA, were obtained with the following

substances: Nitroso R salt,
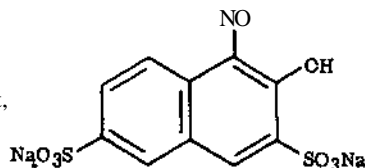
nitrilo triacetic acid, $N(CH_2COOH)_3$;

y-acetaminotropolcme, CH3CCNH —
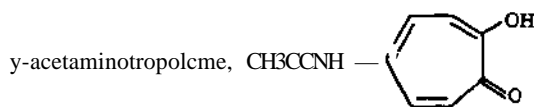
For use in mass culture, Nitroso R salt is especially promising because of its lower price as compared with EDTA.
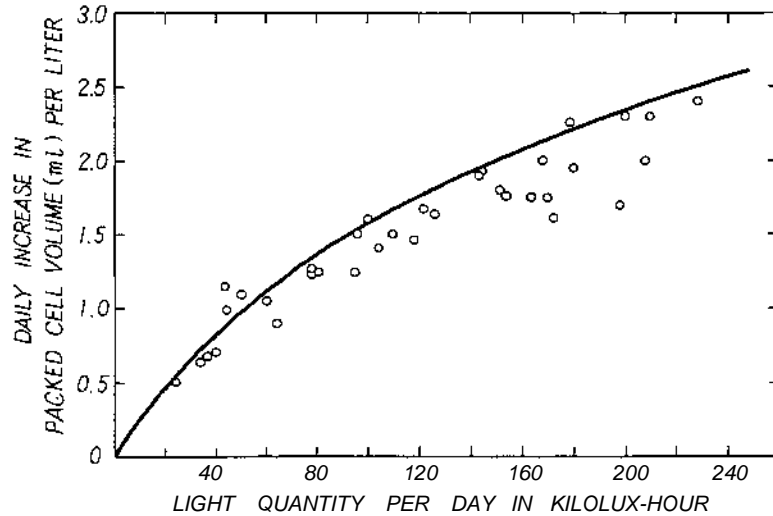
Fig. 11. Daily increase of algal concentration in the outdoor culture as a function of quantity of sunlight in each day. Temperature, 17°-28° C. The curve represents the values calculated by equation (7) in section D.

light intensity (in fig. 6), showing no tendency to light saturation at higher light quantities. The highest yield obtained was 2.4 ml (0.6 g) cells per liter at 228 kilolux-hour per day. The meaning of the continuous curve in the figure will be explained later.

Extinction Coefficient of Cell Suspension of Chlorella

As will be shown in section D, one of the important factors determining the linear growth rate of algae as a function of light intensity is the light transmittance of algal suspensions. The cells used were taken from cultures in the linear phase of growth.

As we have shown elsewhere (chapter 7 in this monograph), the average size and the average chlorophyll content of Chlorella cells vary considerably according to culture conditions such as intensity of available light and temperature. In ordinary cultures, larger cells with less chlorophyll content predominate at earlier stages, and with the progress of culture the proportion of smaller, chlorophyll-rich cells increases till they form the whole population. It is evident that with such changes in cellular characteristics, the extinction coefficient of the cell suspension also changes. For our present purpose, we are interested in the photochemical characteristics of cells in the linear phase, most of which are small and chlorophyll-rich. The cell samples chosen for our measurement were composed practically 100 per cent of such cells.

The extinction coefficient of cell suspensions at various wave lengths was measured with the apparatus shown diagrammatically in figure 12.
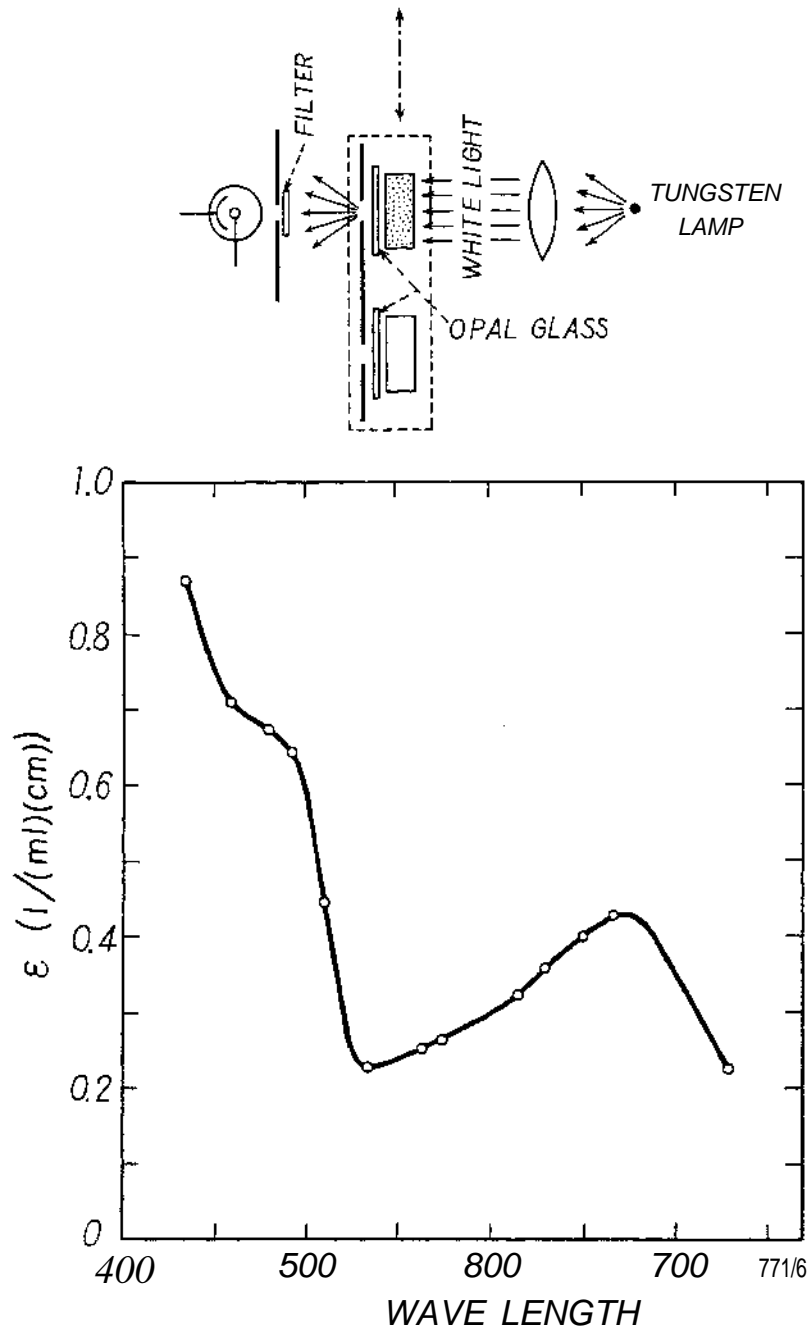
Fig. 12, Extinction coefficients of Chlorella suspension at different waYe lengths, and diagram of apparatus used in the measurement. The suspension used was taken from dense cultures in the linear phase of growth, and was composed practically 100 per cent of smaller, chlorophyll-rich cells.

The use of opal glass in the apparatus afforded the advantage of making the light absorption by cell suspension follow Beer's law within a wide range of VXD.   The curve in the figure represents the extinction coefficient € (= (l/DV)(log IQ/I); V, concentration of cells in terms of packed cell volume per liter; D, thickness of cell suspension in centimeters) measured in the wave-length range 434 to 729 *mfi*.   The average value of € in this range was found to be 0.41 (l/(ml)(cm)). ^

D

Theoretical Considerations

Equations for Growth Rate with Continuous Illumination

The various facts we have observed may be explained by the following considerations. Let us assume an algal culture with a uniform population density, a uniform depth D, and a surface A, on which light of intensity I falls perpendicularly (see fig. 13). If Beer's law is applicable to this system, we may write:

$$i = I\, e^{-6\,V\,x},\tag{2}$$

where i is the intensity of light at the depth x from the surface, £ the extinction coefficient of the algal suspension, and V the population density (in terms of packed cell volume per unit volume of culture). If the quantity of algae contained in the layer A - dx is denoted by $, <f> = VA•dx.

In general, the rate of increase of <£ may be expressed by

$$\frac{d\phi}{dt} = f(i)\,\phi,$$

where f(i) is a function of light intensity and represents the rate constant of exponential growth of algal cells. Experiments summarized in figure 7 show that the function f(i) is approximately of the following form (cf. equation (1)):

$$f(i) = \frac{a\,k\,i}{k + a\,i},$$

where k is the value of f(i) at sufficiently large values of i, and a is the value of df(i)/di at sufficiently low values of i. If the total quantity of algae contained in the whole culture is denoted by $, we·may write

[4] The absorption spectrum of cell suspensions of £W^^ll§--E2I§noldQsa has been measured by Emerson and Lewis [217], who, however, used cells harvested at relatively earlier stages of culture. From their data, the average value of extinction coefficients in the wave-length range 400 to 740 m/i is estimated to be 0.23 (l/(ml)(cm)), a value which agrees with ours in order of magnitude. The discrepancy between their values and ours may have been due to the difference in the proportion of larger and smaller cells in the samples used.
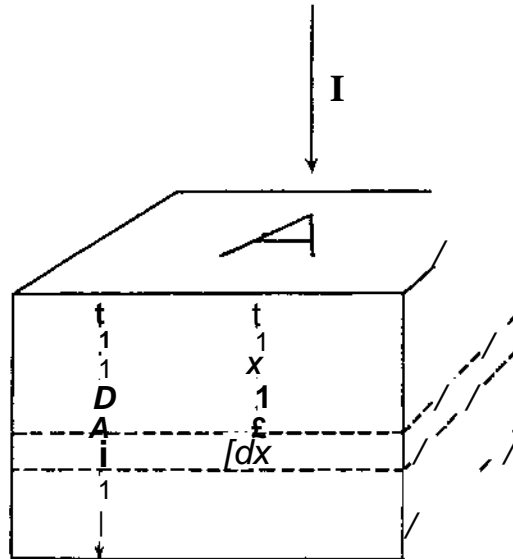
Fig. 13. Schematic diagram of illuminated culture

$$\frac{d\Phi}{dt} = \int_0^D f(i) V A dx$$

$$= \int_0^D \frac{}{k+ai} V A dx .$$

Substituting i from equation (2) we have

$$\frac{d\Phi}{dt} = \int_0^D \frac{a k I e^{-\epsilon V x}}{k + a I e^{-\epsilon V x}} V A dx .$$

Since $d\Phi/dt * A D dV/dt$, it follows that

$$\frac{dV}{dt} = \int_0^D \frac{a k I e^{-\epsilon V x}}{k + a I e^{-\epsilon V x}} \cdot \frac{V}{D} \cdot dx$$

$$= \frac{k}{\epsilon D} \ln \frac{k + a I}{k + a I e^{-\epsilon V D}} . \tag{3}$$

Equation (3) was derived from the preceding one by the use of equation 410 in Pierce's "Short Table of Integrals/' page 54.

If the population density is sufficiently low to make the value $e^{-\epsilon VD}$ approximate 1-ϵVD and to make eVD sufficiently small as compared with (k/al) + 1, we have from equation (3)

$$\frac{dV}{dt} = \frac{akIV}{k+al}$$

or

$$\frac{d\ln V}{dt} = \frac{akl}{k+al}$$

or, in finite fprrii and in terms of Briggsian logarithms,

$$\frac{\Delta \log V}{\Delta t} = \frac{\alpha k_G I}{k_G + \alpha I} , \qquad (4)$$

where $k_G = 0.4343k$ and $a = 0.4343a$.

If, on the other hand, the population density is sufficiently high to make the value ale-^VD negligibly small as compared with k, equation (3) gives

$$\frac{dV}{dt} \quad \frac{k}{\epsilon D} \quad . \quad A \quad \frac{al}{\_} \Big)$$

or, in finite form and in terms of Briggsian logarithms,

$$\frac{\Delta V}{\Delta t} = \frac{5.304 \, k_G}{\epsilon D} \log \left(1 + \frac{\alpha I}{k_G}\right). \qquad (5)$$

Since AV = A<$/AD, we have from equation (5)

$$\frac{\Delta \Phi}{A \Delta t} = \frac{5.304 kc}{\epsilon} \log \left(1 + \frac{\alpha I}{k_G}\right). \qquad (6)$$

Equation (4), which is identical with equation (1), is the expression of the exponential growth rate to be observed at lower population densities, and equations (5) and (6) represent the linear growth rate to be observed at higher population densities. These equations are satisfactorily in line with the following facts established in our experiments:

(1) The exponential growth rate is a function of incident light as expressed in equation (1) (see fig. 7).

(2) The linear growth rate is a logarithmic function of I, showing, in contrast with the exponential growth rate, no "saturation" phenomenon at higher light intensities (see fig. 5). At sufficiently low light intensities, however^ both exponential and linear growth rates are linear functions of I, namely:

$$\left(\frac{\Delta \log V}{\Delta t}\right)_{A_{I\to 0}} = \alpha I, \qquad\qquad I \Delta t A_{I\to 0} \approx \frac{al}{eD}.$$

(3) The higher the light intensity, the higher is the population density at which the transition from exponential phase to linear phase occurs (see fig- 3).

(4) The linear growth rate is inversely proportional to the thickness (D) of the culture solution; in other words, the increase of cells per unit area of illuminated surface is independent of the thickness of the culture solution (see equation (6) and fig. 8).

In the experiments described in section C, D = 2.8 cm, and the values of *k.Q, oc,* and € were estimated to be as follows: kQ = 1.34 (I/day) at 25° C, a= 0.45 (1/day-kilolux), e = 0.41 (l/(cm)(ml)). Applying these values in equations (4) and (5), the values of AlogV/At and AV/At at different light intensities were calculated. As may be seen from figure 14,
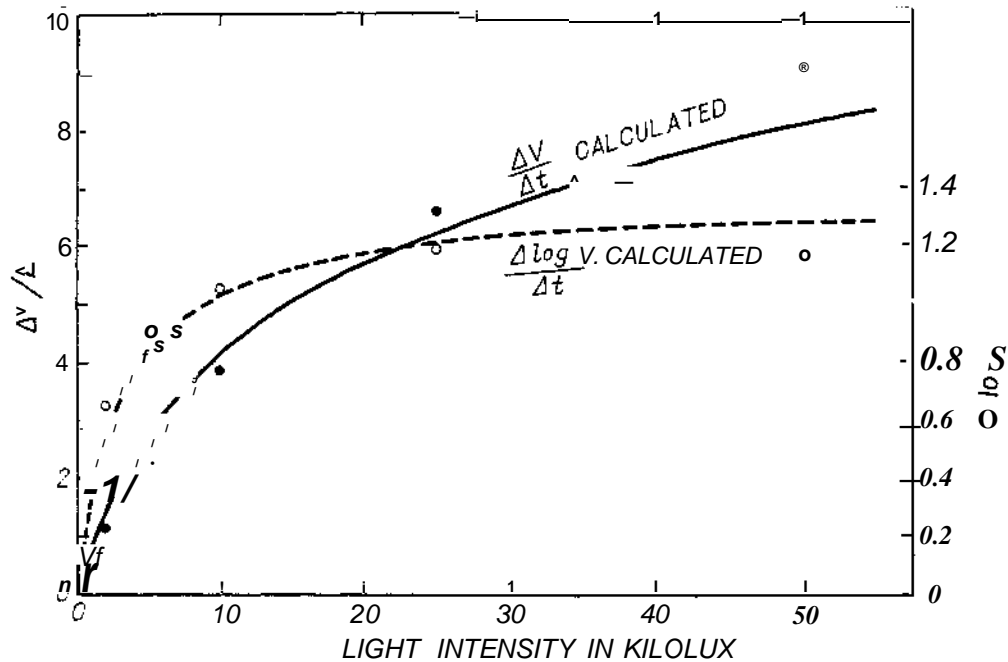
Fi$.\ 14, Fit uf calculations made by equations **(4) and (5) to the experimental points in**

the agreement between calculated and observed values may be regarded as satisfactory for the prediction of growth yields. The discrepancy between the calculated and observed values of AV/At at higher light intensities may have been due to the fact that at those light intensities the culture contained appreciable quantities of larger cells with less chlorophyll content, whereas *the* value € used in the calculations was that for the suspension of smaller,

chlorophyll-rich cells. The value of A$/AAt calculated by equation (6) was 16.9 ml/(day)(m^2), which is in excellent agreement with the observed value, 17.3.

Effect of Diurnal Variation of Illumination

We shall now attempt to extend our considerations to the case of outdoor cultures, and derive the formulas giving the daily yields of algae per unit volume of culture (Y_v) or per unit area of illuminated surface (Y_a) as functions of daily quantity of sunlight. For practical purposes we need to consider only cultures with high population densities in the linear phase of growth.

Let us denote the daily quantity of sunlight (in kilolux-hour) by L and assume, as a first approximation, that the culture is illuminated 12 hours a day, with a mean hourly intensity of L/12. If, as was the case in our outdoor culture, the value L is measured in the open air, and the actual quantity of light at the surface of the culture is a certain fraction (r) of L, the mean intensity of the light which is received by the culture each day may be regarded as rL/12 (in kilolux). According to equations (5) and (6), the daily increases of cells per unit volume of culture and per unit area of illuminated surface are given by the following equations:

$$Y_v = \frac{1}{2} \times \frac{5.3\,k_G}{\epsilon D} \log\left(1 + \frac{\alpha\gamma L}{12\,k_G}\right) \ (ml/(l)(day)), \qquad (7)$$

$$Y_a = \frac{10}{2} \times \frac{5.3\,k_G}{\epsilon} \log\left(1 + \frac{\alpha\gamma L}{12\,k_G}\right) \ (ml/(m2)(day)) \qquad (8)$$

(k in I/day; D in cm; *e* in l/(cm)(ml); *oc* in 1/day-kilolux; L in kilolux-hour.)

In our outdoor experiment, reported in the preceding section, the culture was circulated in a series of glass tubes 3 cm in diameter, with a total illuminated length of 33.3 m. If we assume that the tubes received the sunlight on exactly half of their cylindrical surface, the total illuminated surface of the culture system is calculated to have been 1.57 m2. Since the total volume of culture was 40 liters, the average thickness (D) of the culture is estimated to have been 2.5 cm. The correction factor (r) for the light intensity was 0.64. Using these values for D and r, and applying the already mentioned values of k_G (1.34 at 25° C), *a* (0.45), and €(0.41), the daily yield Y_v as a function of L was calculated according to equation (7).

The curve drawn in figure 11 represents the results of such calculations. It may be seen that the general trend of the experimental results is well depicted by the theoretical curve, though the majority of the observed values are scattered somewhat below the curve. The main reasons for these discrepancies may be sought in the following circumstances: (1) In the calculation we assumed that the temperature was 25° C throughout the

whole course of culture, whereas the actual temperature fluctuated between 17° and 28°; (2) the intensity of light entering the culture tubes through the illuminated surface was assumed to have been uniformly 64 per cent of the measured intensity of sunlight, whereas the actual intensity of available light must have been appreciably lower than that, owing to reflection from the curved surfaces of the tubes. It should also be taken into consideration that the values of kg, a, and £ applied in the calculation were those measured for the culture with "nitrate-A" medium, whereas the outdoor culture was run using "tirea~EH" medium, in which the values must have assumed somewhat different magnitudes. 5

Estimates of Yield in Outdoor Cultures

Among the factors in equation (8), only kQ is dependent on temperature, being 0.092 at 7°, 0.52 at 15°, and 1.34 at 25° C. Applying these values in equation (8), we can calculate approximate values of Ya (yield per unit area and unit time) as a function of light quantity (L) at three different temperatures. The results of such calculations (assuming $ot \sim 0.45$, $€ = 0.41$, r = 0.64) are shown in figure 15, in which the yield is given in three different terms, ml/(m2)(day), g/(m2)(day), and metric tons (dry weight) per acre per year.

In figure 16 are given the average temperature (the mean of many years' measurements) and the average light quantity per day (measured during the period December 1951 to August 1952) for each month of the year in Tokyo. ® On the basis of these figures the monthly yield of algae was roughly estimated according to the theoretical curves given in figure 15. Unfortunately, the estimate cannot yet be made for the whole year owing to the lack of light-quantity data for the period September to November. But it may be reasonable to conclude from these results that, under the conditions considered here, the yield would be minimum in January (only 0.1 ton per acre) and maximum in August (about 1.7 tons per acre), giving an annual yield of approximately 10 tons per acre, a figure considerably smaller than those generally assumed by other authors.

Possible Means for Increasing Yield in Outdoor Cultures

Possible measures to be taken for improving the yield may be considered here briefly:
(1) Artificial regulation of culture temperature, and especially its increase during the cold season. (The above calculation was made on the

[5] Another likely cause for the scattering of points in figure 11 is that the abscissa represents an integration of light intensity and time. High light intensity itself is a primary cause of retardation of growth. Therefore two experiments having the same "light quantity" may have differed considerably in light intensity and hence in daily yield. Also a reason for points falling below the curve may bo inadequacy of average depth.--Ed.
[6] In 1952 there was an abnormally heavy rainy season in early eummer. Normally, the light quantity in June and July is somewhat higher than that recorded m the figure.
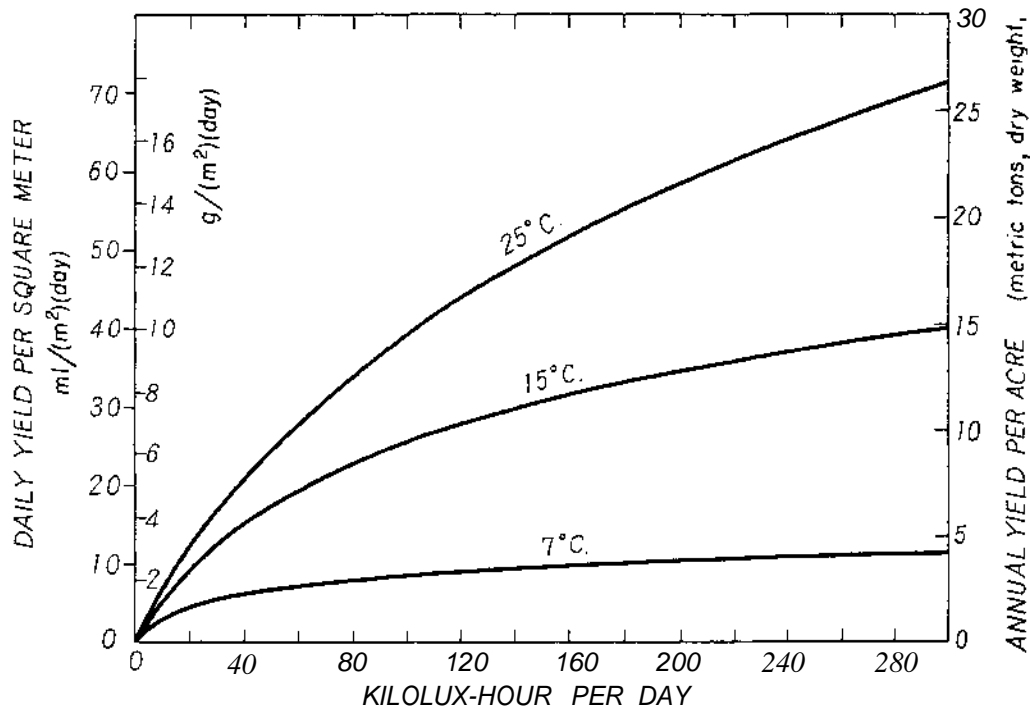
Fig. 15. Yield of algae per unit time and per unit area of illuminated surface as a function of temperature and daily quantity of light. Calculation made by equation (8), using the values $oc = 0.45$, $€ = 0.41$, $r = 0.64$, $k_G = 0.092$ at 7°, 0.52 at 15°, and 1.34 at 25° C.

assumption that the temperature was unregulated and equal to the atmospheric temperature at all seasons.)

(2) Selection of algal strains and culture media giving higher values of kg/e and cc/kQ. A higher value of kQ especially is effective in increasing the yield.

(3) Finding of algal strains which can grow at higher temperatures, and growing them in tropical or subtropical zones where L is very large. Whereas the value kQ, In itself, increases with temperature, temperatures higher than 35° C are injurious to most algae. Most promising in this connection is the recent discovery by Myers' group (see chapter 4, pages 45, 51) of a thermophilic strain of Chlorella which can grow at 39° with a kQ value Of about 2.7.

(4) Vigorous agitation of culture medium so as to cause increased growth by the effect of intermittent Illumination of cells. (See Davis, in chapter 9, pages 135 ff.) As has already been mentioned, the cultures In our laboratory experiments were constantly agitated by aeration with carbon dioxide-enriched air. This agitation, however, apart from supplying carbon dioxide to the culture, served only to keep the algal suspension homogeneous and had no growth-promoting effect by virtue of causing
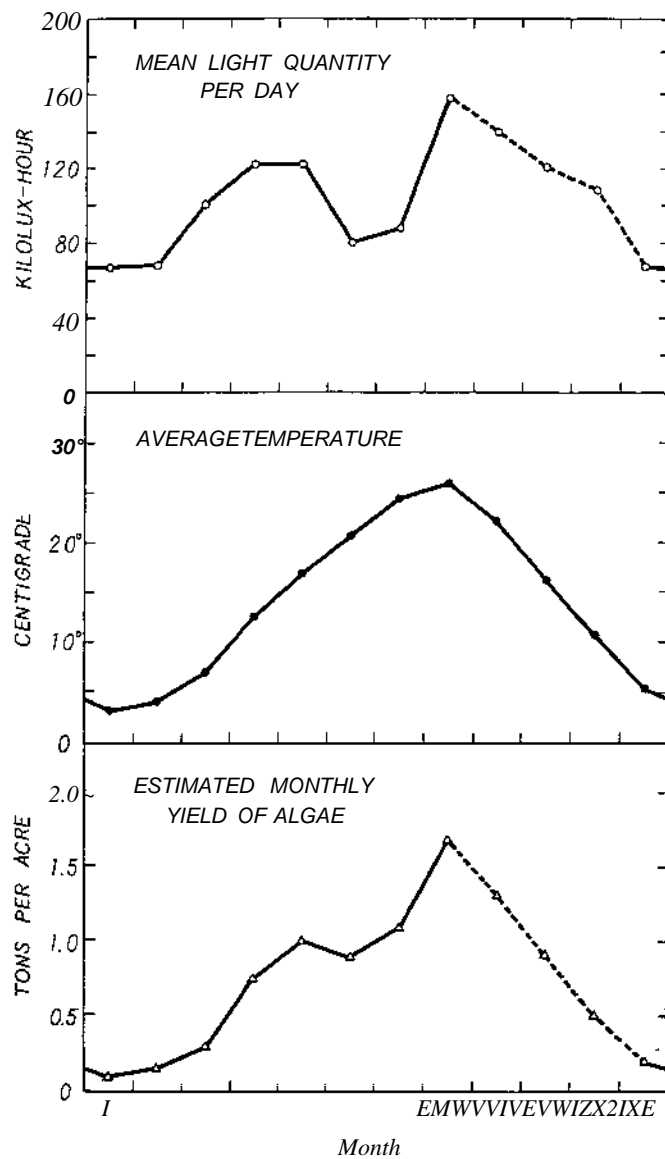
Fig. 16. Mean light quantity per day, average atmospheric temperature, and estimated monthly yield of algae in each month of the year in Tokyo. Light quantity was measured during the period December 1851 to August 1952. The values assumed for September to November are tentative. Temperatures given are the averages of many years' measurements.

intermittent illumination of cells. Experiments not reported here have indicated that the growth-promoting effect of intermittent illumination becomes apparent only when the cell suspension is agitated with very great vigor. It was also deduced from these experiments that the growth-promoting effect of intermittent illumination is insignificant at lower temperatures and lower light intensities. The effect of turbulence in culture on the yield of algae will be discussed in detail on some other occasion.

E

Appendix: Probability of Mutual Shading of Cells, and Estimation of Average Intensity of Light Available to Each Cell in Algal Suspensions

Let us assume a cell suspension with a thickness D along the direction of a light beam, and consider a rectangular box extending in the same direction and with a surface of unit area (1 cm2) through which the light enters perpendicularly (fig. 17). The algal cells suspended in the solution
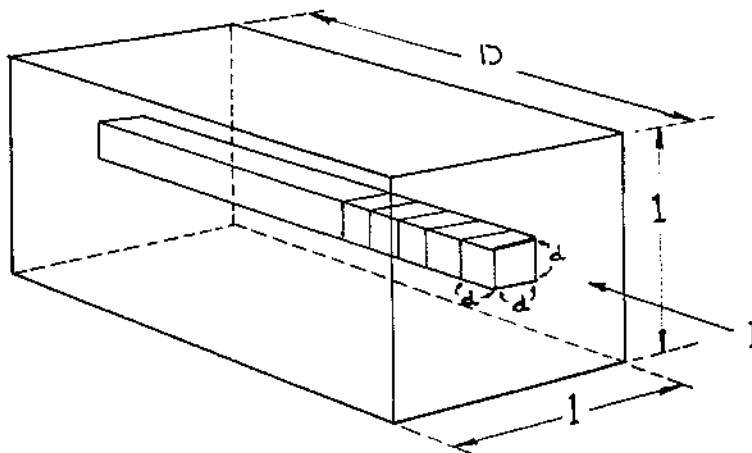
Fig. 17. Diagram of a portion of a cell suspension with a thickness D and an illuminated surface of unit area.

are assumed to be uniform in size, and their form is supposed to be that of a cube with sides of length d. It is further assumed that the rectangular box is composed of cubic compartments^ each having the size of a single algal cell (d3) and being marshaled in rows along the direction of light as shown in the figure. The number of compartments in each row is D/d, and the total number of rows in the box is **l/d2.** This latter value, which

may be denoted by N, is of the order of 10? in the case of our alga. If the population density in the suspension is denoted by c (cell number per milliliter), and the total number of cells contained in the rectangular box by n, then n = cD. Under the experimental conditions to be considered here, the value n is of the order of $10^\wedge$ to 108.

The cells are supposed to be distributed in the solution according to the law of probability. The probability that a row of compartments will contain i cells is given by

$$P_i = \left(\frac{1}{N}\right)^i \left(1-\frac{1}{N}\right)^{n-i} \frac{n^i}{(n-i)!\,i!} \, . \tag{9}$$

If we consider only the caffes in which i is not very large, then i and 1 may be neglected in comparison with n. On the other hand, 1/N is sufficiently small as compared with 1. Therefore, equation (9) may be approximated by

$$P_i \fallingdotseq \frac{1}{i!}\left(\frac{n}{N}\right)^i e^{-n/N}$$

or, if we denote n/N by x,

$$P_i \fallingdotseq^\wedge x V^*. \tag{10}$$

The probability that a row will contain no cell at all is

$$P_0 = e^{-x}.$$

Therefore, the probability that a row will contain one or more cells is

$$P_{i \geq 1} = 1 - e^{-x}.$$

In the rectangular box there are $NP_j^\wedge >^\wedge$ rows which contain one or more cells. Since each of these rows must confain one directly illuminated cell, the fraction (rQ) of directly illuminated cells contained in the rectangular prism is given by

$$\gamma_0 = \frac{NP_{i \geq 1}}{n} = \frac{1}{x}\,d^{-e}) \quad .$$

The number of rows containing two or more cells is

$$NP_{i \geq 2} = N(1 - P_0 - P_1) = N(1 - e^{-x} - xe^{-x})$$

and in each of these rows there must be one cell which is shaded by one directly illuminated cell. The fraction of such "one-fold shaded" cells in the rectangular prism is, therefore,

$$\gamma_1 = \frac{N}{n}(1 - P_0 - P_1) = \frac{1}{x}(1 - e^{-x} - x e^{-x}).$$

In the same manner, the fraction of "j-fold shaded" cells is given by

$$\gamma_j = \frac{N}{n}(-1_0 P_1^P - \frac{P}{...} -)_j$$

$$= \frac{1}{x}\left(1 - e^{-x}\sum_0^j \frac{x^i}{i!}\right)$$

or, since $x \sim n/N = cDd2$,

$$\gamma_j = \frac{1}{cDd^2}\left(1 - e^{-rDH^2}\sum_0^j \frac{(cDd^2)^i}{i!}\right). \tag{ID}$$

If we denote the light transmission (I/IQ) by one single cell by T, the average intensity (I) of light available to each cell in the algal suspension is given by

$$I = I_0 \sum_0^\infty ZT \ y_j . \tag{12}$$

In our experiments using the flask shown in figure 1, D = 2.8 cm. The value d may be considered equal to the diameter of the cells, which, in the case of our alga, averages 3x10-4 or 5.5x10-4 cm. As was set forth above, the average extinction coefficient (e) of a Chlorella suspension (consisting of smaller, chlorophyll-rich cells) is 0.41. If this value may be extrapolated to the light absorption by a unicellular layer of algal cells, the transmission T by a single cell is calculated to be 0.75.

Figure 18 shows the results of calculations by equations (11) and (12) using the above-mentioned values of D and T for the suspension of smaller cells (d •= 3xlO~4). The concentration of cells is given not'only in terms of cell number per liter, but also in terms of packed cell volume (ml) per liter (5.6X10^ cells corresponding to 1 ml packed cell volume). It may be seen that with the increase of cell concentration, the percentage of directly illuminated cells decreases exponentially, while the percentages of one-, two-, or more-fold shaded cells increase in sequence, but, after attaining a certain maximum value, gradually decrease one after another.

At the population density of 0.1 ml packed cell volume (or **5.6X10^** cells) per liter, the percentage of directly illuminated cells is only about 54, but since the rest of the cells are not heavily shaded, the average intensity (Ī) of light available to all cells in the suspension is as much as 85 per cent of that of incident light. A similar calculation made for a suspension of larger cells (d = **5,5X10-4)** shows that at the population

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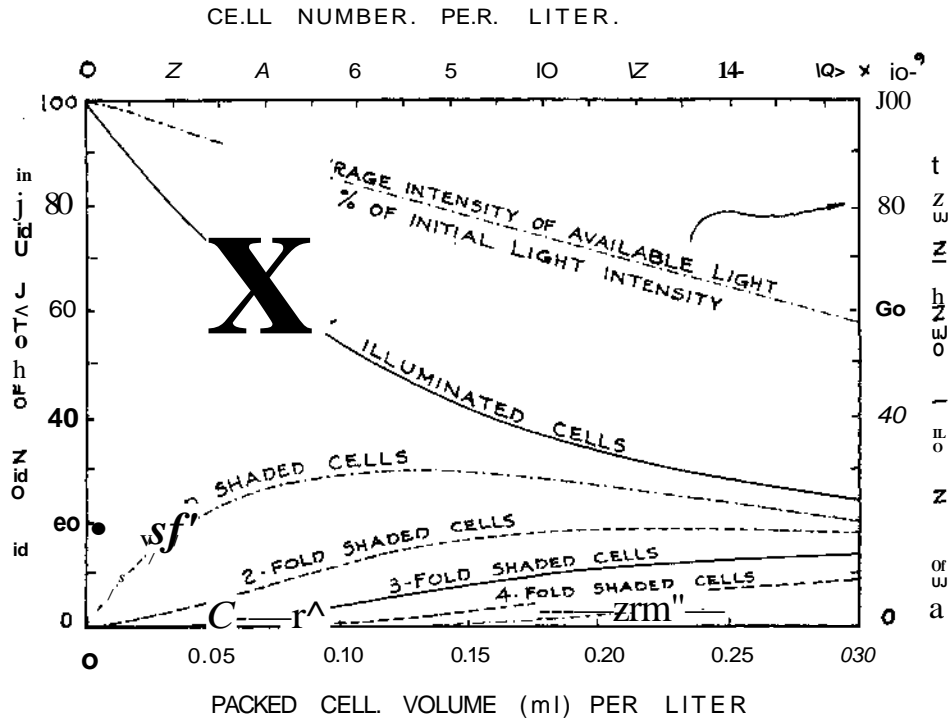
Fig. 18. Percentages of directly illuminated cells and of cells which are shaded by an
increasing number of other cells as functions of population density of cell suspension. Calcu-
lation was made by equation (11), using the values $D = 2.8$ cm, $d = 3 \times 10^{-4}$ cm. The curve at
the top represents the average intensity of light (in percentage of the intensity of incident
light) available to each cell in the suspension, calculated by equation (12) with the assumption
that $T = 0.75$.

density of 0.1 ml packed cell volume (or 0.91×10^9 cells) per liter, the per-
centage of directly illuminated cells is 70, and the average intensity avail-
able ($\bar{I}$) is 90 per cent of that of incident light. It may, therefore, be con-
cluded that in order to make the cells receive an average light intensity
more than 85 per cent of that of incident light, the population density of
the cell suspension should be kept below 0.1 ml packed, cell volume per
liter (when the thickness of the culture solution is 2.8 cm).

The experiments with sufficiently low light intensity show that below
the population density just mentioned, both the rate of photosynthesis and
the rate of growth (referred to a unit quantity of cells) are practically in-
dependent of the cell concentration used in the experiment, whereas a cell
suspension denser than 0.1 ml per liter gave lower rates owing to the ef-
fect of mutual shading in the cell suspension.

PART IV

PILOT-PLANT EXPERIMENTS

Chapter 17

PILOT-PLANT STUDIES IN THE PRODUCTION OF CHLORELLA[1]

When the pilot-plant project was begun in 1951 at Arthur D. Little, Inc., Cambridge, Massachusetts, it had four purposes: (1) to achieve the continuous production of high-protein Chlorella on a larger scale than had been attempted before; (2) to obtain further information that would aid in the appraisal of economic possibilities; (3) to study the conditions of growth suitable for continuous mass culture on a large scale; and (4) to distribute experimental quantities of Chlorella for studies of possible end uses of the product.

Throughout the pilot-plant program the species Chlorella pyrenoidosa (Emerson's strain) was used. Since all previous data had been obtained under essentially pure culture conditions, closed systems that could be readily cleaned or replaced were adopted. Only natural illumination was used for the pilot units.

A
Laboratory and Intermediate-Scale Operations

Agar Slants

The original cultures of Chlorella were supplied by the Carnegie Institution's Department of Plant Biology on agar slants, and new slants were inoculated from the previous slants every week or two.

Flasks

As the first step in preparing the culture for the large-scale experiments, 1-liter Erlenmeyer flasks containing 300 ml of standard pilot-plant medium (see table 1) were inoculated with a small quantity of Chlorella from the slants. These flasks were set up on a shaking rack illuminated from below by three fluorescent tubes (fig. 1), and a 5 per cent CO2-in-air mixture from pressure cylinders was bubbled through the cultures.

Vertical Columns

To produce enough culture for an effective initial concentration in the large growth units, a second intermediate step was used. Ten vertical

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[1] This chapter is based on a report by Arthur D. Little, Inc., Cambridge, Massachusetts, "Pilot-Plant Studies and Economic Evaluation of Mass Culture of Algae," submitted to the Carnegie Institution of Washington on June 24, 1952.
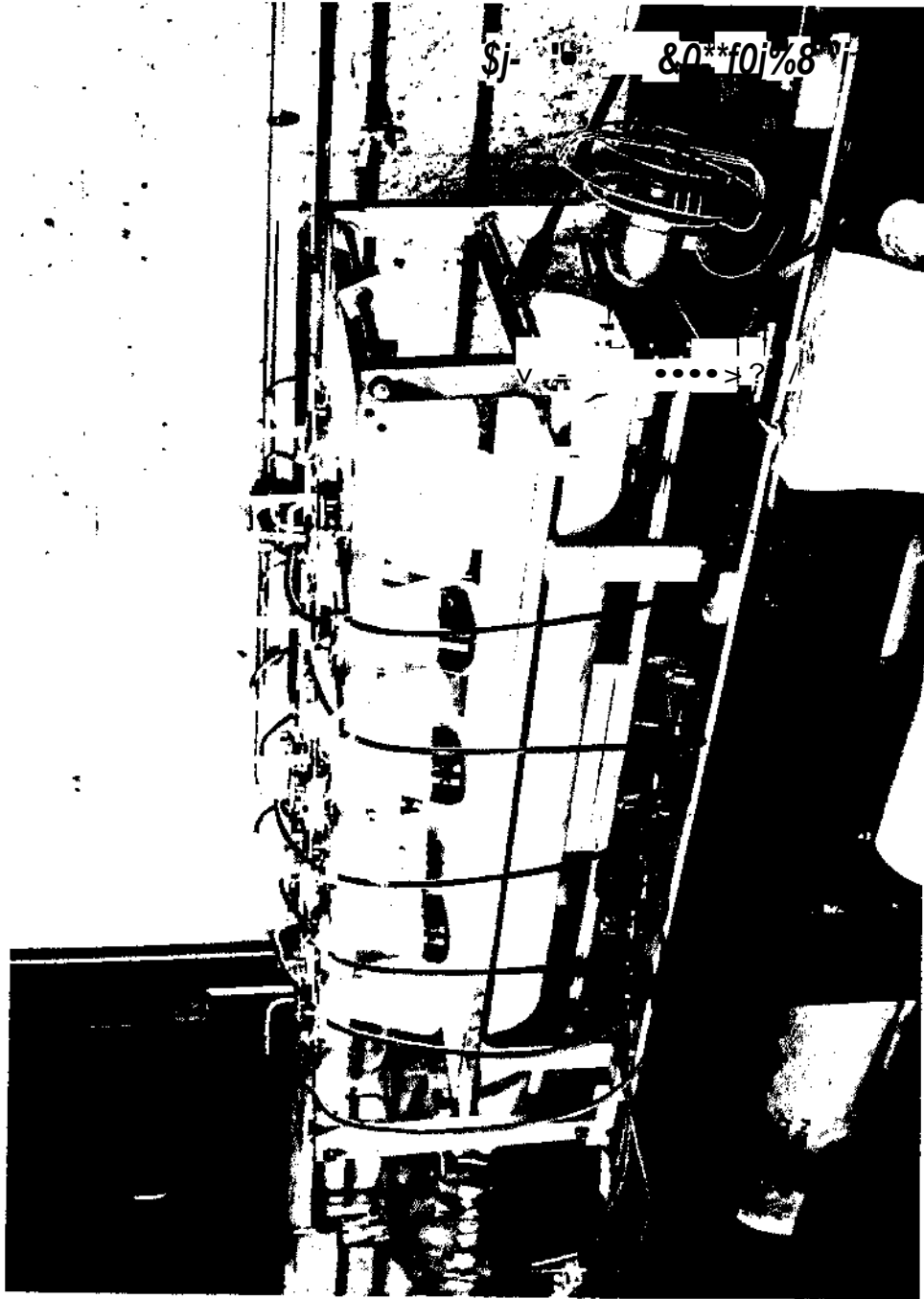
Fig. 1. Flask mixing device for laboratory experiments

Pyrex columns, 4 in. in diameter and 6 ft. long, were installed outdoors against a white board, which faced south for maximum sunlight (fig. 2). The design of these columns was largely based on information from the Stanford Research Institute report [279]. Special conical bottom sections with three-way stopcocks were fabricated. Air with 5 per cent CO_2 was introduced through the stopcocks, which were also used for sampling and draining the columns. Large rubber stoppers at the tops of the columns held 10-mm glass U-tubes reaching to the bottom of the cylindrical tubes for circulation of cooling water. A short 25-mm glass tube inserted in the stopper served as the gas exit and as a support for hanging a thermometer in the culture. The tube was plugged with cotton and capped loosely by a small beaker for protection against rain and dirt. The cooling water for each column was regulated by a needle valve at the top of the board. The gas manifold and individual gas needle valves were placed near the bottom of the columns to facilitate adjustment of the agitation intensity. Loops of polyethylene tubing 0.5 in. in diameter, reaching above the culture level, connected the valves with the column stopcocks to feed gas without risking loss of culture by siphoning. The columns could be sterilized readily with live steam before new culture was added. These units operated satisfactorily.

It had originally been thought that three or four intermediate steps between the agar slants and the 1200-gallon growth units would be required. The successful use of flasks and columns only, as described above, made possible an appreciable saving in time and equipment. These relatively simple techniques were shown to be satisfactory and could be followed in commercial-scale operations. With growth-area units of several thousand square feet, a horizontal unit similar in type and size to the large pilot-plant system might be needed as an additional step.

B

Large-Scale Operations

In carrying out the main project three large-scale culture units were developed and studied.

Unit 1

Large, thin-walled plastic tubes to form channels with simultaneous flow of culture and gas had been suggested by Dr. Davis R. Dewey, of the American Research and Development Corporation, and by Dr. **Vannevar** Bush, of the Carnegie Institution.

Of the possible plastics, polyethylene was available and seemed to provide a suitable combination of light transmission, chemical and physical stability under exposed conditions, freedom from toxicity, and physical strength. The tubing, manufactured by the Plax **Corporation,** had a thickness of 4 mils and when laid flat measured 4 ft. across. It was obtained
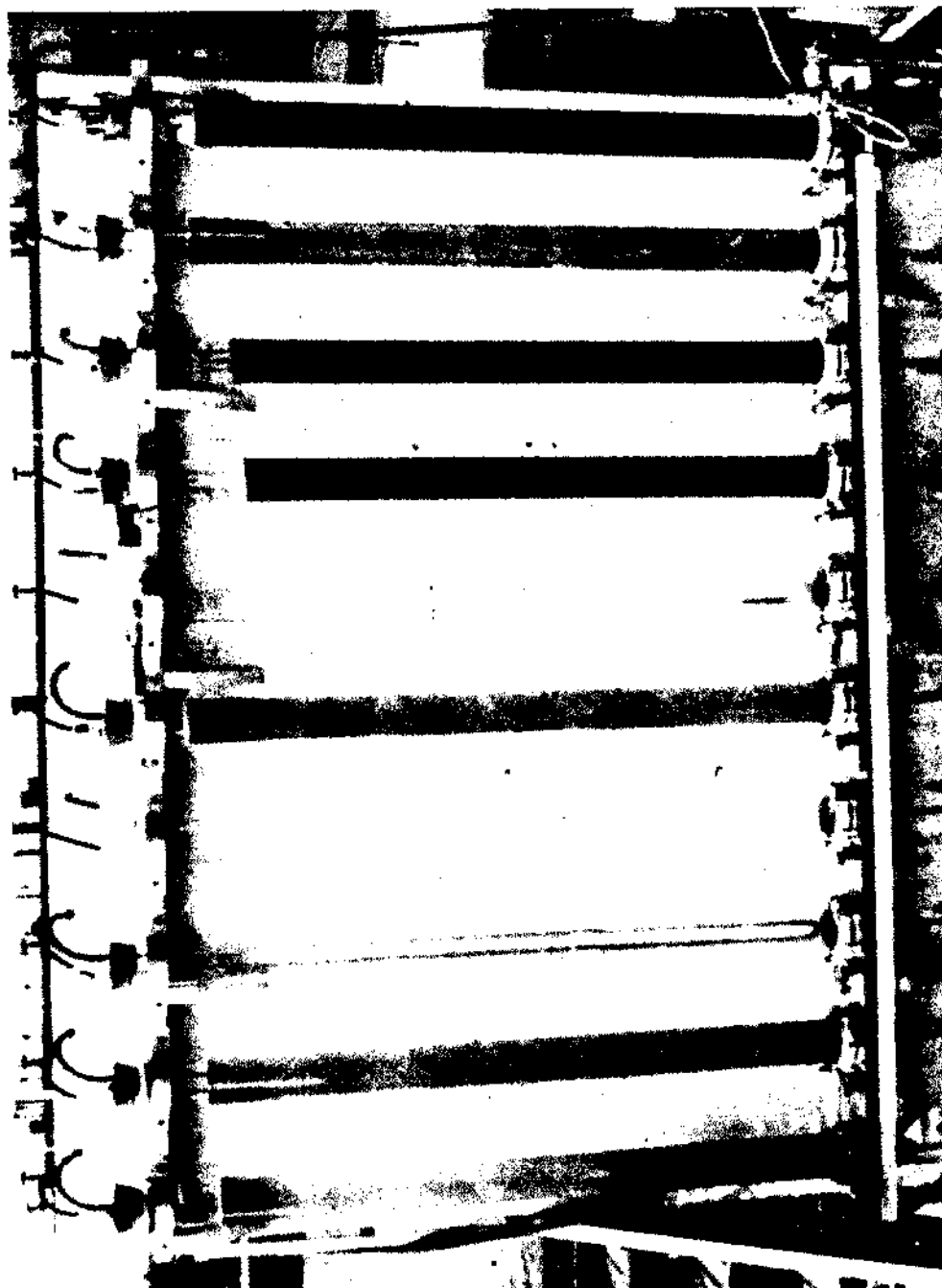
Fig. 2. Vertical Pyrex columns

in rolls of 500 linear feet. Two parallel tables 70 ft. long, 4 ft. wide, and 4 ft. apart, connected at the ends, were set up to support the tubing.

The initial tube installation was in the form of a U, receiving culture by means of a pump from a polyethylene-lined box at the end of one table, and discharging through a flat funnel of polyethylene to the tubing on the other table. Leakage from the seals on the box and funnel was serious. A second suction box, of plywood with sealed joints and coated on the inside with plastic emulsion, was more successful but still not satisfactory.

The 2-in. pump suction and discharge pipes were inserted into the top of the tube through slits in the film near the ends of the tables. The slits were sealed and the film was fastened to the pipes above the liquid level to prevent leakage from the large tubes. Horizontal cross pipes below the culture surface with slots on the lower side were used to distribute the intake and discharge flow across the width of the culture tubes. This method was reasonably satisfactory, except that the suction pipe drew air when the depth of the culture was less than 2 in., reducing the range of depths and velocities which could be investigated.

In the final arrangement (fig. 3), full turns were used at both ends, and the suction and discharge pipes were installed, in the manner described above, close together near the middle of one of the straight runs. The circulating pump was removed from the roof to the second floor of the building to facilitate the operation of the heat exchanger and the harvesting of the product.

The fabrication of the growth chamber from thin-walled tubing presented difficulties, since there was no previous experience on which to draw. For the long straight sections, tubing was laid out directly from the roll. It was found that placing a half inch of water in the tube as it was unrolled made manipulation easier; even so, care was required to prevent misalignment, twisting, and folding.

The first 180° turn was made by cutting the ends of straight tubes at 45° and connecting them with a crosspiece of tubing by diagonal heat seals made with a hot-air welder. Although this method was relatively simple, it was never possible to get an end seal of this type which did not leak. If the plastic in the seal was completely fused, some failures always occurred along the edge of the seal. If the heat was insufficient to cause fusion, some sections of the seal itself would eventually separate. The same problems· were encountered in sealing the tube to the heavy plastic used for the original inlet and outlet fittings. It was also observed that heat seals were unsatisfactory *on* tubing which had been exposed to weather and to culture medium.

In the final installation, the first tube was replaced by a new one with a culture area of approximately 600 sq. ft. This unit had a 180° turn at each end made by sealing a large number of narrow triangular pleats into a continuous tube, using an electrically heated clamp sealer. By completing the first half of the new unit while half the original tube was still in operation, it was possible to transfer a large part of the original culture. Although
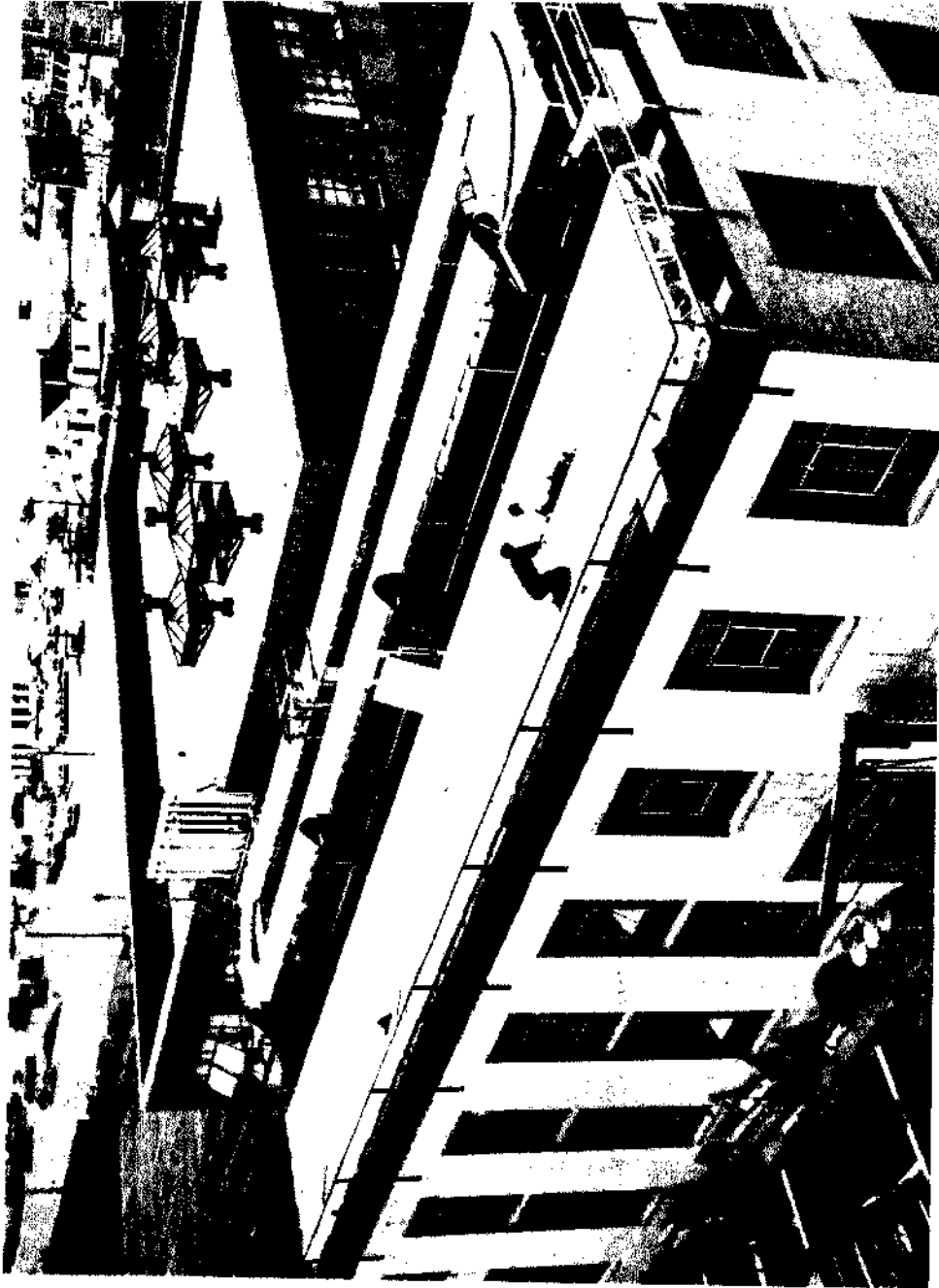
Fig. 3. Pilot-plant installation. Unit 1 in operation, unit 2 under construction in foreground, glass columns in left background.

the pleated construction was more satisfactory than the previous arrangement, it did ultimately give trouble as the pleats gradually separated because of wind and varying gas pressure. In several cases minor leaks developed at pleats.

It is believed that in using thin plastic film it will be advisable to avoid heat seals until a method can be worked out to eliminate seal failures. An alternative might be the use of a wide metal or plastic ring over which the tubing could be clamped to connect straight runs of tubing with prefabricated turns and end sections.

Although the tubing was occasionally punctured, sometimes below the liquid level, tearing was not a problem. Throughout the operations, minor leaks developed in the straight sections of the tubes as well as in the turns, as the result of imperfections in manufacture and of handling. It was found that Minnesota Mining and Manufacturing Electrical Scotch Tape No. 22 is one of the few materials which will seal well on polyethylene, and this kept leakage from being a serious problem.

Stability of plastic tubing. In the operation of unit 1, liquid depths of from 2 to 4 in. were tried, with the gas space taking its own shape. Figure 4 shows a close-up view of the edge of the tube under operating conditions, and a cross section is presented in figure 5. In strong winds, with liquid depths of about 2 in. and an over-all height of 5 or 6 in., the top of the tube showed instability. Liquid depths of more than 3 in., however, caused enough tension in the film to prevent serious effects from moderate winds. Elimination of the gas provided a stable condition at any depth, even with high wind velocity.

Circulation of culture. The circulation velocity selected for unit 1 was slightly above the critical value normally associated with turbulent flow in smooth channels. A centrifugal pump was used to circulate the culture. This unit had a capacity of about 110 gallons per minute (gpm) under the operating conditions, giving a velocity of approximately 0.3 ft./sec in the 4-ft.~wide tube when the culture was 2.5 in. deep. This velocity proved to be too low to maintain complete suspension when contaminants were present (see below under "Results of Unit 1")- The pump served well, giving dependable performance with no indication that its used caused damage to the culture.

Cooling of culture. Since free evaporation of water from the culture was prevented by the closed tube, the considerable amount of energy received from the sun was transformed to heat in the algal suspension. An indirect cooling system was therefore provided.

The first installation provided for cooling by spraying water on part of the top of the tube through 12 fishtail nozzles 3 ft. apart. The first day of operation proved this method to be entirely inadequate, and so for a period the entire tube was covered with cheesecloth kept wet by spraying. Since even this did not hold the temperature in the middle of the day below the desired level of 80° F, the idea of cooling with any type of water spray on the outside of the growth tube was abandoned.

Fig. 4. Close-up of unit 1 tube, showing foam on culture
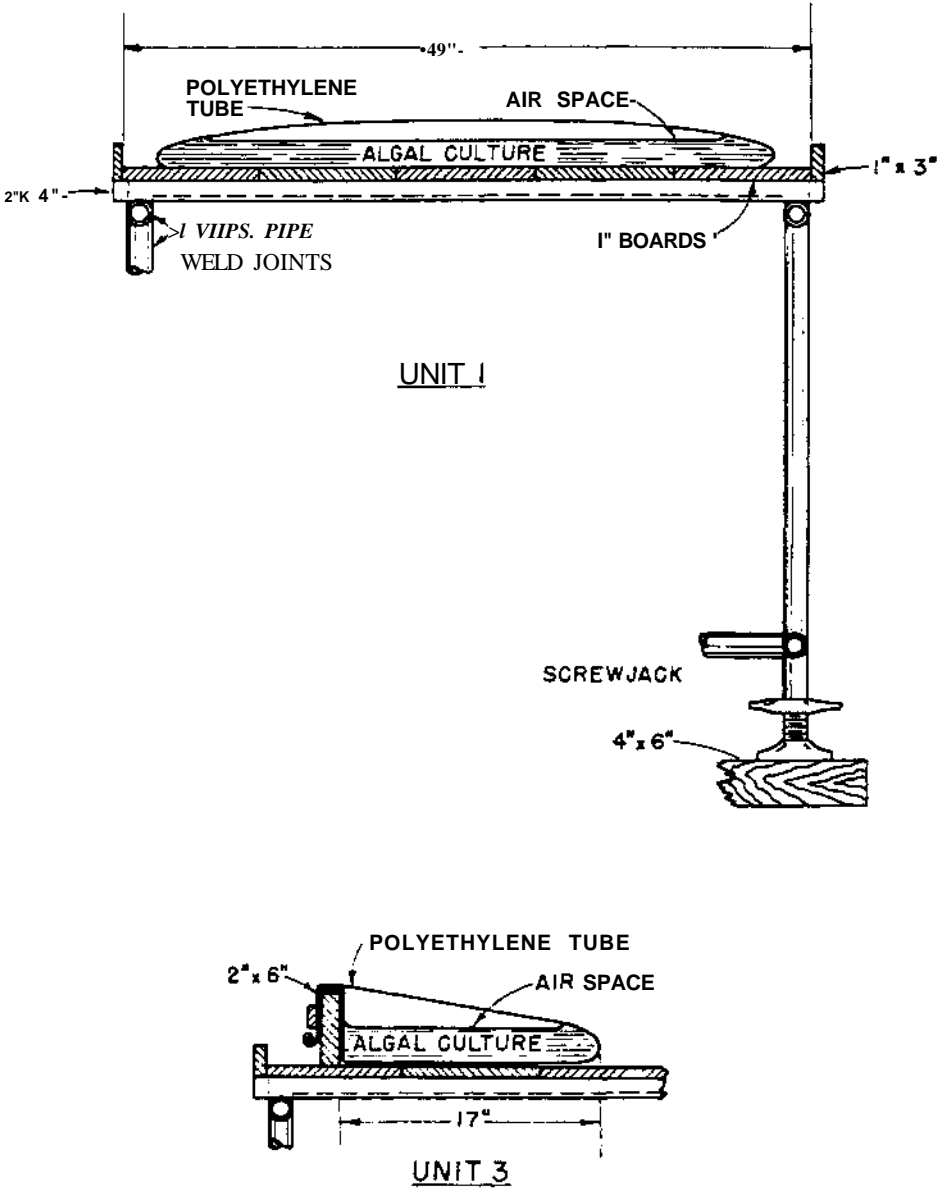
Fig. 5. Cross sections of units 1 and **3**

A heat exchanger with 15 stainless steel plates having a transfer area of 47 sq. ft. was installed (fig. 6) on the floor below the roof and connected in parallel with the pipe returning culture to the unit. About 25 per cent of the culture flow was passed counter current *to* city water. With this apparatus it was possible to keep the culture at temperatures below 80° F, with heat removal as high as 90,000 Btu/hr for short periods.

Harvesting system. A Sharpies Special No. 16 Super Centrifuge having a clarifier bowl 4 in. in diameter was obtained. This unit, driven at 13,000 rpm by a 2-HP motor, performed satisfactorily for all harvesting. Figure 7 shows this unit installed with the bowl on the table in cleaning position. Liquid flow rates as high as 5 gpm were found to be satisfactory for complete removal of the algae. For complete removal of all suspended matter, including bacteria, rates of 1 to 2 gpm were maximum.

The clarified medium from the centrifuge was discharged into a 30-gallon stainless steel receiver. This was connected by means of a 3/4-in. stainless steel pump and a plastic hose so as to permit return of the centrifuged medium to the growth area. The receiver contents could alternatively be drained to the sewer.

Since the capacity of a centrifuge is governed by the maximum liquid flow rate, the time required to harvest a given quantity of algae depends on the concentration in the growth tube. During long runs at low concentrations of algae the bowl was heated by friction enough to cause concern about damage to the product. This would not be a problem at high concentrations or with continuous product discharge, as might be provided for commercial installations.

Nutrients. Carbon dioxide: The major nutrient for algae is carbon dioxide. Five per cent CO_2 in air was used throughout most of the pilot-plant work on the basis of the findings of Spoehr and Milner [151]. The optimum equilibrium concentration has not been determined, particularly when there is high light intensity.

The carbon dioxide-enriched air for the columns and growth units was supplied through a surge tank system (fig. 8). Commercial carbon dioxide from cylinders and compressed air were fed to the tank through special orifices. Since the flow through a well rounded orifice is independent of downstream pressure so long as the downstream pressure is less than half the upstream pressure, the composition of the gas mixture is determined by the orifice area and the upstream pressure. The surge tank operated between pressure limits of 30 and 40 pounds per square inch absolute (psia), with a Pressure-trol switch operating solenoid valves to permit a simultaneous flow of air and carbon dioxide through pressure-regulating valves to their orifices at constant pressure of about 80 psia. The mixture from the surge tank was reduced through a regulator to a distribution-line pressure of 25 psia. The system worked satisfactorily throughout the summer and fall, giving gas of reasonably constant carbon dioxide content.

A small filter of high efficiency, built for the Atomic Energy Commission by the Cambridge Corporation, was used in the discharge line from

Fig. 6. Heat exchanger
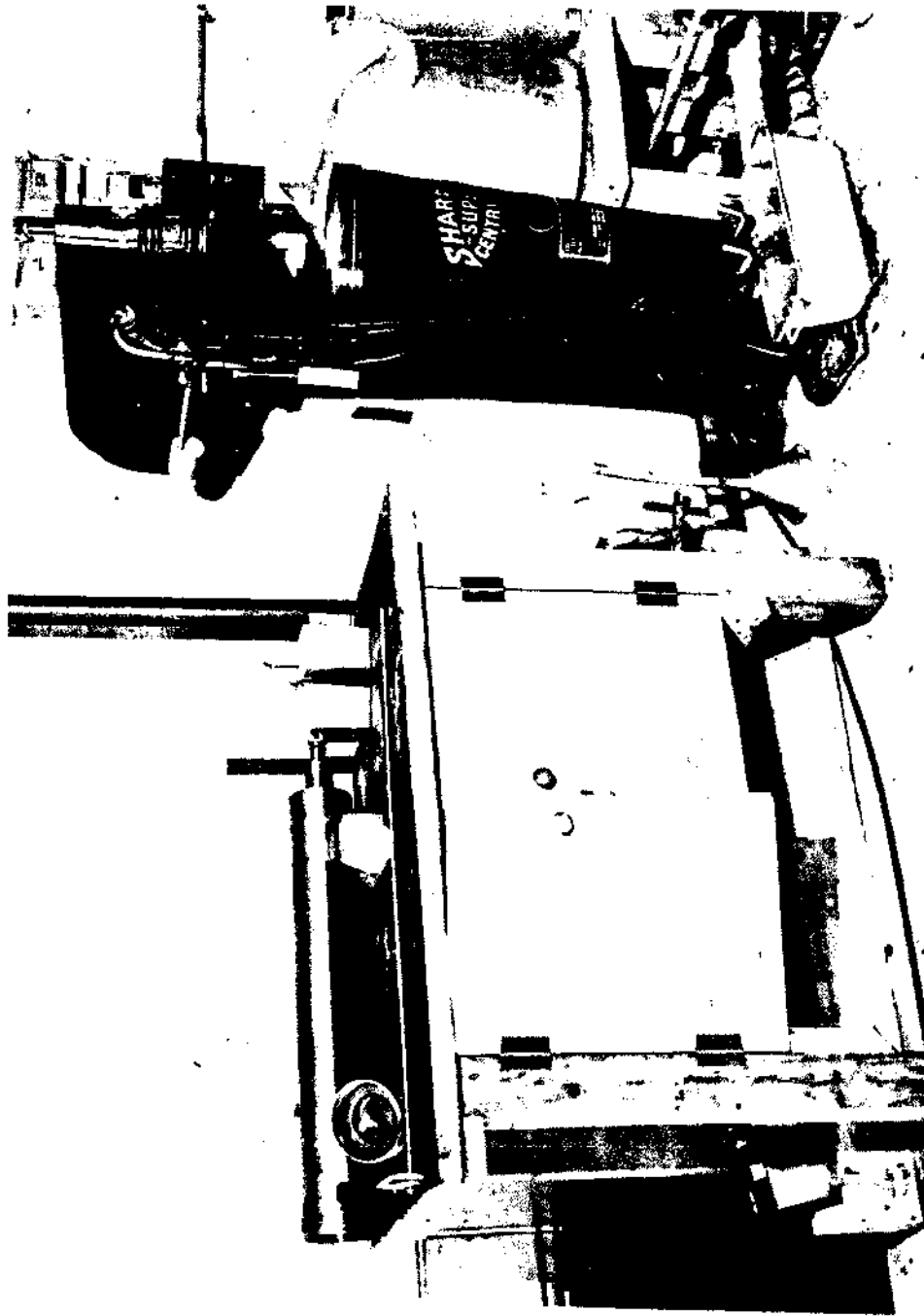
Fig. 7. U S with bowl in cleaning site
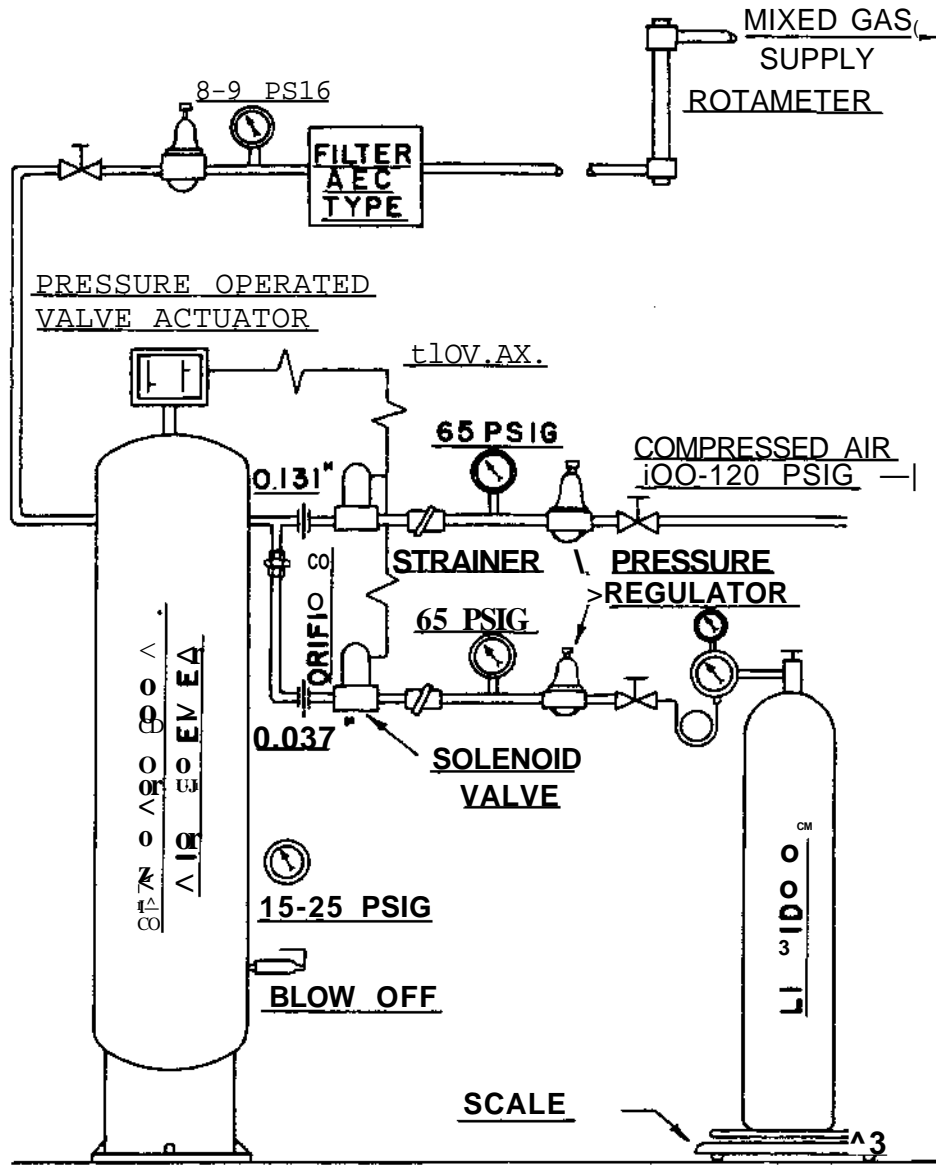
Fig. 8. Diagram of carbon dioxide-air system

the surge tank to prevent contamination by bacteria or other suspended matter.

Plastic garden hose carried the gas from the mixing unit to a rotameter and valve for flow control and then to the inlet tube at the growth area. The gas mixture was fed into unit 1 through a 0.5-in. polyethylene tube inserted through the top of the growth tube beside the pump discharge pipe and sealed in with Electrical Scotch Tape. Gas was released through covered slits in the growth tube near the pump suction pipe.

Table 1
Macroelement solutions

Salt	Ion	A2 [a]		"Standard pilot plant"	
		g/l	ppm	g/l	ppm
KNO$_3$		2.5		2.5	
	K$^+$		970	....	970
	NO$_3$		1530	....	1530
MgSO$_4$·7H$_2$O		5.0		2.5	
	Mg^{++}		492	....	246
	SO$_4^{--}$		1940	....	970
KH$_2$PO$_4$		2.5		1.25	
	K$^+$		720		360
	H2PO4		1780		890
	Total K$^+$		1690	....	1330

[a]Spoehr and Milner [151].

Macroelements: It was decided not to attempt investigation of nutrient combinations. The medium (table 1) suggested by Spoehr and Milner [151] and used successfully by Stanford Research Institute [279] and others was used originally; but after discussion with Myers the concentration of potassium acid phosphate and magnesium sulfate in this medium was reduced one-half. The nitrate concentration of the A2 medium was retained, since there was no evidence of adverse effect, and this concentration was sufficient to produce about 4 g dry Chlorella per liter without further addition, thus providing a factor of safety in operation.

The "standard pilot plant" medium used throughout the program (table 1) was prepared with technical-grade salts. It was found necessary either to filter or to boil the solutions of nutrient salts, particularly of the phosphate, in order to prevent contamination by mold. For large quantities, the salts were dissolved at high concentration in a pressure tank, and the solution was forced through the filter by admitting city water under pressure. For the replacement of small amounts of nutrients during operation, salts were weighed out and dissolved at saturation in water in 12-liter flasks, and the solution was sterilized by blowing steam Into it for about an hour. The operation of unit 1 was not on a large enough scale to require special equipment for the replacement of nutrient.

For all pilot-plant medium, filtered city water was used. Water for use in the flasks was autoclaved, and for the columns it was usually heated with live steam. Microscopic and plate-count tests showed that filtration through Filter Aid and activated carbon on paper reduced the bacteria count by 90 per cent and removed all larger organisms. Although better filtration is possible, this was considered adequate for the large units. Chlorine was not a problem, since Cambridge water is dechlorinated before going to the mains. The average analysis of the water is given in

Table 2
Analysis of Cambridge water [a]

Constituent	ppm	Constituent	ppm
SiO_2	5.8	SO_4^-	26.0
Fe^{+++}	0.14	Cl^-	7.4
Ca^{++}	15.0	NO_3	1.1
Mg^{++}	2.5	Total solids	96
Na^+	5.5	Free NH_3	0.05
K^+	2.0	Albuminoids	0.08
HCO_3 §	22.0	CO_2	0

[a] Averages for 1951 obtained from the City of Cambridge Water Department.

table 2. Since preliminary growth tests with filtered water in flasks showed no difficulties, no corrections were made for mineral content of the water in preparing media. Although analysis varied somewhat from week to week, no operating difficulties were encountered which we attributed to water supply.

Microelements: Traces of certain elements necessary for satisfactory growth were added to the culture. These elements and their role in Chlorella metabolism are described in detail by Kxauss (chapter 8) and Myers (chapter 4). The concentration of microelements supplied initially to the culture in unit 1 is given in table 3. The elements indicated as essential

Table 3
Microelement solutions

Salt	Stock solution	Ion	Final ion conc, in culture (ppm)
H3BO3	14.3	B^{+++}	0.5
MnCl2*4H$_2$O	9.05	Mn^{++}	0.5
ZnSC4*7H$_2$O	1.11	Zn^{++}	0.05
CuSO$_4$-5H$_2$O	0.395	Cu^{++}	0.02
H$_2$MoO4 (87% MOO3)	0.885	Mo^{++++++}	0.01
Fe(NO$_3$)3 • 9H$_2$0	40.4	Fe^{+++}	0.01

In Arnon's A4 solution (Myers, personal communication) were prepared in a single concentrated sterile solution using dilute sulfuric acid, and added directly to the culture. The iron solution was prepared separately. The supply of microelements in the culture was replenished as required.

The pH of the medium for Chlorella, according to earlier work (chapter 8), should be between 5 and 7. In the pilot-plant work the pH was held as close to 6 as possible by adding 5 per cent nitric acid solution.

Results of unit 1.   First tube: In its original form of a U with the pump connected across the open end, unit 1 contained 1000 gallons of standard medium at a depth of about 2.5 in. The first inoculation was made on July 7, 1951, from eight of the glass columns, to give an initial concentration below 0.05 g/L In spite of leakage, requiring the addition of over 800 gallons of medium to replace losses, and other difficulties, * the concentrations of the newly inoculated unit rose to 0.9 g/1 (dry weight) in the first 8 days. Taking into account the estimated losses, the growth averaged about 11 grams per square meter per day (g/(m2)(day)). During this period the weather was fair except on one day. On that day the concentration increase was noticeably lower than on the others. Some settling of algal cells was noted, even in the first few days. With the new culture, stable foam formation was observed as the concentration reached 0.3 to 0.4 g/1.

On the day the unit was inoculated, two of the vertical glass columns were filled with culture from the large tube. On the third and fourth days other columns were filled from the tube. Each time, the material freshly removed from the horizontal tube was appreciably darker than the contents of the columns filled earlier, indicating more rapid growth per unit volume in the horizontal tube than in the vertical columns.

After 13 days of operation the concentration had reached 1.1 g/L Several changes were then made, including the installation of the heat exchanger and pump on the floor below the roof, the shortening of the growth tube to about two-thirds its original length, and the elimination of the original end-fittings. In the next several days an entirely new tube was constructed and most of the original culture was harvested. Few data were obtained during this period, but the actual harvest from the original tube totaled 33 pounds wet, which at an estimated 20 to 25 per cent solids would be equivalent to 7 to 8 pounds dry weight. In addition to this amount, the equivalent of some 2.5 pounds of dry material was transferred to the new tube as culture. Thus the total dry material accounted for in the first 20 days of operation was about 10 pounds, in addition to which an amount perhaps equal,to this was lost through leakage and handling.

Second tube: The replacement tube was filled with 1200 gallons on July 27, using about 400 gallons of the culture from the original tube as inoculant. Regular harvesting was started on July 30, the centrifugate being replaced by fresh medium. On August 10, a recycle of centrifugate to the unit was started. Table 4 presents the growth data from July 30 through September 9, which was a period of fairly consistent operation without serious leakage, temperature-control problems, or mechanical changes. Except on one day, the concentration in the tube was held between 0.3 and 0.5 g/L The concentration figures given are those for the early morning samples, and growth was calculated by adding to the quantity harvested on a given day the calculated change in material in the tube based on the difference between the concentrations on that day and on the following day.

Table 4
Growth data for unit 1

Date (1951)	Weather	Chlorella in tube		Harvest		Growth in 24 hr (lb)	Remarks
		Dry wt. (g/D[a])	Total wt. (lb)	Wet (lb)	Dry[b] (lb)		
July 30.....Fair		0.35	3.5	2.2	0.7	1.2	200 gals, make-up
31.....Fair		0.40	4.0	2.3	0.7	1.2	300 gals, make-up
Aug. 1.....Rain		0.45	4.5	3.7	0.9	0.8	300 gals, make-up
2.....Sunny		0.44	4.4	3.8	0.9	0.5	400 gals, make-up
3.....Partly cloudy		0.40	4.0	7.7	1.9	1.2	400 gals, make-up
4.....Sunny		0.33	3.3	....	....	1.8	
5.....Overcast		0.51	5.1	....	....	0.9	
6.....Sunny		0.60	6.0	6.8	1.7	1.7	400 gals, make-up
7.....Sunny		0.60	6.0	5.5	1.4	-0.1	400 gals, make-up
8.....Rain		0.45	4.5	....		2.0	400 gals, make-up
9.....Sunny		.....	....	6.0	;•;}	(48 hr)	
10.....Cloudy		0.50	5.0	6.5	1.6	0.6	Recycle started
11.....Hazy		0.40	4.0	....	....	1.0	
12.....Hazy		0.50	5.0	....	....	1.8	
13.....Sunny		0.68	6.8	8.5	2.1	0.3	
14.....Sunny		0.50	5.0	8.4	2.11	2.2 (48 hr)	
15.....Hazy		.....	....	7.2	1.8/		
16.....Hazy		0.33	3.3	6.2	1.6	1.2	
17.....Hazy		0.29	2.9	5.2	1.31	2.2 (48 hr)	
18.....Sunny		.....	....	3.5	0.9 J		
19.....Sunny		0.29	2.9	3.4	0.9"!	0.9 (48 hr)	
20.....Hazy		.....	....	....	....}		
21.....Hazy		0.35	2.9	6.4	1.6	1.5	Vol. 1000 gals.
22.....Sunny		0.33	2.8	....	....	-0.2	200 gals, added
23.....Sunny		0.26	2.6	5.5	1.4	2.1	Vol. 1200 gals.
24.....Partly cloudy		0.33	3.3	4.5	1.2	1.4	
25.....Sunny		0.35	3.5	6,9	1.7	2.6	
26.....Partly cloudy		0.44	4.4	....	....	0.9	
27.....Partly cloudy		0.53	5.3	6.9	1.7	1.2	
28.....Hazy		0.48	4.8	7.3	1.8	0.9	
29.....Sunny		0.39	3.9	6.6	1.6	1.5	
30.....Sunny		0.38	3.8	3.4	0.9	LI	
31.....Sunny		0.40	4.0	7.1	1,81	1.3 (48 hr)	
Sept. 1.....Rain		.....	....	....	..../		
2.....Rain		0.35	3.5	4.1	10 1	1.2 (48 hr)	
3.....Cloudy		.....	....	....	—)		
4.....Sunny		0.37	3.7	5.6	1.4	1.7	
5.....Partly cloudy		0.40	4.0	4.5	1.1	0.4	
6.....Cloudy		0.33	3.3	5.7	1.4	1.4	100 gals, added
7.....Partly cloudy		0.33	3.3	5.9	1.5	0.8	Vol. 1200' gals*
8.....Sunny		0.26	2.6	6.7	1.7	1.7	
9.....Sunny		0.26	2.6	4.8	1.2	0.1	
10.....Sunny		0.15	1.5	....	....	....	
Over-all change....			-2,0	180.0	45.0	43.1	

[a] Based on $\frac{\text{dry Weight per } liter}{\text{wet cell volume per 100 ml}}$ = 2.2.

[b] Based on 25 per cent solids in centrifuged algae.

Table 4 shows the variation in the data for individual days. One of the major difficulties was the settling of algae when contaminants were present, which varied from day to day. In spite of attempts to agitate the contents of the tube before sampling, variations such as those on August 21, 22, and 23 occurred. The over-all results, however, indicated correlation with weather conditions. The average growth was 1.03 lb/day for the 42-day period or approximately 9 g/(m2)(day). Over the shorter period August 21 through August 30, the average was 1.3 lb/day or 11 g/(m^2)(day). In general, no reduction in growth was detected during the 30 days of the recycle of centrifugate. As further evidence on this point, daily harvests of about the same size were obtained for the following month.

During the period covered by table 4, contamination by an alga of Chlorococcum type increased. By mid-September the settling and sticking on the sides and top of the tube were serious. Leakage became a problem again, so that frequent additions of medium were made to maintain volume. For this reason the day-to-day data beyond September 10 had little meaning and are not included. By October 5 both end sections were removed because of increasing leakage, and the unit was reduced to one straight run of about 60 ft. with an estimated volume of 400 gallons. This was operated continuously until October 20, when the unit was shut down and the entire contents harvested. The total harvest from September 10 through the shutdown was 89 pounds wet or between 18 and 22 pounds dry. From August 10, when recycle started, approximately 5 g of dry algae had been grown per liter of culture medium.

Total: The total amount of algae actually harvested in the 105 days of operation of unit 1 from July 7 to October 20 was 308 pounds of wet material, the equivalent of between 65 and 75 pounds of dry solids. If we calculate on the basis of 600 sq. ft. of area, the original area of the replacement tube, we get an over-all average of about 6 g/(m2)(day), without taking into account the losses from leakage and handling or the 20 days during which the irradiated area was less than half of 600 sq. ft. It should be pointed out, moreover, that in unit 1 the velocity of flow was never high enough to give complete suspension of algae, particularly when the culture was contaminated with Chlorococcum.

In summary it may be said that the operation of the first unit satisfied the first objective of the program, namely, continuous relatively large-scale production of high-protein Chlorella. It also provided material for end-use studies by several industrial and research organizations, the results of which were reported in chapters 20 and 21.

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A second unit was constructed but never functioned satisfactorily and was abandoned.

EaiLl

The operation of unit 1 indicated that higher flow velocities should be used. Also, greater turbulence was desired as a means of investigating

the effect produced on growth rate by intermittent lighting of Individual cells.   Therefore a third unit was formed by draping a **4-ft.-wide** polyethylene tube over 2"x6" planks set on edge on one of the tables used for unit 1, and fastening the tube so that the actual flow channel was approximately 15 in. wide.   This width of channel was selected in order to utilize the available circulating pump.   Figure 9 shows a close-up of the discharge end of the unit; figure 5 gives a cross-section sketch of the growth area.   A straight run of tubing 71 ft." long was used.   This provided a total illuminated surface area of 98 sq. ft.   The culture was fed into one end and removed from the other end by means of 2-in. plastic pipe.   The suction and discharge pipes entered through the top of the tube in the gas space, as in the final arrangement of unit 1.   An improvement over unit 1 was the use of pools at the ends of the unit which added 3.5 in. of depth for submergence of the inlet and discharge pipes, permitting operation with lesser depths in the main part of the tube.   The gas entered one end through a 0.5-in. polyethylene tube and discharged at the other end through a 1.25-in. polyethylene tube.   The pump and heat exchanger from unit 1 were used.   During November and December the exchanger was used to heat the culture.   Other mechanical arrangements for this unit were similar to those for unit 1.

The only difference in operating procedures between unit 3 and unit 1 came about during the later phases of the work, when severe deficiency of some micronutrients was indicated by flask experiments.   Sediment in the concentrated microelement solution originally made up for unit 1 suggested coprecipitation of some of the metals, and therefore individual solutions of the microelements were prepared.   Although the addition of small amounts of individual microelements did not appear to solve the problem, it was recognized that storing standardized solutions of the individual elements and mixing them just before use is preferable to maintaining a single concentrated solution.   The use of the sequestering agent ethylenediamine tetraacetic acid (EDTA) was initiated, and much higher concentrations of microelements were used, as shown in table 5.   Since calcium chloride was included in the recommended mixture, a small amount of it was added, although flask experiments did not indicate that it w^s necessary.

The culture velocity was about 1 ft./sec at a depth of 2.5 in.   The loss in head over the 70-ft. length varied from about 0.75 in. at 2 in. average depth to about 0.25 in. at 3 in. average depth.   Tests of flow pattern were made before inoculation, using baffles of various kinds with colored confetti suspended in the water for visual observation.   Baffles more **than** 0.5 in, high showed too great a head drop to be practical for this operation, but 0.5-in. baffles at 45° to the direction of flow caused a good cross-flow pattern without serious loss in head.   Sufficient growth data were not obtained to indicate the value of baffles.

Results of unit 3.   The operation of unit 3 with average velocities from 1 to 1.5 ft./sec was satisfactory, and no settling occurred.   The **further** objective of investigating the effect of turbulence on growth, **however,** was not achieved.
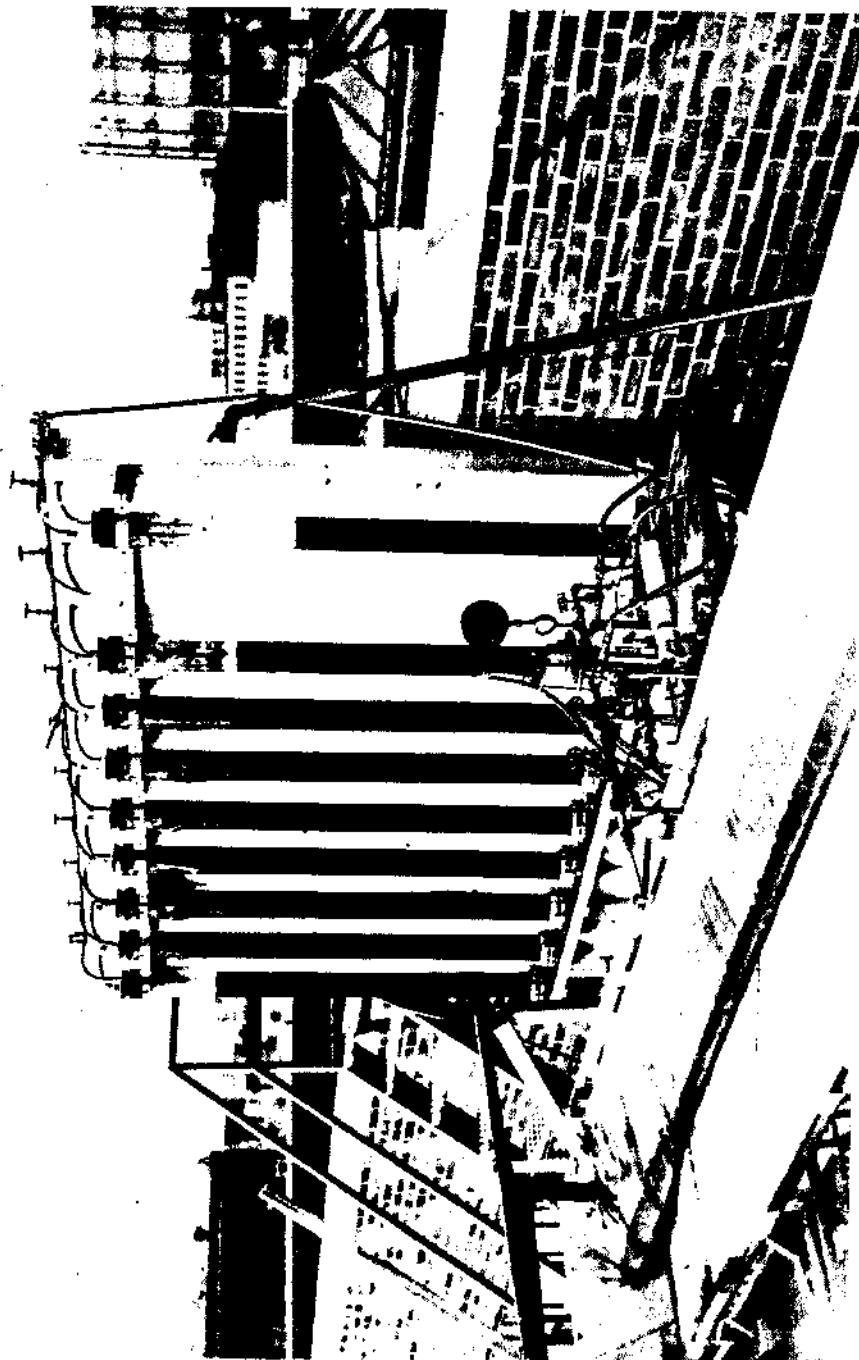
Fig. 9. Close-up of discharge end of unit 3, and vertical columns

Table 5
Microelement solutions for use with EDTA[a]

Salt	Solvent	Stock solution (g/D	Ion	Final ion conc, in culture (pprn)
CaCl$_2$.....................H$_2$C		8.35	**Ca^{++}**	30
Hutner's formula:				
H3BO3................H$_2$C		11.42	**B^{+++}**	20
FeSO$_4$-7H$_2$O.........dil. **H$_2$SO$_4$**		4.98	**Fe^{++}**	10
ZnSC$_4$-7H2O[b]......dil. H$_2$SO$_4$		8.82	Zn^{++}	20
MnCl$_2$-4H$_2$O.........dil. H$_2$SO$_4$		1.44	Mn+ +	4
H$_2$MoC$_4$(87% M0O3). dil. H$_2$SO$_4$		0.71	Mo^{++++++}	4
CuSO$_4$-5H$_2$O.........dil. H$_2$3C$_4$		1.57	Cu+ +	4
Co(NO$_3$)$_2$ • 6H$_2$O......dil. H$_2$SC$_4$		0.49	C$_0$ ++	1
EDTA stock solution .... IN KOH		100	EDTA____	500

[a] Myers, personal communication.
[b] The five salts may be made up in a single solution, or separately for mixing when needed.

The unit was inoculated at a concentration of about 0.04 g/1 on October 28 with healthy culture from two of the vertical columns. This culture showed no contamination with Chlorococcum or protozoa. The Volume of the culture was about 175 gallons with a depth of a little over 2.5 in. The pumping rate was 110 gpm, giving an average velocity slightly over 1 ft./ sec. Figure 10, in three parts, presents the history of unit 3 on the basis of change in wet cell volume and also shows the hours of sunlight per day and estimated daily growth. The latter figures were based on changes in culture volume as well as in concentration of algae. The figures for daily growth should not be considered accurate. There were difficulties in sampling, and variations in ratio of dry weight to wet cell volume were observed.

The first few days of growth appeared to be normal. Within a week after the start, a severe storm with gusts reaching 90 miles per hour drove gravel from a neighboring roof against the tube, making many small puncture holes. Some of the stones penetrated the tube. Thereafter contamination occurred, coupled with appreciable leakage, which was made up from fresh culture medium. The holes were patched before medium was added. Growth continued rapidly for the following 10 days, with a few values higher than 10 g/(m^2)(day) in spite of short days and some cloudy weather.

WET CELL VOLUME   (ML/IOO ML)   HOURS OF SUNLIGHT   GROWTH (G / M *, A.M.-AM.)
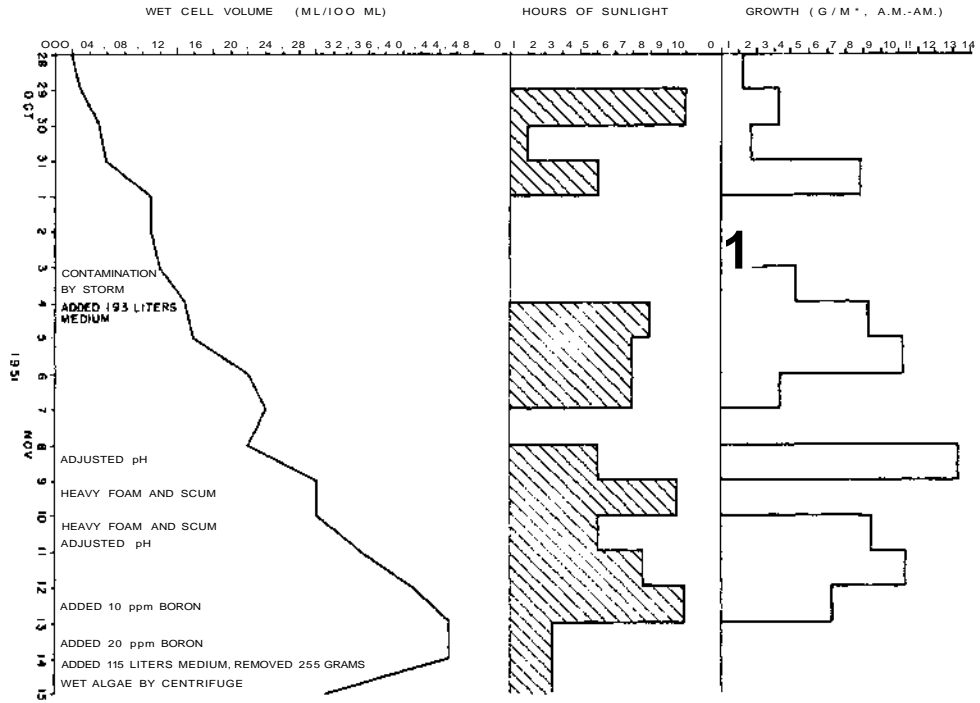
Fig. 10A. Growth data for unit 3

WET CELL VOLUME   (ML/100 ML)   HOURS OF SUNLIGHT   GROWTH CG/M*. A M.- A M)
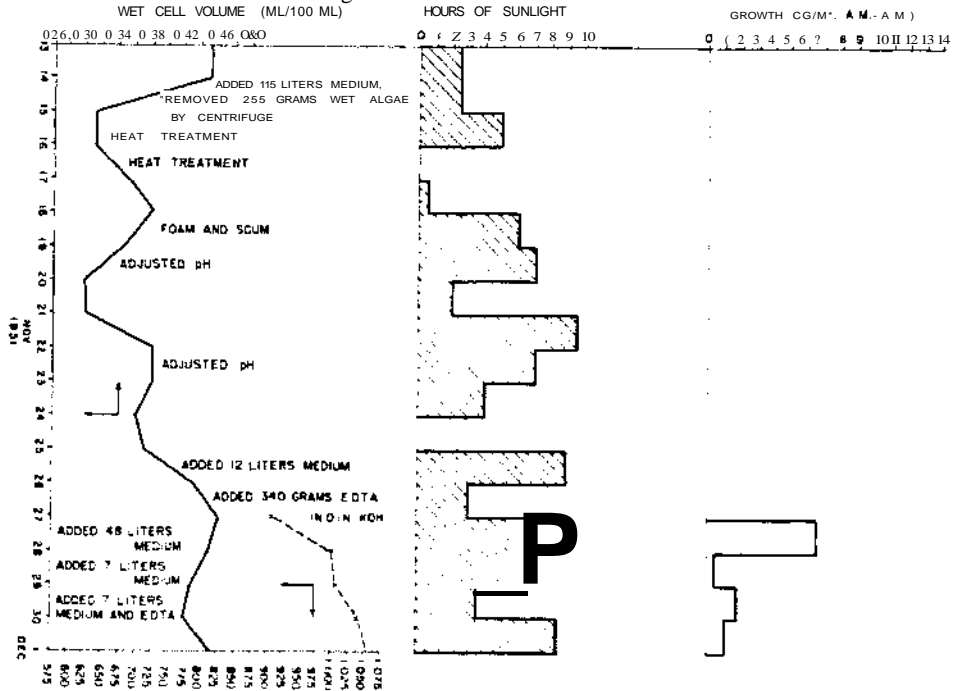
Fig. 10B. Growth data for unit 3
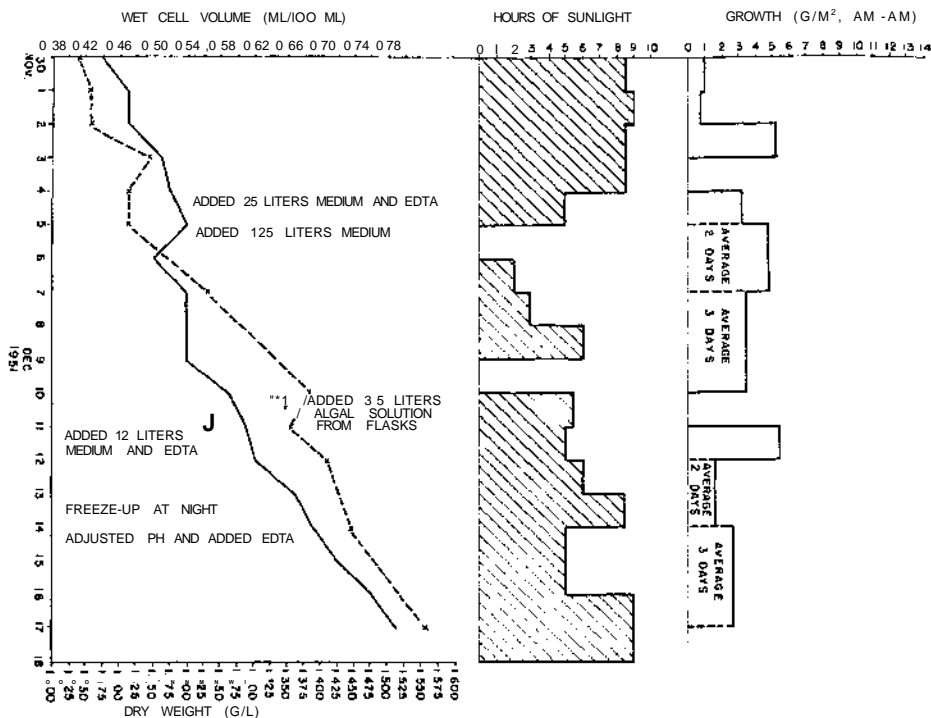
Fig. IOC. Growth data for unit 3

During this period infestation by rotifers became serious. The Chlorella concentration reached a maximum of about 0.9 to 1.0 g/1 dry weight (wet cell volume 0.45 ml/100 ml). Since no chemical means of selectively destroying the rotifers was known, centrifuging at higher flows to remove them was tested. About 55 g of dry algae were harvested and an additional quantity was lost in handling. This loss, combined with the replacement of a considerable volume of medium, reduced the wet cell volume to 0.31 ml/100 ml. Although the centrifuge was satisfactory for selective removal of contaminants, it was not suitable for large volumes. Therefore, the culture was heated to 45° C on November 15 and 16, and the rotifers were killed.

It appeared, however, in the following two weeks that the Chlorella was not healthy and that the ratio of dry weight to wet cell volume was low. This condition may have resulted from the heating and a simultaneous deficiency of microelements. The cause of the deficiency, if it existed, is not clear• Some of the microelements may have been removed by precipitation or taken out of solution by the rotifers. Throughout the period adjustments were being made in microelements, and on November 26 the system was converted to a high level of microelements with EDTA. The conversion was followed by a gradual readjustment of the ratio of dry weight to wet cell volume as shown by daily dry-weight determinations. This appears in the selected data given in table 6.

Table 6

Relation of wet cell volume to dry weight

(Samples from unit 3)

Date (1951)		Wet cell vol. (ml/100 ml)	Dl Twt. (g/1)	Factor
Nov. 27,	a.m.	0.46	0.9085	1.98
	p.m.	0.50	1.094	2.19
Nov. 29,	a.m.	0.43	1.018	2.36
	p.m.	0.41	1.030	2.51
Nov. 30,	a.m.	0.42	1.030	2.45
	p.m.	0.43	1.033	2.42
Dec. 1,	a.m.	0.45	1.056	2.35
	p.m.	0.45	1.072	2.39
Dec. 2,	a.m.	0.45	1.058	2.35
	p.m.	0.46	1.176	2.54
Dec. 3,	a.m.	0.50	1.150	2.30
	p.m.	0.50	1.164	2.34
Dec. 4,	a.m.	0.50	1.118	2.23
	p.m.	0.51	1.118	2.20
Dec. 7,	a.m.	0.52	1.232	2.37
	p.m.	0.52	1.221	2.35
Dec. 10,	a.m.	0.57	1.384	2.43
	p.m.	0.58	1.409	2.43
Dec. 11,	a.m.	0.59	1.358	2.30
	p.m.	0.60	1.458	2.43
Dec. 14,	a.m.	0.66	1.442	2.19
	p.m.	0.68	1.517	2.23

We may use the dry-weight estimates in figure 10C to study the growth rate qualitatively. During the last period of the operation of unit 3, however, the weather was inclement, with many days of only partial sunshine and some days with none. As in unit 1, growth did not always appear to correlate with hours of sunlight; this is indicated in figure 10. At one time the unit experienced a 5-in. snowfall, which it weathered successfully. On another occasion the circulation failed, and some ice formed on the surface of the culture. By December 18, much later than the planned termination of the operation, the weather as well as increasing mechanical problems made it seem advisable to shut down.

The final harvest was 7 pounds 14 ounces of wet material, which on the basis of the ratio existing at that time corresponded to a dry weight of approximately 1.8 pounds. In addition to this harvest there had been appre-

ciable losses, on the order of 400 to 500 liters of culture or almost the volume of the unit.

C

Supplementary Studies

Vertical Columns

Since the vertical columns bore no architectural relation to the units which are envisioned for large-scale growth, no attempt was made to measure the growth rate in them. This had been done previously at the Stanford Research Institute [279]. With an excess of gas for agitation and southern exposure of the column mounting, growth was rapid and healthy and the units were found to be adequate to produce and maintain culture for inoculation of larger units. Occasionally columns became contaminated, but in general their operation was normal.

Studies in Flasks

Nutrients. Throughout the program, flasks were used for supplemental tests as well as for growing fresh cultures from slants. In the early stages of the program, various types of nutrient were examined. Some tests on microelement requirements were made, which confirmed data in the literature. The flasks also served to confirm the use of ethylenediamine tetra-acetic acid (EDTA) as a chelating agent to sequester microelements at relatively high concentrations while providing solution concentrations below toxic levels. No harmful effect on Chlorella was noted, and there was indication that growth was more rapid. Flask studies of the medium in which algae had been grown in the pilot plant gave indication as to whether it was still suitable for the healthy growth of Chlorella.

Flow Study

A flow study was made by Dr. Arthur T. Ippen, professor of hydro-dynamics at the Massachusetts Institute of Technology. One purpose of the study was to assist in exploring the relation of turbulent flow to inter-mittent lighting of Chlorella cells. The analysis indicated that for a flow in a strongly turbulent condition, the distribution of algae from top to bottom of a culture 1 to 5 in. deep would be uniform. No basis exists for exactly predicting the statistical path of particles due to turbulent flow, but it may be reasonable to assume that the transfer of particles is pro-portional to the root mean square of the vertical velocity fluctuation due to turbulence. The mean path for a complete cycle from the top surface to the bottom of the channel and back to the surface can then be calculated* Particles will have paths both shorter and longer than this, but the calcu-lated mean is adequate to give the order of magnitude of the effect, Ippen

calculated cycle time as a function of velocity and depth (as given in fig. 11). The number of cycles per unit length is a function of depth only, but the length of time of one cycle varies with average velocity. Thus the actual intermittence of lighting is a function of depth, velocity, and distance of light penetration. The last factor depends on algal concentration. For long runs of plastic tubing, the drop in head due to fluid friction might prove excessive. The curves of figure 12 show the head loss per 500-ft. length of 4-ft.-wide tubing as calculated from Ippen's data.

A further limitation on the velocity of flow pointed out by Ippen is the so-called Froude number, a measure of surface stability. Velocities in excess of a critical value would result in surface undulations or standing waves, accompanied by surging. The limitations imposed by this phenomenon restrict linear velocities-to less than 1.5 ft./sec for channels 3 in. deep. The lines labeled $\bar{V}$crit on figures 11 and 12 indicate these limitations.

D

Handling the Product

Freezing

The freshly harvested material as it came from the centrifuge, containing between 20 and 25 per cent solids, was a heavy paste. Brief laboratory tests on freshly harvested material showed that it developed a strong odor after a few hours' exposure at room temperature. Most of the material produced in the pilot plant, therefore, was packed in l|-pint cartons and frozen immediately upon removal from the centrifuge bowl, and so stored. No loss of frozen material by spoilage was noted. Much of the material was shipped in frozen condition, packed in solid carbon dioxide.

Drying immediately after harvest was investigated, as it was considered to be of interest in any commercial process. Three drying methods were explored.

Spray Drying

Tests in a small spray drier at Bowen Engineering Corporation resulted in a product that was satisfactory as to appearance and odor. No operating problems were encountered when inlet temperatures of 300°, 400°, and 500° F and corresponding discharge temperatures of 150°, 165°, and 185° F were used. To permit feeding into the drier, the thawed paste was diluted from 25 per cent solids to 17 per cent solids. Sufficient information was obtained to confirm the feasibility of the methods and to permit preliminary estimates of the cost of commercial operation. No study was made of the effect of drying temperature on vitamins and other unstable constituents.
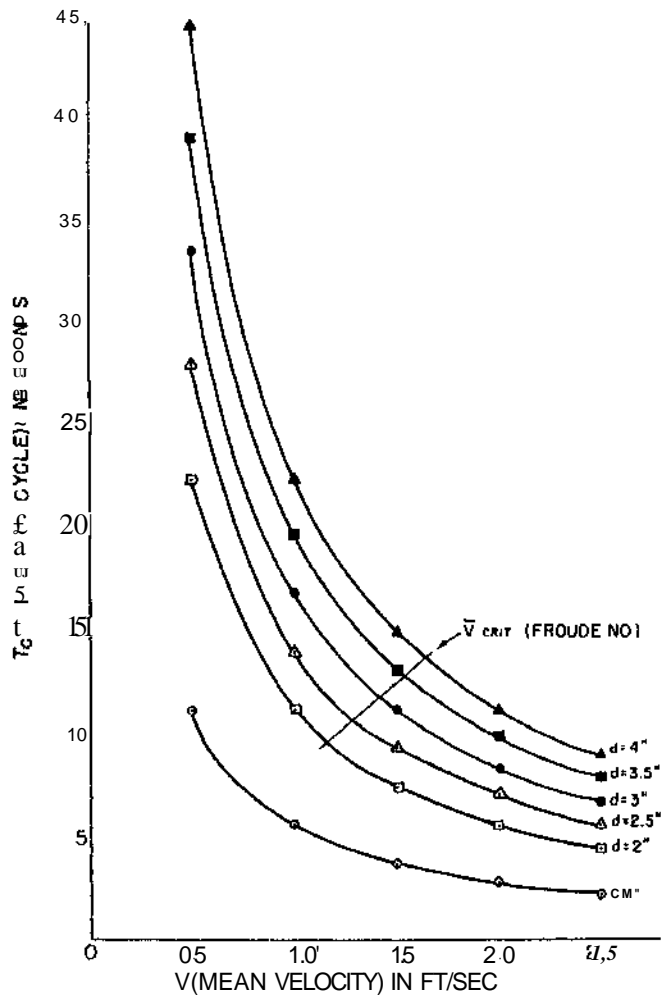
Fig, 11. Time cycles for turbulent flow in tubes
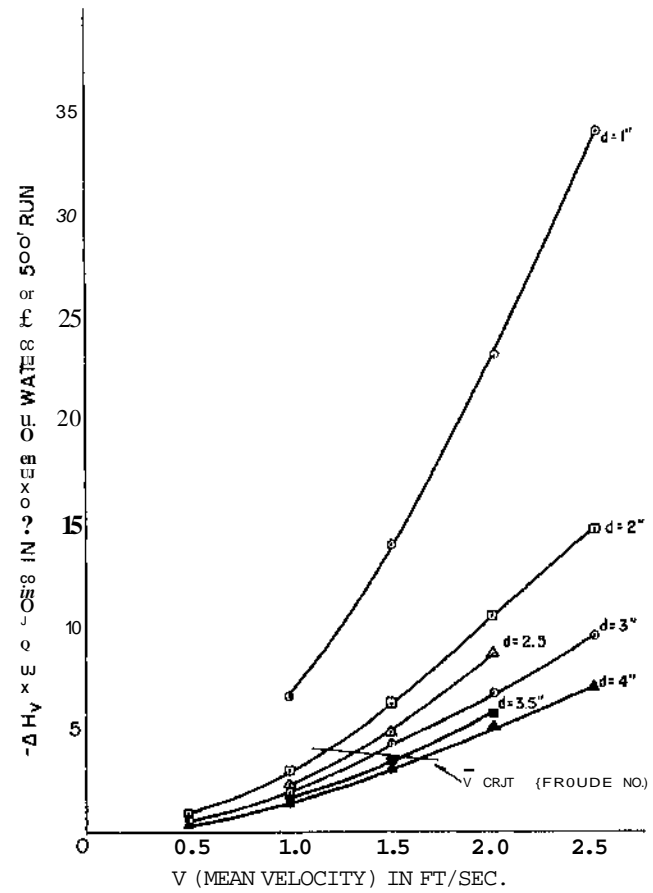
Fig. 12, Head loss for turbulent flow in tubes

Lyophilization

About 25 pounds of dry algae were prepared as a dark green, flaky, dried product by the use of a stainless steel vacuum belt drier at the Department of Food Technology of the Massachusetts Institute of Technology. The drying operation was normal in all respects. Though this method of drying from the frozen state is known to result in a good product with a minimum of decomposition, it is not likely to be used commercially because of its high cost.

Solvent Drying with Extraction

The VioBin Corporation generously agreed to test their azeotropic process, described by Levin and Lerman [245], for simultaneous defatting and dehydrating by means of organic solvents. The product obtained was light gray-green in color and had a somewhat milder flavor than the lyophilized product. In addition, a fat and pigment fraction of about 10 per cent of the dry weight of the algae was obtained which was very dark in color.

VioBin Corporation reported that the technique would be suitable for Chlorella, that the product would have good keeping qualities as compared with dry whole algae, and that the cost of drying on a large scale would be approximately 1 cent per pound of wet product as harvested. The use of mild hydrolysis to increase the fat removal is not believed to be necessary. It appears that the process would be useful, since the remaining protein would have high food value.

Hygroscopicity

The product from all three of the drying processes tested was hygroscopic. Samples of powder produced by each method were exposed to saturated air at room temperature in a desiccator. After 24 hours, the spray-dried material had increased 24 per cent in weight, the lyophilized product 37 per cent, and the VioBin sample 17 per cent. Other investigators have referred to the hygroscopic character of dried Chlorella as unfavorable to the particular applications with which4hey were concerned. The appearance of frozen Chlorella cells on thawing suggests extensive rupture of cell walls in the freezing process. It is possible that if fresh cells were dried they might remain intact and the hygroscopicity might thus be reduced.

E

Measurement Techniques

Determination of pH

Throughout the program, the hydrogen-ion concentration of cultures was determined several times daily with a standard **Beckman** laboratory pH meter.

Determination of Algal Concentration

Early in the work several determinations of wet cell volume were made in 100-ml Goetz tubes under laboratory centrifuging conditions. This work and available information from other laboratories indicated that wet cell volume was a sufficiently accurate index for determining population density of Chlorella. For a 20-minute centrifuging period at 2000 rpm, 1 ml of packed cells per 100 ml of culture corresponded to 2.2 g of dry solids per liter of culture.

Later, however, during the operation of unit 3, it was found that the conversion factor from wet cell volume to dry weight varied appreciably. Therefore, dry-weight measurements of algal concentrations were made for the last 20 days of operation of unit 3 by centrifuging 200 ml of culture, washing the cells with distilled water, centrifuging again, and transferring the concentrated cells to weighing bottles. Practically all the remaining water was removed in an oven at 50° C, and the drying was then carried to constant weight over concentrated sulfuric acid in a desiccator at room temperature. The finding of variations in the factor casts doubt on the accuracy of many determinations of algal concentration based on wet cell volumes.

Determination of Carbon Dioxide Concentration

The carbon dioxide content of the culture medium was not determined on a regular schedule. An analysis of culture was made with a conventional Van Slyke apparatus to check applicability. The correlation was satisfactory, and it is believed that the method can be used in future studies.

A Baird Associates Model A-929 Infrared Gas Analyzer was installed late in the summer. This was equipped with a Leeds and Northrup Model S Micromax four-point recorder and valving arrangement to provide automatic changing of sample source every 10 minutes. Three different samples plus cylinder nitrogen as a standard could be analyzed successively in an hour. Only a few readings were made. The results confirmed the satisfactory operation of the supply unit but did not determine the rate of consumption of carbon dioxide by the culture. On a few days, when growth conditions were good, reductions of 1 or 2 per cent in carbon dioxide concentration in the gas between the inlet and outlet of unit 1 were noted.

Determination of Concentration of Major Nutrients

In general the major nutrients were replaced on the basis of estimates of the amounts removed with material harvested. Occasional analyses of the culture were carried out by conventional quantitative methods to check the correctness of these estimates.

Although additions of nutrients were not frequently made in the pilot plant, the simplest method of operation on a large scale would seem to be to add nutrient materials continuously at a low rate and thus maintain a constant concentration. Daily calculation based on the amount of harvest would be a simple routine procedure which could be supplemented by analytical checks.

Determination of Microelement Concentration

The determination of the microelements in the culture is more difficult than that of the major elements, but it is believed that spectrographic analysis may be used successfully. The function of EDTA is discussed by Krauss in chapter 8. In one spectrographic analysis, made before the use of EDTA was begun, the concentrations of some of the elements could not be measured. The consumption of microelements is slow enough so that determinations need not be made frequently.

Visual Observation

Throughout the work frequent observations of culture samples were made under a microscope, using magnifications of 200 and 880 times. These observations were particularly important during periods when large contaminants were present, since these could be counted and a record kept of the effect of countermeasures. Standard plate-count techniques for the determination of bacteria seem satisfactory for routine use.

Illumination and Weather Data

Frequent outdoor light-intensity measurements for rough guidance of the program were made with a Weston Illumination Meter which had a range from 0 to 10,000 f.c. and could also be used for measuring intensities of artificial light for flask experiments. Since normal daylight varies so widely in intensity, however, no attempts were made to use these readings for correlating growth data. Published weather summaries provided by the U. S. Weather Bureau were used for general appraisal of the data. No determination was made of the actual intensity of light at the culture surface inside the growth tubes[9] nor was the amount of reflection from the plastic and liquid surfaces determined. New polyethylene film showed a transmission of 85 per cent, and samples exposed as part of unit 3 for 2 months showed somewhat lower transmissions, from 11 to 82 per cent. These values were determined in a standard Fisher comparator unit using light of 4250 Å.

F

Discussion of Results

Plastic Growth Tubing

The results with units 1 and 3 confirm the effectiveness of plastic tubes for the growth of Chlorella. Although several problems remain to be solved, it is believed that commercial operation with tubes of this type is feasible.

Auxiliary Equipment

The pilot-plant operation demonstrated that standard available types of equipment can be used for all auxiliary purposes. Circulation, cooling, harvesting, drying, and other operations involving the handling of cultures and products did not present any unusual engineering problems, nor did they affect the algae. Materials of construction are important; several plastics, stainless steel, and glass are known to be satisfactory.

Nutrient Medium

The composition of medium was not studied as a variable. Brief tests were made to confirm the standard pilot-plant medium, which was adapted from small-scale operations reported by Spoehr and Milner [151]. Micro-element composition both with and without EDTA was suggested by Myers (see tables 3 and 5 above) on the basis of work in his laboratory and elsewhere. Pilot-plant results did not demonstrate higher growth rates with EDTA, but the Department of Plant Biology (private communication, 1951) obtained some data suggesting that these can be expected. Though the pilot-plant results suggested that the composition of the medium was satisfactory, there is no reason to believe it was the best or cheapest possible. Other concentrations should be investigated, with emphasis on the minimum concentration feasible, assuming continuous replacement of material removed with the harvest. The suitability of less expensive sources of nitrogen such as ammonia or urea should be confirmed on a large scale. Flask tests on ammonia during this work were not conclusive because of insolubility of some of the resulting salts. The importance of microelements has been demonstrated, but the present lack of detailed data on specific elements and concentrations makes continued study desirable.

Carbon Dioxide

The growth data did not appear to indicate carbon dioxide deficiency at any time* All operations were performed with culture in contact with 5 per cent CO_2 in air. A brief study with 10 per cent CO_2 was inconclusive. The economics of operation suggests investigation of the lowest practical carbon dioxide concentration during growth in plastic tubes, where equi-

librium is probably not achieved. The effect of carbon dioxide concentrations during darkness has not been sufficiently studied to provide a basis for effective commercial operating procedures.

Recycle of Medium

The economics of algal culturr requires the conservation of water and nutrients by returning to the culture the centrifugate from harvesting. Re-use of medium had been previously considered, but there was some evidence to suggest that it could not be accomplished without special treatment. The pilot-plant experience with recycle carried out continuously for over two months, however, demonstrated that re-use without special precautions is a practical procedure. Old medium after centrifuging was used to grow fresh cells in flasks, and the results showed good growth with healthy cell division. In a few cases during the operation of units 1 and 3 some color appeared in the centrifuged medium, but after a few days of operation this cleared up, apparently with the help of bacteria, which reached high levels of concentration during these periods. The medium used in unit 1 grew a total amount of algae equivalent to 5 g/1 (dry weight); this yield is not so high as some attained elsewhere without harvesting.

Contamination

The pilot-plant data indicate that the presence of some bacteria may not constitute a critical effect in large-scale culture operation. Table 7 shows the data on bacterial counts taken in units 1 and 3 at intervals.

Table 7

Bacteria count

Unit 1		Unit 3	
Date (1951)	Colonies/ml culture	Date (1951)	Colonies/ml culture
Sept. 12	4,800,000	Oct. 30	6,500,000
19	7,525,000	Nov. 6	9,850,000
24	11,750,000	15	101,500,000
28	17,650,000	21	37,500,000
Oct. 18	102,500,000	27	5,750,000
		Dec. 3	9,850,000
		12	23,250,000
		17	3,725,000

These indicate that concentration of bacteria remains at a level which depends on the condition of the culture, and not on its age. The contaminants likely to cause serious trouble appear to be rotifers and other large organisms which can actually devour algae. Protozoa such as Amoeba and Vorticella, as well as molds, apparently did not have the capacity to grow

rapidly in healthy cultures, although their presence under certain conditions might be harmful. Algae which are not adaptable to the type of operation considered in the pilot plant can be a serious problem, as was demonstrated by the infestation of unit 1 with a Chlorococcum-type species. These algae upset operations by sticking to the walls of the tube and by settling out on the bottom.

Since the major contamination problems are expected to result from large organisms or algae which form clumps, it is believed that means of control can be developed. Though the use of heat was successful in destroying the rotifers in unit 3, it had an adverse effect on the algae. Also the cost involved would be high on a unit basis. On the other hand, selective centrifuging can be used, as was demonstrated in unit 3. This method is also adaptable to removal of clumping algae which are still suspended. Settling tests with the Chlorococcum indicated very rapid fall relative to Chlorella. Thus the use of an intermediate centrifugal force on the bulk of the culture should allow removal of a small side stream which would contain all the heavy organisms and clumped algae. This side stream could be further processed for harvesting. Other agents, such as antibiotics and high concentrations of boron, were tried briefly but were unsuccessful.

The operation of the pilot plant has clearly shown the advisability of immediate rejection of heavily contaminated cultures and the necessity of providing suitable means for cleaning out the culture unit after such a mishap before a new inoculum is introduced.

Temperature

Temperature was not studied as a variable in the pilot-plant work, although at times it was impossible to control it. With temperatures somewhat above 80° F on hot, clear days, there was no apparent damage to the algae^ but data from earlier programs suggest that growth rate is reduced when the temperature is 85° F or above. When temperatures over 110° F were used to destroy rotifers, the algae were not killed, although their growth was apparently inhibited for a time. Data from the Carnegie Department of Plant Biology indicate that daily temperature variations have a definite effect on growth rate. Night temperatures of about 60° to 70° F and 85° daytime temperature appear to cause better growth than constant-temperature operation at 77°. Although a night temperature of 60° F with a daytime temperature of 80° F was tried in unit 3, the results were inconclusive.

Higher temperatures of operation of culture would permit the use of a simpler and less expensive cooling mechanism. The optimum of 80° F for the Chlorella strain used here is low considering the water temperatures available for cooling in southern climates. The strains recently reported by Myers (chapter 4) as capable of high growth rates at temperatures as high as 102° F are promising and should certainly be tested in future pilot-plant studies.

Illumination

In the pilot plant the only source of illumination was natural sunlight. The light actually reaching the culture, reduced through reflection and absorption by the polyethylene film, was not measured. Furthermore, the variations in light intensity from dawn to dusk, plus day-to-day and hour-to-hour variations due to haze and cloudiness, made it difficult to correlate growth rate with light intensity in any quantitative sense. It was, however, possible to note gross differences in growth from day to day on the basis of the general weather conditions. Growth was generally, though not consistently, better on clear, sunny days {see fig. 10A, B, C).

Concentration of Algae

The highest concentration of Chlorella attained was the 1.5 g/1 maximum in unit 3 (see table 6). There was no evidence at this point that concentration was affecting growth adversely. It had been hoped that concentrations above 2 g/1 could be obtained in order to have a light penetration of only a small percentage of the total depth, to assist turbulence in producing an intermittent-light effect. There appeared to be no evidence from the pilot-plant units, the vertical columns, or the flasks that such concentrations would be harmful. Successful operation at concentrations considerably above this has been reported by Myers and the Department of Plant Biology.

The observation that a stable foam formed as a result of agitation of aeration of cultures at concentrations of algae above 0.3 to 0,4 g/1 is of some importance from the operational point of view. The layer of foam which tended to form could reduce the amount of light reaching the culture. In the pilot plant two anti-foam agents were used successfully after laboratory tests indicated that they were nontoxic. These materials, both previously used by Stanford Research Institute [279], were Dow-Corning Antifoam A, a silicone, and Span 85 produced by Atlas Powder Company. The optimum, quantities of these materials and the best methods of using them in large-scale operation were not worked out. These factors should be considered further, since they might be economically important.

Hydrodᣳnamic Problems

Since the present concept of large-scale culture equipment involves long runs of plastic tubing, it is important to know the effects of liquid velocity and depth on the loss in head? so that systems may be properly designed to run at approximately constant depth. No quantitative measurements were made in the pilot plant. The data provided by **Ippen's** calculations are believed to be sufficient for the present phase of the work. Confirming data should be obtained in later pilot-plant studies.

Ippen's data on head loss (fig. 12) show that this loss is an important consideration at velocities much above 1 ft./**sec,** and for runs of several

thousand feet could total several feet. In order to maintain a uniform depth, the surface under the tube might be graded either continuously or in relatively short steps. Since a minimum head drop is desirable, the ultimate choice of velocity and length of run will result from an economic balance between the advantages of high velocities and the cost of pumping and installation.

A more serious limitation, however, may be the surface instability above the critical Froude number. Operation above the critical velocity would result in surging which might decrease tube life. Calculations based on Ippen's data show that for 3 in. depth the maximum velocity will be less than 1.5 ft./sec, and even at a depth of 5 in., which is believed to be the greatest practical for the tubes, velocities must be kept below 1.8 ft./sec. The head losses corresponding to these maximum velocities are 0.59 and 0.55 ft. per 1000 ft. of tube. These values are indicated in figures 11 and 12. Since the data provided by Ippen are for ideal smooth-surface channels with free liquid surface, they should ultimately be checked by experiments when actual tubes are laid out under the conditions of commercial operation. Though it is not expected that deviations will be large, it may be necessary to provide for the increased power load and greater head drop.

Intermittent Light by Turbulent Flow

The laboratory data on the effect of flashing light on growth per unit of light energy (Kok, chapter 6; Myers, chapter 4) suggested to Dr. Vannevar Bush that a similar effect might be obtained with steady illumination by moving the algae in and out of the light. Use of the motion involved in turbulent flow appears to be a practical means for achieving this result. Although this effect, may have been achieved to some extent in unit 3, the low light intensities and generally unsatisfactory operating conditions prevented any definite conclusions. A rough analysis did suggest that the yield per unit of solar energy received was somewhat higher in unit 3 than in unit 1.

Ippen's data, based on hydrodynamic theory, suggest that time cycles for statistical movement of an algal cell from the bottom to the top of the stream and return would be between 10 and 20 sec in the range of velocities and depths which are now considered practical. If the concentration of algae were maintained at a level such that the light penetrated only about 10 per cent of the depth, then the average length of exposure of a cell to light would range from 1 to 2 sec. On the basis of laboratory data gathered by Kok, this exposure time appears to be 100 times as long as necessary to achieve the important increase in yield per unit of input energy realized by Kok. On the other hand, the data of Burk and coworkers [197] suggest that Intermittent exposure periods of perhaps as long as 30 sec can successfully be used in achieving the intermittent-light effect.

In general, the results from the pilot plant provided no new evidence *on* turbulent flow as a means for achieving increased growth by intermittent

illumination. On the other hand, there were no data to suggest that it may not be possible to obtain such an effect. In experiments at the Carnegie Department of Plant Biology (chapter 9, pages 135-138), a 70 per cent increase in yield was brought about by culture turbulence. Turbulence is one of the more important factors to be studied in future work, and it seems that the approach should be twofold. First, the effect of length of periods of intermittence of light on growth should be investigated in small laboratory-scale devices with flashing lights. Secondly, data on the effect of turbulence on the exposure of individual algal cells are needed.

Growth Inhibition and Variation in Cell Density

A general opinion was formed during the program that growth rate is temporarily slowed after sudden changes in environment. Although no quantitative data can be cited on this phenomenon, several times during the pilot-plant program unexpectedly low yields were obtained. It is believed that these periods of inhibited growth followed adjustment of pH, large temperature changes, addition of large quantities of fresh medium, and other such occurrences.

The phenomenon of variation in the density of individual cells with environmental changes is believed to be significant. Early experiments with cultures from flasks and unit 1 had established that 1.0 ml wet cells obtained by centrifuging in a 100-ml Goetz tube at 2000 rpm for 20 minutes was equivalent to 0.22-0.23 g dry cells. This ratio checked well with those reported by other investigators and was, therefore, used with measurements of wet cell volume as the only check on concentration during most of the program. Dry-weight determinations made during the latter part of the operation of unit 3 (figure IOC and table 6), however, showed that the dry weight of 1.0 ml of wet cells varied from 0.2 to 0.26 g. It was also observed in both unit 1 and unit 3 that the wet cell volume of an early morning sample was usually higher than that of the last sample of the previous day, the difference varying somewhat with environmental factors on the previous day.

The same factors which appear to cause temporary inhibition of growth seem to be factors in variation of the volume-to-weight ratio, although again the data are not conclusive. During periods when growth was inhibited by overheating, by cooling to near the freezing point, or by additions of salts or acid for adjustment of medium, the ratio tended to decrease below the supposedly normal level of about 0.22. EDTA with high microelement concentrations was added on November 26, and the ratio, as is shown in table 6, increased to nearly 0.26 over a period of a few days. Though the factor never became constant, most of the subsequent readings were between 0.23 and 0.25.

These observations suggest that fairly substantial errors can result through the use of the wet-cell-volume method of determining growth, if the weight-to-volume ratio is changing during the course of an experiment.

It therefore seems important that frequent checks by the gravimetric determination of dry weight of cells should be made during any operation.

yield per Unit Area

Taking into account the amount of energy received by a unit of area from the sun during the year in a location having conditions of average suitability for algal culture, and also considering the mechanism of photosynthesis as it is now known, it has been estimated that, with complete utilization of the visible spectrum, it should be possible to produce approximately 110 g of dry algae per square meter per day. Even under the best of conditions, this yield has never been approached. The operation of unit 1 yielded an over-all average of about 6 g/(m2)(day), figuring the area at its original 600 sq.ft. and taking no account of losses from leakage and handling or of the fact that for 20 days only 300 sq. ft. were in actual operation. For a 42-day period during which operating difficulties were at a minimum and the area was complete, the average yield was approximately 9 g/(m2)(day). For a shorter period of 10 days, 11 g/(m2)(day) were obtained. In unit 3, individual daily estimates higher than 10 g/(m2)(day) were recorded in spite of the relatively short days and generally poor weather. Following the destruction of rotifers by heating, the weather was particularly unsatisfactory for growth, and few pertinent growth data could be obtained. The over-all average for the 52 days was approximately 2 g/($_m$2)(day).

There are several factors which could not be included in the pilot-plant studies which should lead to an increase in yield. The first of these, of course, is improved weather conditions with a higher percentage of sunlight, such as might be found in the southern or southwestern part of the United States. In addition, use of the faster-growing strains of algae recently isolated by Myers would presumably add to the yield. The evidence suggests that these strains are capable of higher growth rates because they can utilize light up to a higher intensity than can the Chlorella strain used in this work. Recent experiments at the Department of Plant Biology have indicated that the high-temperature strain of Chlorella at 102° F gives the same yield as C. pyrenoidosa at 78° F, at the high cell densities used in mass culture (see chapter 9, pages 134-135). Furthermore, the intermittent-light effect due to turbulence may contribute to increased growth; this was not demonstrated. In order to obtain this increase it is believed that algal concentrations on the order of 2 to 5 g/1 will be necessary, and these were not achieved in the pilot units.

It is interesting to speculate, on the basis of pilot-plant data, as to the potential yield. Since there are many uncertainties, an exact analysis is not justified. On the 5 days of unit 3 from December 5 to 9, the yield was 4 g/(m^2)(day). This is believed to be a reasonable average figure. During this period the average sunlight was about 3 hours per day, as compared with 12 to 14 hours during good weather in the summer, which should give

a growth increase of from 4 to 5 times. Even with a daily average of 11 hours of sunlight as in the southwestern part of the United States, the factor should considerably exceed 2.5. And the annual average of total daily energy received per unit area is more than 2.5 times that of the winter solstice period when the data were gathered.

If no consideration is given to the new strains of algae and the effects of turbulent flow, optimum nutrient and temperature conditions, and other factors that should help to increase growth rate, correction only for hours of sunlight and amount of incident energy indicates a yield of 25 g/(m2) (day). Allowing for deviations from the assumptions used, it seems safe, on the basis of the other potentially favorable factors, to conclude that 20 g/(m2)(day) can be realized under advantageous climatic conditions with the type of equipment and the methods of operation used in the pilot plant. This yield approximates 2"g/(sq.ft.)(day), or 100 lb/(acre)(day) or 17.5 tons/(acre)(year). The 11 g/(m2)(day) figure for 10 days in unit 1 at low concentrations and without benefit of uniform suspension or high degree of turbulence is believed to be further confirmation of this conclusion. The unexplored possibilities leave a reasonable expectation that this figure may be improved, and this should be an objective of future work. Half this yield, however, more nearly reflects the actual experimental results obtained.

Chapter 18

PRE-PILOT-PLANT EXPERIMENTS ON ALGAL MASS CULTURE[1]

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Guided by the experience gained in the basic studies reported in chapter 16 of this monograph, we constructed a culture unit of pre-pilot-plant scale in order to get further information concerning the practice of large-scale algal mass culture. Although the experiments thus far conducted with this unit are preliminary, it may be of some use to present here a description of the unit and some of the results obtained to date.

A
Construction of the Unit

The unit consists of three main parts: the growth unit, the control house, and the gas-exchange tower. The general construction of the whole unit is illustrated in figures 1 and 2, the latter being a diagrammatic representation of the former.

Growth Unit

The growth unit consists of a concrete trough 15 m long, 1 m wide, and 20 cm deep, and an attached concrete sump 0.8 m long, 1 m wide, and 1.1 m deep; see figures 3 and 4. The trough has a level bottom and the top is covered with a polyethylene plastic sheet[2] (not shown in figs. 1, 2, and 4), which is supported by a number of arch-shaped iron frames attached to the walls of the trough (see fig. 4). The sump is covered with a metal lid (not shown in fig. 2). Between the trough and the sump there is a **wooden baffle,** by means of which the depth of the culture can be regulated between 2 and 15 cm.

The culture enters the trough through two openings at one end and goes out through an opening at the bottom of the sump at the other end. In one longitudinal wall of the trough there are several openings, through which the carbon dioxide-enriched air coming from the **gas-exchange** tower is

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* See Foreword, page iv.

[2] The plastic sheeting used was the same as that employed fey Arthur D. Little, Inc., in their work on algal mass culture (see chapter 17). We are indebted to them for this material.

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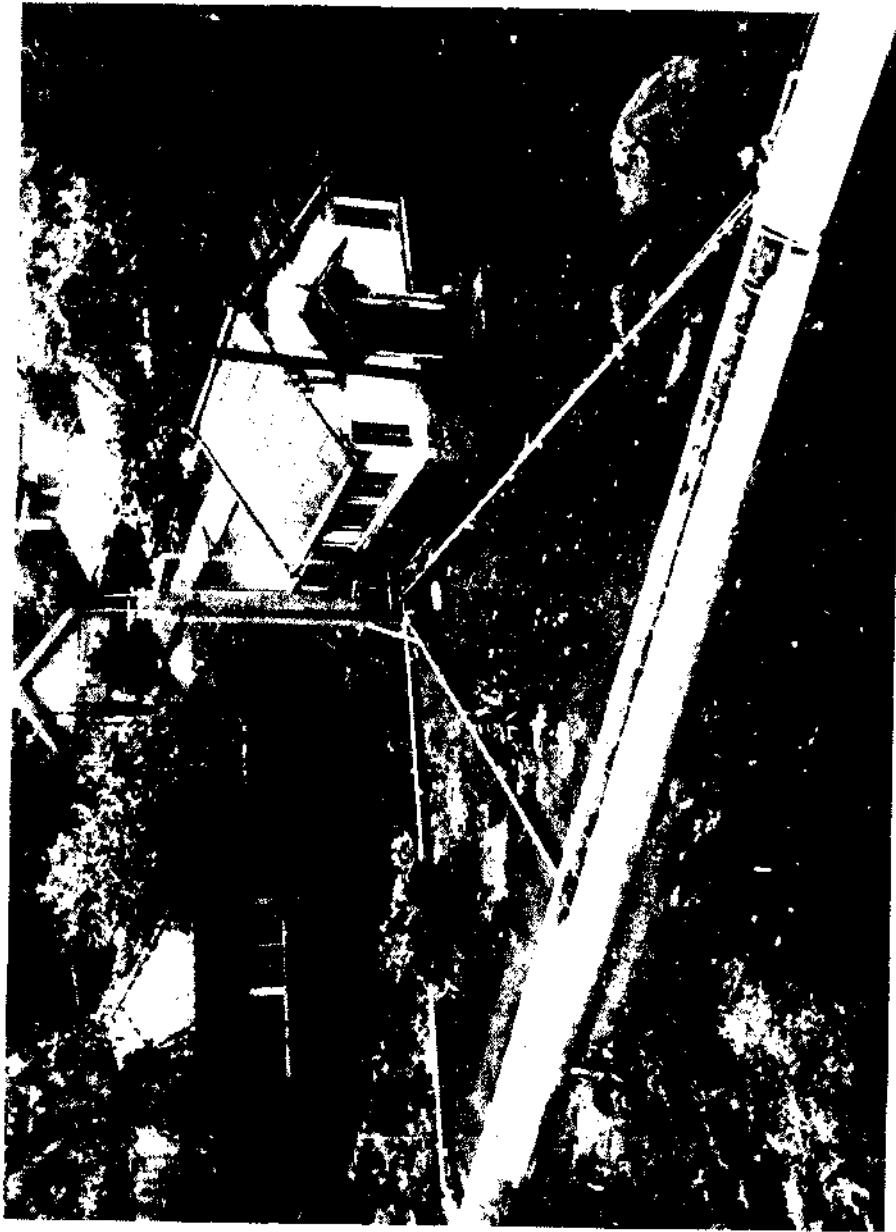
Fig. 1. General view of the unit
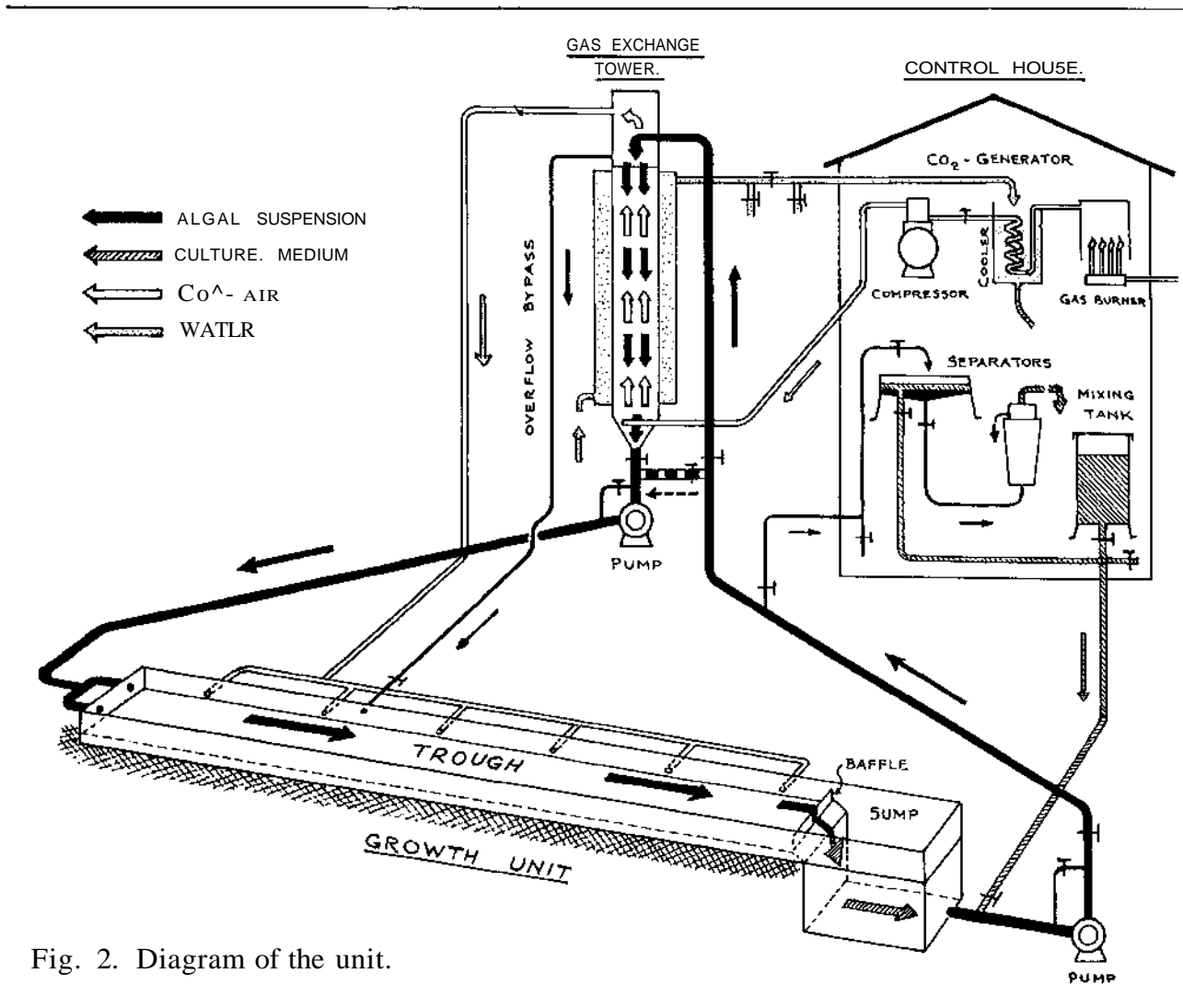
Fig. 2. Diagram of the unit.

Fig. 3. Growth unit with the trough cover fitted with plastic sheeting
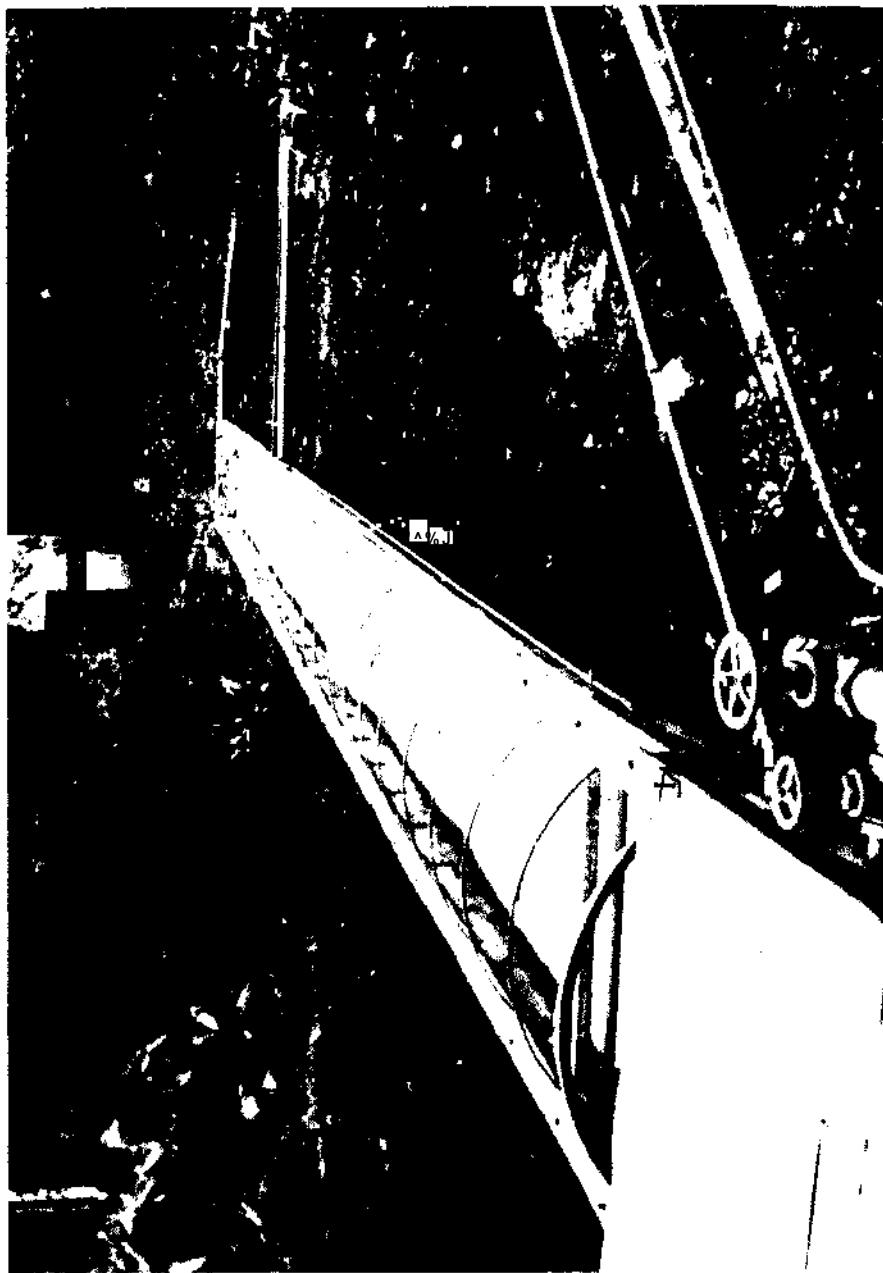
Fig. 4. Growth unit with the trough uncovered

bubbled into the culture. There is also an opening to admit the overflow of culture from the upper part of the tower.

Gas-Exchange Tower

The gas-exchange tower (fig. 5) is 4.25 m high and 0.5 m in diameter and is encircled with a cooling water jacket which can serve also as a heater for the culture in cold seasons. The culture and carbon dioxide-enriched air enter and leave the tower countercurrent as indicated in figure 2. The tower contains no packing, and the carbon dioxide in air is simply bubbled through the culture. The volume of the culture staying in the tower is 600 liters, the overflow being led to the trough as shown in the figure.

Control House

In the control house there are three units: a carbon dioxide generator, separators of algal cells, and a mixing tank for culture medium.

The air is enriched with carbon dioxide generated by burning coal gas. The gas stream is cooled by water, and sent to the tower by means of a compressor. This system feeds to the tower air containing 2.5 to 9.0 per cent CO_2 at a rate of about 24 liters per minute. [3]

To separate the algal cells from the medium, the culture is first introduced into a sedimentation vat, a flat funnel-shaped vessel with a diameter of 1.8 m and a depth of 35 cm at the center and 20 cm at the periphery. The supernatant fluid is fed back to the main circuit of the culture, and the sedimenting thick cell suspension is led to a Sharpies centrifuge. The clear medium separated from the cells is recycled to the main culture after its nutrient salt content has been adjusted.

The mixing tank for the culture medium is a drum of 200 liters capacity.

The culture is circulated in the system by two pumps (each driven by a 3-hp motor) as illustrated in figure 2. It can be circulated in two ways: either through the gas-exchange tower as indicated in the figure, or through the bypass shown in the middle of the figure. In the latter case, the carbon dioxide-enriched air is supplied to the culture in the trough only.

The total volume of culture may be varied between 950 and 3000 liters when the tower is used, or between 350 and 2400 liters when the tower is not used, according to the depth of the culture in the trough as well as the amount of the culture contained in the sump. The maximum rate of circulation of culture is 500 to 600 liters per minute, so that its linear velocity in the trough varies between 6 and 45 cm/sec according to the depth of the culture in the trough (15 to 2 cm).

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[3]
°According to the Tokyo Gas Company, the composition of the gas they supply is as follows (percent): H_2 3C.1, N2 23.0, CO 18,5, CH_4 15.2,-CO2 6.4, C2 3.8, C3H3 3.0.
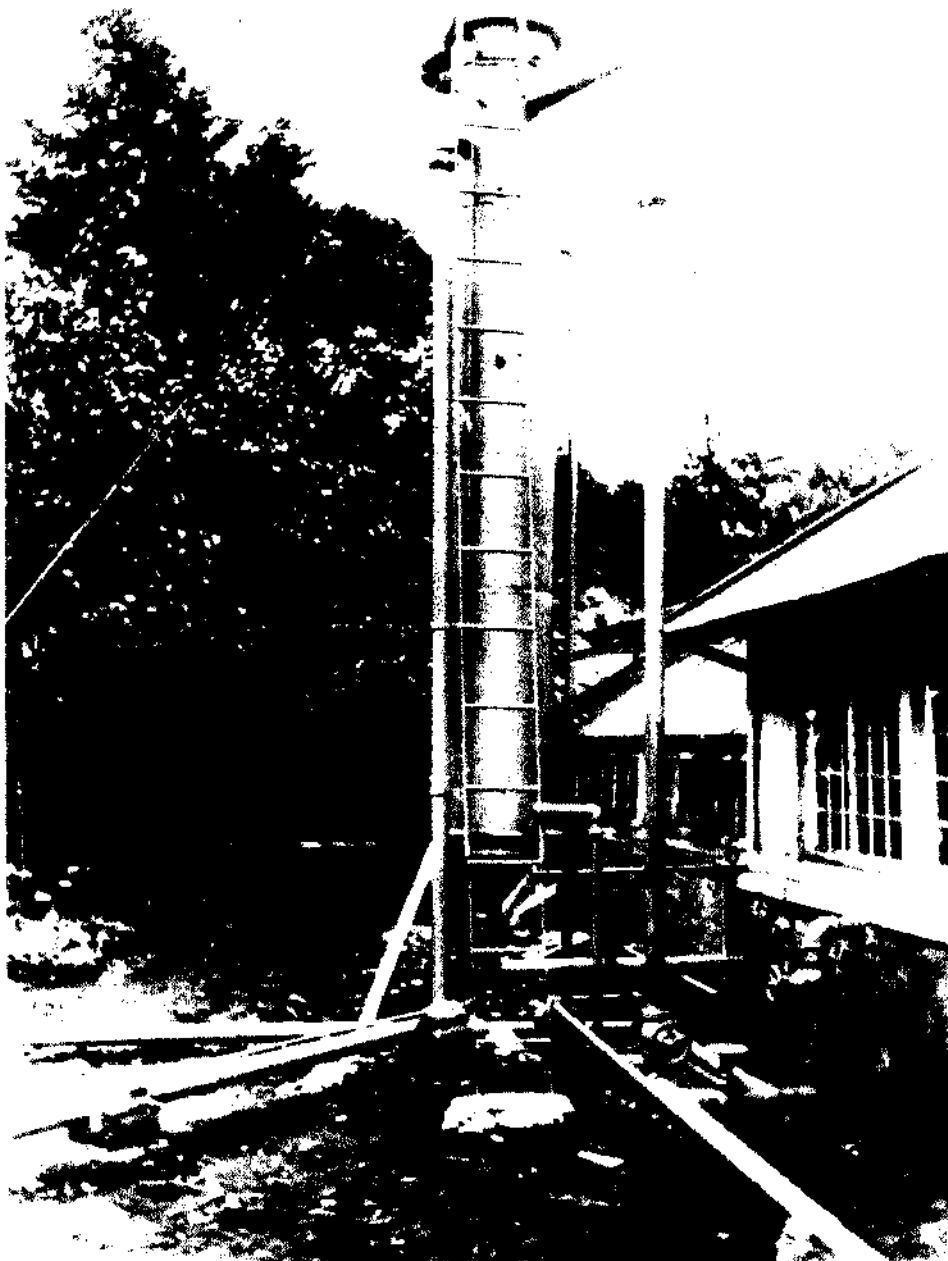
Fig. 5. Gas-exchange tower

The inner diameter of the main pipe is 3 inches. All pipes are of iron, and the inner surface of the pumps is partly of iron and partly of Babbitt metal. The concrete of the trough and sump was found to exert, immediately after their construction, an inhibitory effect upon algal growth, but the unfavorable effect totally disappeared when the concrete had been kept in contact with water and culture medium for about two months.

B

Results of Operation

A culture of Chlorella ellipsoidea run during the period from February 18 to 28, 1953, gave the following results.

Culture Conditions

The medium contained, per 1000 liters: urea, 3 kg; $MgSO_4 \cdot 7H_2O$, 2.5 kg; KCl, 670 g; H_3PO_4 (sp. gr. 1.74), 610 ml; NaOH, 440 g; ^{26407}-10 H_2O, 177 g; $CaCl_2 \cdot 2H_2O$, 110 g; $FeSO_4 \cdot 7H_2O$, 50 g; $ZnCl_2$, 41.6 g; ethylenediamine tetraacetic acid (di-sodium salt), 37.2 g; $CuSO_4 \cdot 5H_2O$, 15.9 g; $MnSO_4 \cdot H_2O$, 12.3 g; $3(NH_4)_2O \cdot 7MoO_3 \cdot 4H_2O$, 7.4 g; $CoCl_2 \cdot 6H_2O$, 4.7 g. The pH was 5.2. All chemicals used for the macroelements were of "technical" quality.

The total volume of the culture was 1200 liters, and its depth in the trough 3 cm (linear velocity of flow in the trough, 30 cm/sec). Under stationary conditions, the culture was distributed as follows: 450 liters in the trough, 150 liters in the sump, and 600 liters in the tower. Culture was circulated continuously, day and night, through the tower. The cooling jacket on the tower was left empty during the experiment.

The temperature of the culture was about 6° C at night and in the morning, and between 13° and 16° C in the middle of the day. The average light quantity per day was 44 kilolux-hour, measured in the open air. The intensity of sunlight was reduced to about 70 per cent by transmission through the plastic sheet. The initial population density was 0.48 ml/1 packed cell volume.

Results

The increase in population density in 10 days was 1.72 ml/1 packed cell volume or 0.43 g/1 dry weight. The yield from the whole culture system per day was 206 ml packed cell volume or 52 g dry weight. The yield per unit area of illuminated surface per day was 14 ral/m^ packed cell volume or 3.5 g/m2 dry weight. As far as microscopic observation of the algal suspension went, there was no recognizable contamination by other microorganisms.

According to the calculation made in chapter 16, figure 15, the daily yield (dry weight) at the light quantity of 44 kilolux-hour per day is expected

to be 1.7 to 4.1 g/m^2 in the temperature range from 7° to 15° C. The experimental result is roughly in agreement with these theoretical figures.

In the course of operation, we found several defects in the construction of our unit. It was found that, although the average linear velocity of flow in the trough was as much as 30 cm/sec, a certain quantity of algal cells precipitated here and there on the bottom of the trough owing to the unevenness of flow. This may be avoided either by increasing the velocity of circulation or by giving the trough a slight tilt along the direction of the flow. The cover of plastic sheeting also caused some trouble. It was found to be resistant to heaps of snow, at least up to 30 cm thickness, but was easily broken by such mechanical strains as those caused by dogs walking or running on it, as often occurred during our experiments. The inner surface of the sheeting was often covered with condensing water droplets, which appreciably reduced the available light intensity. Improvements are necessary to eliminate these shortcomings.

PART V

POSSIBLE USES OF MICROSCOPIC ALGAE

Chapter 19

THE CHEMICAL COMPOSITION OF ALGAE

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This chapter deals primarily with the composition of the green unicellular fresh-water algae. Very little information on composition is available for fresh-water algae other than the unicellular green ones and a few diatoms. The reviews by Smith [277] and Strain [282] include information on the components of marine algae, such as alginic acid, laminarin, agar, mannitol, and iodine, to mention but a small number.

In the following discussion emphasis is placed on work giving quantitative information about the amount or composition of algal constituents. References to the mere presence in algae of various constituents are noted briefly.

A

Composition of Plant Materials

Elementary Composition

A comparison of the elementary composition of marine algae, the leaves of land plants, and fresh-water algae shows that these materials fall into three somewhat distinct groups. For these determinations the marine algae were collected from the beach near Half Moon Bay, California, and are, of course, a tiny sample of the many species of marine algae. The leaves of land plants were collected at random from plants growing in the open without any experimental control. The fresh-water algae were grown in pure culture in the laboratory. Table 1 shows the chemical analyses.

Because of the wide variation in the ash content of these plant materials, 3 to 46 per cent, a comparison of the composition of the organic portion of their substance is greatly facilitated by converting the percentages of carbon, hydrogen, and nitrogen to an ash-free basis. In going from the marine algae to leaves of land plants to fresh-water algae in table 1, an over-all increase is noted in the percentages of carbon, hydrogen, and nitrogen. The difference between the sum of these percentages and 100 per cent, Which is the percentage of oxygen, shows a progressive

Table 1

Elementary analysis of some marine algae, leaves of land plants, and some fresh-water algae

Form	Ash[a] (%dry wt-)	Analysis (% ash-free dry wt.)			R-value
		C	H	N	
Marine algae:					
Gigartina Agardhii	17.82	42.90	6.27	5.83	29.85
Ulva sp	18.72	44.36	6.09	4.87	30.53
Amphipleura ratilans	46.24	46.52	6.48	5.67	33.67
Macrocystis pyriferab	37.66	46.02	6.82	6.60	32.24
Navicula torquatum	35.10	48.45	6.72	8.80	36.22
Egregia Menziesii[b]	31.74	49.97	6.29	5.39	36.27
Land-plant leaves:					
Corn	5.39	46.69	6.04	2.02	31.88
Begonia	12.92	45.73	6.39	3.04	32.00
Pine	3.13	51.83	6.52	2.40	37.74
Alfalfa	10.31	49.90	6.86	7.39	38.00
Castor	13.62	51.12	6.58	5.63	38.02
Sunflower	14.09	51.04	6.29	9.87	38.37
Nasturtium	9.58	50.46	6.91	6.84	38.45
Tobacco	11.80	50.87	6.78	7.70	38.77
Flax	12.25	51.91	7.01	7.11	40.08
Cedar	5.53	53.58	7.08	2.91	40.75
Fresh-water algae:					
Chlamydomonas sp	4.74	47.46	6.82	5.81	35.27
Anabaenopsis sp	9.35	49.57	7.00	7.28	37.88
Oikomonas termo	5.08	51.76	7.60	5.36	40.85
Stichococcus bacillaris	f 6.50	52.65	6.99	9.96	41.44
	\ 11.24	57.14	8.12	3.61	46.51
Chlorellapyrenoidosa	/ 3*45	49,51	6,78	9,31	37,92
	\ 3.46	70.17	10.53	1.43	63.33

[a] Analyses given in table 3 of chapter 8 show that the ash from corn and that from fresh-water algae contain about the same amounts of the same inorganic constituents.

^Only the blades of these two large algae were analyzed.

decrease within each group in going from top to bottom of the table: from 45 per cent oxygen in Gigartina to 18 per cent in the last sample of Chlorella.

Degree of Reduction

A decrease in the oxygen content of organic material denotes an increase in its degree of reduction, as shown by the R-values in the last column of table 1. The concept of R-value is explained in a paper by Spoehr and Milner [151], who devised it to express the level of reduction of the total content of organic matter in plant material. The formula for calculation of R-value is:

$$R\text{-value} = \frac{(\%^{Cx2}{}^{-664} + \%HX7,936 - \%O) \times 100}{398.9}.$$

The scale of R-values runs from zero for carbon dioxide to 100 for methane. The R-value, being calculated by a method similar to that used for calculating heats of combustion of pure compounds, should be proportional to the heat of combustion of the plant material. This was found to be true by Kok (unpublished data), who determined both R-value and heat of combustion of some of his samples of Chlorella.

The plant materials of highest and lowest R-value in each of the three groups in table 1 are compared in table 2 with some familiar sources of

Table 2

Elementary analysis, R-value, and heat of combustion of some plant materials compared with other substances

Material	Analysis (% ash-free dry wt.)			R—value	Heat of combustion[a] (kcal/g)
	C	H	N		
Glucose	40.00	6.71	0	26.69	3.74
Starch	44.44	6.22	0	29.70	4.18
Gigartina	42.90	6.27	5.83	29.85	. . . . . .
Egregia	49.97	6.29	5.39	36.27	. . . . . .
Corn leaf	46.69	6.04	2.02	31.88	. . . . . .
Wood[b]	49.52	5.99	0.62	34.00	4.77
Cedar leaf	53.58	7.08	2.91	40.75	. . . .
Chlamydomonas	47.46	6.82	5.81	35.27	. . . .
Stichococcus	57.14	8.12	3.61	46.51	. . . .
Protein[c]	51.3	6.8	18.8	42.23	5.7
Chlorella pyrenoidosa	$\begin{cases} 49.51 \\ 70.17 \end{cases}$	$\begin{matrix} 6.78 \\ 10.53 \end{matrix}$	$\begin{matrix} 9.31 \\ 1.43 \end{matrix}$	$\begin{matrix} 37.92 \\ 63.33 \end{matrix}$	. . . .
Fat[d]	73.61	11.26	0	67.77	9.3
Petroleum, crude	87.94	11.21	. . . . .	80.82	10.5
Methane	74.87	25.13	0	100.00	13.14

[a] The heats of combustion are those given in the Handbook of Chemistry and Physics (Chemical Rubber Publishing Co., 23d edition).

b Analysis for maple, heat of combustion of beech or birch.

c Analysis for edestin, heat of combustion of albumin.

d Analysis for castor oil, heat of combustion of olive oil.

food and fuel. The elementary analysis and R-value are shown for each material. The heat of combustion, where it is known, is shown for comparison with the R-value. See section B of chapter 5 for a determination of the heat of combustion of Chlorella. The elementary analyses in tables 1 and 2 are by Milner, mostly unpublished work. Inspection of the figures in table 2 shows how the analysis and R-value of plant materials may vary,

depending on whether low R-value carbohydrate, intermediate R-value pro-
tein, or high R-value fat is the major component.

Protein, Carbohydrate, and Lipide Content

The protein content is commonly calculated to be 6.25 x percentage of
nitrogen. When the R-value and protein content of a plant material are
known, its composition in terms of protein, carbohydrate, and lipide may
be calculated [151]. It is assumed that average plant protein, carbohy-
drate, and lipide have R-values of 42, 28, and 67.5 respectively. Two
simultaneous equations can be written:

$$\%PX42 + \%CX28 + \%LX67.5 = \text{R-value X } 100\%$$

$$\%P + \%C + \%L = 100\%,$$

where P, C, and L signify protein, carbohydrate, and lipide. Substituting
the known R-value and percentage of protein, algebraic solution of the two
equations gives the percentages of carbohydrate and lipide.

In order to show the wide variation in the protein-carbohydrate-lipide
composition of plants in the natural condition, and the even wider varia-
tion induced experimentally in Chlorella, table 3 is presented. The plant
materials are those in table 1, with the addition of two more examples of
Chiorella in order to show the highest and lowest observed percentages
of protein, carbohydrate, and lipide in samples of this alga grown under
different conditions.

The examples of marine algae given in table 3 contain about one-third
to one-half protein, very little lipide, and much carbohydrate. The calcu-
lated lipide content of the marine algae may be low, and the carbohydrate
high, because in some of these plants a substantial fraction of the carbo-
hydrate is probably polyuronide. Polyuronide has the R-value 22.7, instead
of 28 which was used for carbohydrate in the calculation. If we call all the
carbohydrate in Gigartina polyuronide, its calculated composition will be
0.3 per cent lipide with 63.3 per cent carbohydrate and^Se.4 per cent pro-
tein. Making the same assumption for Ulva, we have 5.0 per cent lipide,
64.6 per cent carbohydrate, and 30.4 per cent protein.

The protein content of leaves of land plants varies from one-eighth in
corn to nearly two-thirds in sunflower. The R-values of these leaves are
in general higher than those of the marine algae, because of the presence
of relatively more iipide in the leaves.

Effect of .Environment

As a group-the fresh-water algae shown in table 3 have a higher R-
value, hence higher energy content, than the marine algae or leaves of
land plants. The examples of Stichococcus represent two out of five cul-
tures of different age and grown under different light intensity. The five

Table 3
Calculated composition of some marine algae, leaves of land plants, and fresh-water algae

	R- value	Calculated composition (% ash-free dry wt.)		
		Protein	Carbohydrate	Lipide
Marine algae:				
Gigartina Agardhii................	29.85	36.4	63.6	trace
Ulva sp...........................	30.53	30.4	69.6	trace
Amphipleura rutilans.............	33.67	35.4	62.8	1.8
Macrocystis pyrifera.............	34.24	41.2	57.6	1.2
Navicula torquatum..............	36.22	55.0	43.7	1.3
Egregia Menziesii................	36.27	33.7	57.2	9.1
Land-plant leaves:				
Corn.............................	31.88	12.6	82.0	5.4
Begonia..........................	32.00	19.0	77.6	3.4
Pine.............................	37.74	15.0	65.7	19.3
Alfalfa...........................	38.00	46.2	44.9	8.9
Castor...........................	38.02	35.2	51.9	12.9
Sunflower........................	38.37	61.7	33.9	4.4
Nasturtium.......................	38.45	42.8	45.9	11.3
Tobacco..........................	38.77	48.1	4.1.7	10.2
Flax.............................	40.08	44.4	40.8	14.8
Cedar............................	40.75	18.2	56.0	25.8
Fresh-water algae:				
Chlamydomonas sp...............	35.27	36.3	58.2	5.5
Anabaenopsis sp..................	37.88	45.5	45.6	8.9
Oikomonas termo.................	40.85	33.5	45.8	20.7
Stichococcus bacillaris .... {	41 44	62.3	25.8	11.9
	46.51	22.6	38.5	38.9
	{ 37.92	58.0	37.5	4.5
Chlorella pyrenoidosa.............	42 41	88.2	6.6	5.2
	61.87	7.3	9.5	83.2
	63.33	8.7	5.7	85.6

R-values were 41.44, 42.83, 43.89, 45.77, and 46.15, and the five composi-
tions varied between the limits shown. Hence, an analysis of a single sam-
ple of a given plant must not be considered representative of all samples
of the same species, because composition can change in response to change
in environment.

The range of variation observed in the composition of Chlorella prob-
ably has not been exceeded with any other plant material. It is not thought
that this is a property peculiar to Chlorella. No other plant species has
been subjected to so extensive experimentation with regard to the effect of
environmental conditions on chemical composition. If one were to investi-
gate thoroughly the effect of environmental conditions on the composition
of other green unicellular algae, it seems likely that one or more species
might show as much variation in composition as is found in Chlorella.

Over three hundred pure cultures of <u>Chlorella pyrenoidosa</u> were grown in the study of its composition in relation to environment. No two cultures had exactly the same elementary analysis, hence chemical composition. The variation in any one determined quantity appeared to be distributed fairly evenly over its range. Perhaps as good a way as any to indicate the variation observed is to cite the upper- and lowermost values determined for each quantity. Ash varied between 1.36 and 20.21 per cent of the dry weight. Carbon, hydrogen, nitrogen, and oxygen by difference are expressed as percentages of the ash-free dry weight. Carbon varied from 49.51 to 70.71 per cent, hydrogen from 6.78 to 10.53 per cent, nitrogen from 1.17 to 14.11 per cent, and oxygen from 17.87 to 34.40 per cent. The range of R-values ·was from 37.92 to 63.33. The calculated compositions ranged from 7.3 to 88 per cent protein, from 5.7 to 38 per cent carbohydrate, and from 4.5 to 86 per cent lipide. (See also table 4.)

Ketchum and Redfield [68] also grew algae under different environmental conditions, determined the elementary composition and R-value, and calculated the protein, carbohydrate, and lipide content. They grew and analyzed <u>Stichococcus bacillaris</u>, <u>Chlorella pyrenoidosa</u>, <u>C. vulgaris</u>, <u>Scenedesmus obliquus</u>, <u>S. vasiliensis</u>, and <u>Nitzschia Closteriurn</u>. Their values for analysis and calculated composition of <u>Chlorella pyrenoidosa</u> and <u>Stichococcus bacillaris</u> fall within the range for the same two species given above. The elementary analyses and calculated compositions of all six species of algae were roughly the same.

B

Lipides in Algae *

Much more attention has been given to the fat content of algae than to the carbohydrate and protein fractions. In 1921 Beijerinck [188(a)j observed that as the fixed nitrogen content of the medium ran low in old cultures of diatoms, fat accumulated in the cells. Since then, accumulation of microscopically visible droplets of fat in diatoms has been reported by a number of investigators.

Lipides in Chlorella

In order to determine the composition of the lipides of <u>Chlorella pyrenoidosa</u>, Milner [91] grew four lots of the alga under different conditions. These lots had the R-values, calculated compositions, and actual lipide contents shown in table 4.

The total iipide content of each lot of Chlorella was extracted with solvents, then dried to constant weight. The solvent-free extracted lipide expressed as percentage of the dry weight of the Chlorella, which is listed in the last column,, is in good agreement with the calculated value. This

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[1] *See* chapter 12 for new data *by* Fogg and Collyer.--Ed.

Table 4

R-value, calculated composition, and actual lipide content of four lots of Chlorella
grown under different conditions

Lot no.	R-value	Calculated composition (% ash-free dry wt.)			Lipide found
		Protein	Carbohydrate	Lipide	
1	42.45	46.4	33.4	20.2	23.4
2	45.74	27.3	37.5	35.2	33.2
3	54.87	13.1	23.5	63.4	63.0
4	59.56	7J3	15^0	77J.	75.5

confirms the validity of the method described in the preceding section
for calculating the composition of plant material.

The total lipide from each lot of Chlorella was saponified and sepa-
rated into three fractions: fatty acids, unsaponifiable matter, and water-
soluble saponification products. The amounts of these fractions are shown
on lines 2, 3, and 4 of table 5. Total fatty acids from each lot of cells were

Table 5

Analysis of the lipides of Chlorella

Analysis	Chlorella lot no.			
	1	2	3	4
1. Total lipide (% of Chlorella)	23.37	33.17	62.96	75.51
Composition of total lipide				
2. Fatty acids (% of lipide)	28.0	49.5	83.0	86.8
3. Unsaponifiable fraction (% of lipide)	12.0	7.7	3.3	3.3
4. Water-soluble, after saponification (% of lipide)	60.0	42.8	13.7	9.9
5. Calculated fat <% of Chlorella)	6.85	17.2	54.7	68.6
Analysis of total fatty acids				
6. Palmitic acid, C_{16} (%)	16.6	10.9	7.9	11.4
7. Stearic acid, C_{18} (%)	0.4	4.1	3.9	3.5
8. C_{16} unsaturatett acids (%)	29.1	18.3	27.2	18.0
9. C_{18} unsaturated acids (%)	53.9	66.7	60.9	67.1
10. Total fatty acids (%)	100.0	100.0	99.9	100.0
11. Equivalent weight	269.5	273.6	272.7	274.1
12. Iodine no. (Hanus)	163.1	143.8	143.6	125.3
Degree of unsaturation				
13. C_{16} + C_{18} unsaturated acids	-4.4 H	-3.6 H	-3.7 H	-3.2 H
14. C_{16} unsaturated acids	-4.1 H	....	-4.4 H	....
15. Cjg unsaturated acids	-4.5 H	....	-3.4 H	....

separated into saturated and unsaturated acids by the usual lead soap pro-
cedure. The separation of Cjg from C^g acids of lots 1 and 3 was made
by fractional distillation of their methyl esters at reduced pressure. From
lots 2 and 4 of Chlorella the quantity of fatty acids obtained was too small
to perform the methyl ester fractionation in the distillation apparatus used,

therefore only the lead soap separation into saturated and unsaturated acids was made, and the quantity of Ci6 and Ci8 acids in each fraction was determined by titration. The quantity of fatty acids having less than 16 or more than 18 carbon atoms, if any, was too small to be determined in the quantities of lipides available for analysis. Therefore in table 5 the sum of $C\backslash Q$ and CJS acids is taken to be 100 per cent of the fatty acids from each lot of Chlorella.

Analysis of the total lipides (lines 2, 3, and 4) shows a marked increase in the fatty acid content as the total amount of lipide increases, accompanied by a corresponding decrease in the unsaponifiable fraction. The increase in lipide content of Chlorella is mainly due to the accumulation of fatty acids. No significant accumulation of hydrocarbons can have occurred.

It would have been of interest to determine the nature of the considerable quantity of water-soluble saponification products (line 4), which were discarded. Chlorophyll degradation products could account for about half of this fraction for lots 1 and 2. The chlorophyll content of Chlorella decreases rapidly as the lipide content increases. Lot 1 of Chlorella had 6 per cent of the dry weight as chlorophyll, whereas lot 4 had only 0.03 per cent. Glycerol would constitute part of the material included in line 4, a substantial part of the total for lots 3 and 4.

From the total fatty acid content of each lot of cells, the corresponding quantity of triglyceride was calculated. This calculated fat content is shown in line 5 for comparison with the lipide content shown in line 1. As the total lipide content increases from 23.37 to 75.51 per cent of the Chlorella, true fat increases from 6.85 to 68.6 per cent of the cell mass. Or, the true fat content of the total lipide fraction varies from 29 to 91 per cent.

Chlorella fats are highly unsaturated; but as the lipide content increases, there is a significant decrease in the degree of unsaturation of the fatty acids (lines 12 to 15). The average equivalent weight of the fatty acids (line 11) is almost equal in lots 2, 3, and 4, and is slightly lower in lot 1. With the exception of the acids from lot 1, the percentage of the different **fractions-** (lines 6 to 9) does not show a clearly defined relation to the change in fatty acid content of the Chlorella. Most of the saturated acid is palmitic, with only a small amount of stearic acid.

Unsaturation is expressed in lines 13 to 15 as the deficiency in number of hydrogen atoms required for saturation of the carbon chain of the acid. That is, one double bond between carbons is equivalent to -2H, two double bonds to -4H, three double bonds to -6H, and so on. A remarkable feature of the fatty acids from Chlorella is the great unsaturation of the Cjg liquid acids (line 14). The iodine number, **217.2** (not shown in table), of the Cig unsaturated acids from lot 3 requires the presence of at least 17 per cent of triply unsaturated acids in this fraction. The occurrence of triply unsaturated Cjg acids in plant fats is rare. Triply unsaturated Cjg is required only *in* lot 1 in order to account for the iodine number of that fraction. **Comparison** of the acids from lots 1 and 3 (lines 13 to 15) shows that the over-all decrease in unsaturation with increase in fat content can be attributed to the Cjg acids, since the **Cjg** acids of lot 3 are even more unsaturated than those of lot 1,

Lipides in Other Algae

Lovern [81] gives analyses of the fatty acids of three green algae other than Chlorella. The fat content of the algae is not specified, but the composition of the total fatty acids is expressed in a way similar to that used in table 5. Lovern's values are shown in table 6. Note that $C^\wedge$ and C20 acids, which are not found in Chlorella, make up 11 to 22 per cent of the total fatty acids in these algae. Acids with less than 14 or more than 20 carbon atoms, except in Oedogonium, were not found in the three algae analyzed by Lovern.

Table 6

Composition of fatty acids of algae (from Lovern's data)

Acids	Percentage of total fatty acids in		
	Nitella opaca	Oedogonium sp.	Cladophora sauteri
C14 saturated 6		2	12
unsaturated . . . . 3 (-2.0 H)		0	trace
C^g saturated 18		20	10
unsaturated . . . . 34 (-2.5 H)		32 (-3.1H)	19 (-4.7 H)
C^g saturated 3		1	2
unsaturated . . . . 23 (-4.5 H)		35 (-4.6 H)	49 (-3.8 H)
C20 unsaturated . . . . .13 (-5.8 H)		9 (-? H)	8 (-7.1 H)
C22 unsaturated		1 (-? H)	...

Lovern also presents analyses of the fatty acids of five marine algae, which do not differ materially from the analyses shown in table 6 except that the marine algae have relatively more C20 acids and less Cjg acids than the fresh-water algae. Attention is directed to the occurrence in Cladophora of highly unsaturated Cjg acid, similar in quantity and degree of unsaturation to that found in lot 3 of Chlorella, table 5.

Deuticke, Kathen, and Harder [31] report analyses of the lipides from Chlorella sp., Scenedesmus sp., and a diatom, Nitzschia palea. The total lipides were obtained by extraction of the algae with ether. The authors do not state the percentage of total lipide isolated from the cells. The components of the total lipide, estimated by an adsorption procedure, are shown in table 1 of chapter 10. Etheric oils amounting to 1.5, 2, and 9.5 per cent of the total lipides from Chlorella, Scenedesmus, and Nitzschia, respectively, were separated by steam distillation. The iodine numbers of the triglycerides were 93-168 for Chlorella, 136-139 for Scenedesmus, and 86-116 for Nitzschia. The corresponding isocyanate numbers were 59-94, 85-90, and 59-64. It is stated that the isocyanate numbers continued to increase upon elution of the fats from an alumina adsorption column.

The saponification numbers of the triglyderides were 188, 189, and 192 respectively. From this the authors conclude that all the fatty acids are

about Cjg- They postulate the composition of the fats from each of the three algae. Chlorella is thought to contain two fats: stearoleolinolein and trilinolein. Scenedesmus is said to contain but one fat, stearodilinolein. Two fats are given for Nitzschia: stearoleolinolein and stearodilinolein.

Harder and von Witsch [54] describe the mass culture of diatoms under conditions which increase the fat content of the organisms. 2 They also state [55] that in young diatom cultures the cells have 4.7 to 10.8 per cent fat, whereas in old cultures the fat content amounts to 40 to 50 per cent of the dry weight of cells. Photomicrographs showing fat globules in the diatoms are included in their paper.

Mazur and Clarke [248] analyzed the fats from marine diatoms. They found a large fraction of the total fatty acids to exist in the free state.

C

Proteins and Amino Acids in Algae

Protein appears to be the most abundant constituent of the unicellular green algae, with the exception of the high-lipide cultures which have been described. The identities and properties of the proteins of algae have not been determined. It is not known whether different species contain different proteins, nor whether within a single species there is a change in the nature of the proteins accompanying a change in the total protein content. In the case of low- and high-lipide Chlorella (table 3), the latter situation is considered unlikely because the actual mass of protein in a culture does not appear to change much as the cells go from the normal 50 per cent protein, 20 per cent lipide state to the high-lipide, low-protein state.

All the essential amino acids were found to be present in an aggregate amount equal to 42 per cent of the protein content of a sample of Chlorella pyrenoidosa. This sample, which had a calculated protein content of about 50 per cent and R-value about 42, was analyzed by Merck and Company (private communication). Eny [37] also has identified some of the amino acids of Chlorella.

Fow'den [41] has identified and determined the relative quantities of twenty-three amino acids from Chlorella pyrenoidosa, C. vulgaris, and a blue-green alga, Anabaena cylindrica. He determined the free amino acids in an alcohol extract, then determined the combined amino acids after hydrolysis of the insoluble portion. The amino acids were separated and identified by paper chromatography. All the essential amino acids occurred in each of the three algae. Fowden's results are presented in table 7.

Identification of amino acids and sugars formed by photosynthesis in Scenedesmus, Chlorella, and higher plants was made by Calvin et al. [199] through use of tracer techniques.

※ A summary of this work is presented by these **authors** in chapter 10.--Ed.

Table 7
Amino acids of algae (Fowden's analysis)

Amino acid	Chlorella pyrenoidosa		Chlorella vulgaris		Anabaena cylindrica	
	Free	Comb.	Free	Comb.	Free	Comb.
Cystine	+	+	+	+	++	+
Aspartic acid	+	++	+	+	++	+ +
Glutamic acid	++	++	+ + +	+ +	+ + +	+
Glycine	++	++	++	+ +	+ +	+ +
Serine	++	++	++	+ +	+ +	+ +
Asparagine	-	-	+	-	-	-
Threonine[3]	+	+ +	++	+ +	+	+ +
Alanine	+++	+ + +	+ + +	+ + +	+ +	+ + +
Glutamine	+	-	+	-	+	-
Citrulline	+	-	++	-	-	-
/3»Alanine	-	-	+	-	-	-
Tyrosine	+	++	+	++	+	+ +
y-Amino-butyric acid	+	-	+ + +	-	-	-
Lysine[a]	+	+ + +	+	+	+	+
Histidine[a]	-	+	>	+		- + +
Arginine[a]	+	++	+	++	+	+ +
Proline	+	++	++	++	-	+
Methionine[a]	+	+	+	+	+	+
Valine[a]	++	++	+	++	+	+ +
Phenylalanine[a]	+	+	+	+	+	+
Isoleucine[a]	+	++	+	+	-	+ +
Leucine[a]	++	++	++	++	+	+ +
Tryptophane[a]	-	+	-	+	-	+

[a] Essential amino acid.

D

Carbohydrates in Algae

Little is known of the identity of the various components of the carbohydrate fraction of the unicellular green algae. This may be because carbohydrate is the least abundant of the major constituents, hence of less interest than the fat or protein. Starch (Milner? unpublished work) and sucrose [91] have been isolated from Chlorella pyrenoidosa and identified.

Brown [14] determined the quantities of several classes of carbohydrate present in Scenedesmus obliquus. The values of four samples were: free reducing sugar, 0.1-0.5 per cent; sucrose, 0.8-3.4 per cent; oligosaccharide other than sucrose, 0.7-1.2 per cent; water-soluble polysaccharide, 0.1-0.6 per cent; insoluble polysaccharide, 15.1-16.9 per cent; total carbohydrate, 17.8-20.2 per cent of the dry weight of the alga. Between 10 and 15 per cent of the oligosaccharide other than sucrose was pentose. There was 10-20 per cent pentose from the water-insoluble polysaccharide.

References to the carbohydrate components of marine algae may be found in the review by Smith [277]. See also the work of Calvin et aL [199].

E

Minor Components of Algae

Minerals

Not much attention has been paid to the composition of the ash of algae. An important factor influencing the ash content of Chlorella is the composition and concentration of the mineral nutrient solution [68, 151]. In a given medium, the ash content of the cells showed a decrease as the age of the culture, and the total amount of organic matter produced, increased [151], Some exceptions to this are noted by Scott [149], who investigated the change in composition of the ash of Chlorella as influenced by varying the ratios of calcium, magnesium, potassium, and sodium ions in the medium.

Sterols

A number of investigators have reported the presence of sterol in algae, with no indication of the kind or quantity. Chondrillasterol, which is of potential usefulness for cortisone synthesis, was found by Bergmann and Feeney [191] to constitute about 0.2 per cent of the dry weight of Scenedesmus obliquus, with little of any other sterol present. Chlorella (unpublished) appears to contain about 0.1-0.2 per cent sterol, of a kind not of interest for cortisone synthesis. 3   in comparison with these figures, the sterol content up to 3.6 per cent of the total lipide of Chlorella, Scenedesmus, and Nitzschia reported by Deuticke et aL [31] (see table 1 in chapter 10 of this monograph) appears to be too high.

Pigments

There is a large body of literature dealing with plant pigments, including those of algae. With one or two exceptions the subject of pigments falls outside the scope of this chapter. The interested reader is referred to the reviews of the subject by Strain [282], Cook [211], and Carter, Heilbronn, and Lythgoe [207]. Two pigments of interest from the standpoint of utilization of algal products are chlorophyll and carotene. Haskin [226], using a spectrophotometric method, made pigment analyses of Chlorella pyrenoidosa. On the basis of the dry weight of cells, he found 1.97-2.04 per cent chlorophyll a, 0.55-0.58 per cent chlorophyll b, 0.267-0.310 per cent xanthophyll, and 0.0441-0.0448 per cent carotenes. Analyses of Chlorella grown under different environmental conditions Fl51] showed

^ A current search for sterols in algae that could be used in this synthesis is described in chapter 22.--Ed.

that on the basis of dry weight of cells the chlorophyll $\underline{a}$ + $\underline{b}$ varied from 6 per cent to less than 0.01 per cent. Carotenes in the same samples varied from 0.16 per cent to less than 0.002 per cent. The carotene was principally the beta isomer, the precursor of vitamin A.

Vitamins ^

The vitamin A content of algae may be inferred from the carotene content where that is known. Thus, Chlorella can be a very rich source of this vitamin.

Von Witsch [175] has determined the quantity of vitamin Bj in three algae. He found in the case of Chlorella that the vitamin Bj content varies widely with the conditions of growth and the age of the culture. He also determined fat content of the algae. Expressing the vitamin Bi content in gammas per gram of dry cells and the fat in percentage of dry weight of cells, he found in a young Chlorella culture with low light intensity and without added carbon dioxide 18.Qy Bj and 10 per cent fat. The other extreme, an old culture grown with high light intensity and 1 per cent CO_2, had 1.8y Bj and 34 per cent fat. Values are given for other conditions. In Scenedesmus sp. the Bj content was 1.5-6.5y/g. Young cultures of Nitzschia palea had 4-5.5y/g, and old cultures 0.25-0.38y/g of Bj. Gerdes [223] gives vitamin Bj analyses of 6 fresh-water and 31 marine species of algae. The Bj content varies between 0.35 and 10.5y/g dry weight.

Chlorella contains from 0.3 to 0.6 mg ascorbic acid (vitamin C) per gram of fresh cells [201(a)]. This is about equal to the vitamin C content of lemon juice, one of the recognized best sources of this vitamin. The vitamin C on a dry-weight basis would be 0.12-0.24 per cent, but unfortunately much of the vitamin is lost on drying, even in the absence of oxygen. The loss is also rapid on storage of fresh Chlorella.

Dam [25] determined the vitamin K in Chlorella vulgaris and in four species of photosynthetic bacteria. The bacteria had a vitamin K content of 0.4-1.0y/g dry weight. Chlorella had six times as much, 6y/g.

F

Distribution of Algal Components

In the literature there are several hundred papers dealing with substances found in algae. Listed below are a number of representative substances, with an indication of the algae in which they have been found. Most of this information was taken from standard reference works on algae: Manual of Phycolpgy, edited by Gilbert M Smith [276]; Morphologic und Biologie der Algen, by Friedrich Oltmanns [250]; and Freshwater Algae of the United States, by Gilbert M. Smith [275]. Substances mentioned earlier in this chapter are included. The information on antibiotics is from a recent paper by Pratt et al. [262].

4 **Further** data for vitamin content of Chtareila are given ie chapter 20.--Ed.

The substances found in algae are listed below in alphabetical order. The name of a substance is preceded in most cases by an Arabic number to indicate the occurrence of that substance in a list of algae which follows. Some of the substances are of such general, limited, or uncertain distribution among algae as to make it undesirable to assign key numbers to them. In these cases the distribution of the substance may be indicated better by words.

Following the list of substances is a skeleton outline of the algae, following the usual taxonomic arrangement but including only those in which substances of the first list have been found. All algae are separated into seven divisions, indicated in the list by Roman numbers and capitalized names. Each division is separated into classes, with names ending in -eae or -ae. Each class contains one or more orders, with names ending in -ales. Sixty or more orders are known, about half of which are listed here. The names of the hundreds of families composing the orders are omitted. Out of the thousands of genera into which the families are divided, only the genera of algae mentioned in this chapter are listed under their respective orders.

Cross reference between the two lists, to show in which algae the substances have been found, is by means of the Arabic and Roman numbers. The Arabic number preceding the name of a substance identifies that substance in the algal list and is placed after the class or order of algae in which the substance is found. A Roman number following the name of a substance refers to the division to which belong algae containing that substance. Two examples will illustrate the use of the key numbers.

In the alphabetical list of substances, 14-Chitin-I means that the number 14 is placed after every class or order of algae in which chitin is found. The I means that chitin is found in algae of division I (Chlorophyta). The number 14 is then found following the orders Oedogoniales and Cladophorales, which contain chitin. A second example: What substances have been found in a given order of algae, say, Ulvales? The numbers 4 6 18 40 55 follow the name Ulvales in the list of algae. These numbers in the first list identify antibiotics, ascorbic acid, dehydrogenase, oxidase, and vitamins respectively. In addition to these compounds, algae of the order Ulvales might be expected also to contain the substances of more general distribution in the class, Chlorophyceae, to which Ulvales belong.

Al^iab^icaLI^sLoLSubstances with Their Key Numbers

1-Agar-VII
2-Algin, Alginic acid-V
Algulose, probably synonymous with cellulose
3-Anthocyanin-I
4-Antibiotics-I, V, VII
5-Arabinose? combined-VI
6-Ascorbic acid-I

7-Auxins-I, V

Bromine is accumulated, to a less extent than iodine, by some marine algae.

8-Calcium compounds-I, III, V, VII

9-Callose instead of cellulose in cell wall-I

10-Carbon monoxide, 5 to 10 per cent of the gas in floats of Nereocystis-V

11-Carbohydrase-V

Carotenes: beta carotene is found in all seven divisions; alpha and epsilon carotene and flavacin occur infrequently.

12-Carrageenin, not identical with agar but very similar-VII

Catalase is widespread among algae.

13-Cellulose-I, IV, V, VI, VII

14-Chitin-I

15-Chloride is accumulated in cell sap-I

Chlorophyll a is found in all seven divisions; each division except CYANO-PHYTA contains one other chlorophyll, which may be b, c, d, or e.

Crocetin, Crocin, see Sexual substances

16-Cyanophycean starch, differs from both starch and glycogen, but called glycogen by some workers-VI

17-Cytochrome c-VII

18-Dehydrogenase-I

19-Diastase, Dextrinase-I, VII

20-Dulcitol-VII

21-Fats, Oils-I, III, IV, V, VI

22-Ferric compounds, principally in cell wall-I, II, IH

23-Floridean starch, allied to starch and glycogen but not identical with either, hydrolyzes to glucose-VII

24-Floridose or Floridoside, soluble carbohydrate composed of one molecule each of galactose and glycerol-VII

Fucin closely resembles algin.

25-Fucoidin, the calcium salt of "Fucoidins&ure," hydrolyzes to yield the methylpentose, Fucose-V

26-Fucosan, a brownish water-soluble pigment resembling tannins but not precipitated with ferric chloride-V

Fuscochlorin, Fuscorhodin, pigments

Gamones, see Sexual substances

Gloeocapsin, a pigment

27-Glucose-I, V

28-Glycogen (see Cyanophycean starch)-HI, VI, VII

Gynotermones, see Sexual substances

29-Hematochrome-I, II

30-Hemicellulose-I, VI

31-Indole acetic acid (auxin)-I, V

32-Iodine-I, V

Iron, see Ferric compounds

33-Laminarin, a polysaccharide found only in V, built of possibly 16 glucose units, with a disaccharide, Laminariose, corresponding to the maltose of starch or the cellobiose of cellulose.

34-Leucosin, a material of unknown structure, assumed to be carbohydrate-III

35-Lipase-I, V, VII

36-Mannitol-V

37-Mucilage-I, V

38-Mucus bodies-II

39-Nitrate is concentrated in cell sap 500 to 2000 times its concentration in the surrounding water-I

Oils, see Fats

40-Oxidases-I, VII

41-Paramylum, an insoluble carbohydrate related to starch, hydrolyzes to glucose-II

42-Pectic compounds, Pectin, Pectose-I, III, V, VI, VII

43-Pentose-I, V

Peroxidase is present in most algae.

Phycobilins: Phycocyanin and Phycoerythrin are pigments of VI, VII.

Phycoporphyrin, a pigment in I

Picrocrocin, see Sexual substances

Pigments: the name, nature, and distribution of 44 algal pigments is given by Strain [284].

44-Polypeptide, octapeptide of glutamic acid-V

45-Potassium is concentrated in cell sap of some algae-I, V

46-Proteinase-V

Protocrocin, see Sexual substances

Quercitin, see Sexual substances

Rutin, see Sexual substances

Safranal, see Sexual substances

47-Sexual substances-L These are: Protocrocin, Picrocrocin, Crocin, Safranal, cis- and trans-Crocetin dimethyl ester, also Rutin and Quercitin. They influence the motility and mating reactions of Chlamydomonas. They are also referred to as gamones or termones.

48-Silica, mostly in silicified cell walls-Ill

49-Sodium is accumulated in some algae-I

50-Sorbitol-VII

51-Starch-I, IV

52-Sterols-I, in, V

53-Sucrose-I

Termones, see Sexual substances

54-Trehalose-VII

55-Vitamins-I, HI, V, Vn

56-Vblutin (metachromatin)-I, III

Xanthophylls: each division of algae contains one or more of the some 20 xanthophylls; none is present in all the divisions.

Algae in Which Substances Listed Above Are Found

I. CHLOROPHYTA (mostly fresh-water algae, some marine; all green,
        many unicellular, some large)
    Chlorophyceae 13 19 21 29 35 42 51 (10 orders)
            Volvocales 8 22 29 47 56
                    Chlamydomonas
            Ulotrichales 29 55
                    Stichococcus
            Ulvales 4 6 18 40 55
                    Ulva
            Cedogoniales 14 30
                    Oedogonium
            Cladophorales 14 55
                    Cladophora
            Chlorococcales 4 6 15 27 43 45 49 52 53 55
                    Chlorella
                    Scenedesmus
            Siphonales 7 8 9 31 32 39 43 44 55
            Zygnematales 3 8 22 37 55
    Charophyceae
            Charales 8 13 15 45 49 51 52
                    Nitella
II. EUGLENOPHYTA (green algae, largely fresh-water)
    Euglenophyceae 22 38 41
            Euglenales 29
            Colaciales 29
III. CHRYSOPHYTA (yellowish-green to golden-brown algae, largely
        fresh-water but many marine forms)
    Xanthophyceae (Heterokontae) 21 34 42 48 (5 orders)
            Heterococcales 22
            Heterosiphonales 55
    Chrysophyceae 21 34 42 48 (5 orders)
            Chrysomonadales 8 28
                    Oikomonas
    Bacillariophyceae (diatoms) 21 34 42 48 56 (2 orders)
            Pennales 52 55
                    Amphipleura
                    Navicula
                    Nitzschia
IV. PYRROPHYTA (yellowish-green to dark-brown algae, many marine)
    Dinophyceae 21 51 (10 orders)
            Dinococcales 13
    Cryptophyceae 21 51 (2 orders)
V. PHAEOPHYTA (golden-brown algae, nearly all marine, mostly large)
    Isogeneratae 2 13 21 28 33 42 (5 orders)
            Ectocarpales 27 55

V. PHAEOPHYTA--<u>Continued</u>
  Isogeneratae- -<u>Continued</u>
      Sphacelariales 55
      Dictyotales 8 36
  Heterogeneratae 2 13 21 26 33 42 (5 orders)
      Chordariales 35 46
      Desmarestiales 7 31
      Punctariales 55
      Laminariales 2 4 7 10 11 13 25 27 31 32 35 36 37 45 55
        Macrocystis
        Nereocystis
        Egregia
  Cyclosporeae 2 13 21 26 33 42
      Fucales 4 7 13 27 31 36 37 43 44 52
VI. CYANOPHYTA (blue-green algae, both fresh-water and marine)
  Myxophyceae 13 16 21 28 42 (3 orders)
      Oscillatoriales 5 30
        Anabaena
        Anabaenopsis
VII. EHODOPHYTA (red algae, mostly marine)
  Rhodophyceae 12 13 19 23 24 28 35 42 (10 orders)
      Bangiales 17 55
      Nemalionales 54 55
      Gelidiales 1
      Cryptonemiales 4 8
      Gigartinales 1 4 40 55
        Gigartina
      Rhodymeniales 4 24
      Ceramiales 20 50 55

    This review of the chemical composition of algae is by no means complete. As was stated at the beginning, the emphasis is on what is known of the quantitative composition of the green unicellular fresh-water algae. The marine algae are mentioned only briefly, and the multicellular fresh-water algae and orders other than the greens have been considered to be outside the scope of this chapter.

Chapter 20

NUTRITIONAL VALUE OF MICROSCOPIC ALGAE

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Present knowledge of the nutritional value of algae is limited, largely because of the lack of experimental material. The small amount of quantitative information available applies only to animal nutrition. It is supplemented by assays of Chlorella for different nutritional factors and by some qualitative observations concerning the use of algae in human nutrition.

A

Nutritional Factors in Chlorella

Protein

Interest in the use of microscopic algae as food concerns their high protein content. As may be seen from table 3 of chapter 19, the protein content of microscopic fresh-water algae is extremely variable. In the case of Chlorella pyrenoidosa, at least, this variability is directly related to environmental conditions during growth; and it is possible to control those conditions so that a protein content of at least 50 per cent is regularly attained [151].

This protein content is appreciably higher than that of the best vegetable sources of proteins used in animal feeds, as shown in table 1. Animal sources of protein, such as tankage and fish meal, are the only ones that have a higher content. Nut meats used as human foods contain only 8 to 34 per cent protein.

Amino Acids

High protein content is not sufficient to ensure a nutritious food. The protein must contain a suitable balance of the essential amino acids. One way of determining whether such a balance is present is by feeding tests with experimental animals; some preliminary results of such tests will be

Table 1

Protein content of Chlorella compared with that of typical animal feeds

Product	Av. Protein content (%)
Fish meal	60
Chlorella	50
Brewers' yeast (USP)	48
Torula yeast (fodder grade)	48
Soybean meal (typical seed meal)	44
Dried skim milk	36
Brewers' dried grains	25
Wheat (typical grain)	12

presented in the next section. Another method is by assay of the protein for individual amino acids.

Two such assays 1 of _Chlorella pyrenoidosa_ (Emerson strain) are reported in table 2. The "pilot-plant sample" was some of the material grown in July 1951, as reported in chapter 17. With the exception of the

Table 2

Amino acid assay of dried Chlorella

Nutrient	Pilot-plant sample[a] (%)	Laboratory sample[b] (%)	Torula yeast[c] (%)
Crude protein	44.0	40.0	. . . .
Arginine	2.06	2.39	3.61
Histidine	0.62	0.65	1.31
Ifioleucine	1.75	1.69	3.75
Leucine	3.79	1.99	3.57
Lysine	2.06	2.43	4.14
Methionine	0.36	0.57	0.84
Phenylalanine	1.81	2.14	2.41
Threonine	2.12	1.91	2.58
Tryptophane	0.80	0.41	0.66
Valine	2.47	2.67	2.98
Glycine	2.20	0.22	

[a]Microbiological assay by Food Research Laboratories, Inc., reported to Carnegie Institution of Washington by Kenneth Morganridge, chief chemist, March 27, 1952.
[b]Mean values of two samples grown by Spoehr and Miner [151]; microbiological assay by Merck and Company, Inc., 1949.
[c] From [234], All results reported on basis of torula yeast as received.

quantities for methionine, leucine, and tryptophane, the two assays agree reasonably well. Table 2 also shows for comparison a reported assay of torula yeast [234].

From the results for the pilot-plant sample the Food Research Laboratories drew the following conclusion (stated in a report to the Carnegie

* Additional data have been reported by Fowden [221a] since this chapter was written.

Institution of Washington, March 27, 1952): "The Essential Amino Acid Index is calculated to be 62. This index has been found useful in predicting the biological value of protein in certain cases. 2 it compares the test sample with whole egg protein which is given a value of 100. For comparison, the essential amino acids of most animal proteins range between 80 and 90. Cereal proteins fall generally between 60 and 80. An index of 62, as found for this sample of Chlorella, places it in a class with white flour, corn gluten and peanut meal."

One of the major deficiencies in the amino acids of Chlorella is the low methionine content. The same deficiency is found regularly in the other vegetable protein foods. Fortunately this amino acid is readily available as a synthetic product made from nonagricultural materials. Synthetic methionine is now being added to some commercial chick feeds. In considering Chlorella as a possible feed for cattle, however, this deficiency can be disregarded, for ruminants synthesize their own essential amino acids.

Vitamin Content

Data concerning the vitamin content of algae, except for a few scattered figures reported in section E of chapter 19, are limited to the two assays of Chlorella pyrenoidosa summarized in table 3. The "pilot-plant sample"

Table 3
Vitamin assay of dried Chlorella

Vitamin	Pilot-plant sample [a]	Laboratory sample [u]
Carotene (mg/lb)		218.0
Thiamin (mg/lb)	11.0	4.5
Riboflavin (mg/lb)	26.2	16.3
Niacin (mg/lb)	54.0	109.0
Pyridoxine (mg/lb)		10.4
Pantothenic acid (mg/lb)	3.6	9.1
Choline (mg/lb)		1370.0
Biotin (mcg/lb)		67.0
Vitamin B12 (mcg/lb)	45.0	10.0
Lipoic acid (acetate units/mb)	15	. . . .

[a] Microbiological assay by Drs. J. J. Mayernik and David Hendlin, of Merck and Company, Inc.. January 1952.
[t] Grown by Stanford Research Institute in continuous dilution culture apparatus; assay by Curtis and Tompkins, San Francisco. Data from [279].

was another portion of the same material used for the amino acid assay of table 2. For those vitamins determined in both samples the agreement is not very close. This is not surprising in view of the finding, presented in chapter 10, that the thiamin content of Scenedesmus depends markedly on the age of the culture and on environmental factors.

[2] See [252].-- Ed.

In spite of the disagreement between the two samples, it is fair to conclude that dried Chlorella could be an important source of vitamins in the diet of human beings. Comparison of the vitamin content of Chlorella with the minimum daily requirements for vitamins shows that approximately a quarter of a pound of dried Chlorella would provide more than the minimum requirement of all except vitamin C. Although this vitamin is present in large quantity in the freshly harvested algae [201(b)]> it is largely lost in drying.

Among the objectives of future algal culture experiments might be the determination of the range of vitamin content among different species. It would also be of interest to learn the variability in content of different vitamins in several species with changes in the conditions of culture.

B

Animal-Feeding Tests

Rat-Feeding Tests

The first animal-feeding test of dried algae was made at the Stanford Research Institute in the summer of 1949, using Chlorella pyrenoidosa grown in the pre-pilot-plant studies there [279]. Because of the limited amount of Chlorella available, only five rats were fed a diet containing the algae. Their growth, from the age of 25 days to 56 days, was compared with that of two groups of matching control animals. The main control group of eight rats was fed a stock diet known to be adequate. The secondary control group of three rats was fed an experimental diet that was the same as the one containing Chlorella except that all the protein (18 per cent) was derived from casein.

The average gain in weight of the control group was uniform throughout the period of the test. During the first two weeks the casein group and the Chlorella group increased in weight only about half as much as the main control group, and the increases were less regular. During the remaining three weeks of the test the casein group increased at about the same rate as the main control group, whereas the rate of increase for the Chlorella group was only one-third as great.

Near the end of the test the Chlorella diet for one rat was supplemented with methionine. A rapid increase in growth rate was observed.

Two features of this feeding trial confuse its interpretation. Difficulty was encountered in maintaining a uniform temperature in the cages, and on one night when the temperature reached 91° F, one of the rats on the Chlorella diet died. A more serious defect in the report of these tests is the absence of information about how much the rats ate. Not only is it impossible to compute the protein efficiency ratio, but also one cannot tell whether the lack of gain in weight resulted partly from insufficient ingestion of food. The rats had unlimited access to the feed, but the ones on the Chlorella diet may not have eaten enough, either because of an unpalatable

flavor or because of mechanical difficulty caused by the hygroscopicity of the dried feed. (This latter condition was observed in the chick-feeding tests reported in the next subsection.) Therefore, no firm conclusions are possible from this preliminary rat-feeding test.

A second rat-feeding test was carried out in 1951 in England, using Chlorella vulgaris. Details of the trial have not been published, but the summary given in section D of chapter 13 of this monograph indicates that on the basis of protein efficiency, the Chlorella was slightly superior to dried brewers' yeast but inferior to dried skim milk. It may be significant that in this test the diet contained only 17 per cent Chlorella, which was less than half the proportion used in the Stanford tests.

A few adult rats were given standard rations containing various amounts of dry Chlorella ovfer a period of about one week at Arthur D. Little, Inc. In some cases individual rats were given a choice of standard ration with and without Chlorella. The results showed that there was no definite preference or dislike for Chlorelia.

Chick-Feeding Tests

Some of the Chlorella grown in the pilot plant (described in chapter 17) during the summer of 1951 was fed to groups of New Hampshire chicks by Professor G. F. Combs, of the Poultry Department of the University of Maryland. Various amounts of dried Chlorella and other supplements were added to a basal diet consisting largely of ground yellow corn and soybean oil meal. This basal diet was known from previous work to be suboptimal as a source of vitamin A, riboflavin, niacin, and vitamin B12 during the early growth of chicks. The Chlorella used was the "pilot-plant sample** for which the amino acid and vitamin assays are given in tables 2 and 3.

The results of this test are summarized graphically in figure 1. As Combs [210] has reported: "The inclusion of 10% Chlorella to the basal diet in place of an equal amount of soybean meal resulted in a very marked increase in growth and improvement in feed efficiency. This improvement is attributed primarily to the high carotene content of the Chlorella although important quantities of several B-compiex vitamins are also supplied by this level of Chlorella."

The only adverse effect observed was a strong tendency to impaction of the beaks of the chicks, caused by the hygroscopicity of the dried Chlorella. This mechanical impediment interfered with feeding, and hence the chicks could not always eat as much as they wanted to. It was so serious with a group fed 20 per cent Chlorella that their experimental feeding was discontinued after two weeks.

The hygroscopicity of the Chlorella used in these trials may have resulted partly from rupture of the cell walls, because it had been frozen before drying. Drying of the algae immediately after harvesting might give a less hygroscopic product. Also the algae could be mixed with another constituent of an animal feed before drying, in order to obtain a less hygroscopic material. Therefore, Combs concluded: "Although a

278

260

254

235

224

98

Basal plus methionine and vitamins 1% and D and B complex

Basal

Chlorella

Chlorella plus 0.1% methionine

Chlorella plus 0.2% methionine

Chlorella plus 0.6% methionine plus D and B complex vitamins

A | D

A' | B' | C' | D'

Basal   Chlorella Added
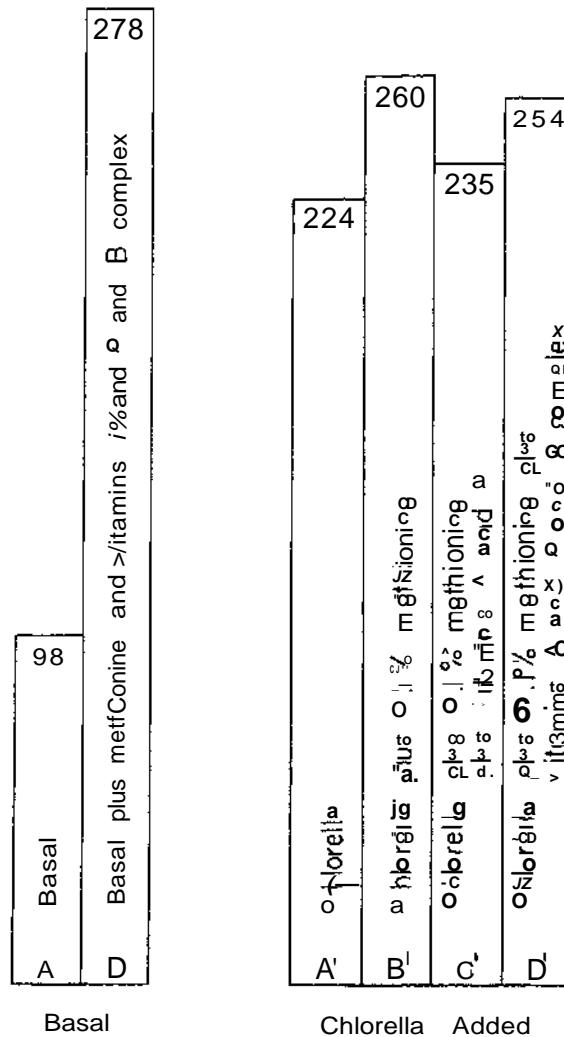
Fig. 1. Chick-feeding test of dried Chlorella. Average gain in weight (in grams) of groups of 16 chicks after 4 weeks' feeding on corn and soybean meal ration, in which about one-third the soybean meal was replaced by an equal weight of dried Chlorella (equal to 10 per cent of total weight of ration). Diets designated by the same letters were identical, except that the one with a prime contained the Chlorelia.

mechanical difficulty was observed in the use of the vacuum-dried algae, the results demonstrate clearly that dried Chlorella may serve as a source of certain dietary nutrients for the chick" [210].

A second chick-feeding experiment was carried out during 1952 by the Ralston Purina Company, using vacuum-dried Chlorella from the pilot plant. Their reports (private communications, July and August 1952) stated that chicks fed with standard broiler ration supplemented with 2.5 per cent of Chlorella gained 706 g in 6 weeks as compared with 755 g for

the broiler ration alone. The weight of feed (1.98 pounds) required per pound of weight gained was identical for the two groups of chicks. No explanation is offered for the depression of rate of growth of the chicks fed Chlorella, although apparently they did not take as much food as the control group. At the low concentrations of Chlorella used, the problem of impaction of the beaks was not evident as it was at the University of Maryland. In a cecond experiment, older birds received a 0.37 per cent Chlorella diet for 4 weeks, starting at 7 weeks old. Here the Chlorella-fed lot gained 960 g as against 965 for the control group, the ratio of weight of feed to weight gained being 2.98 and 2.99, respectively. It was concluded that at these low levels of Chlorella content there was no practical difference in older birds.

Summary

In the rat- and chick-feeding tests reported here the results have been reasonably encouraging. All the tests have been preliminary, and improved methods of preparation as well as further variations in the composition of the rations may well show more favorable results. In no case has there been evidence of toxicity or other adverse reaction, or of refusal to take the rations containing Chlorella. One of the objectives of further work on the culture of algae should be clarification of the nutritional properties of material grown and processed in various ways.

<div align="center">C</div>

Algae as Human Food

Although there has not yet been opportunity for feeding tests of microscopic algae with human subjects, there are a few preliminary pieces of information relating to human consumption of algae.

In the first place, it is well known [208] that for centuries oriental peoples have eaten seaweeds, which are macroscopic algae. Some of them are considered a delicacy. The evidence given in table 3 of chapter 19 on the chemical composition of algae indicates that the marine algae and fresh-water algae are not distinctly different with respect to the proportions of proteins, carbohydrates, and lipides, for both classes show wide variations. From this limited evidence we can at least conclude that since it is known that certain species of marine algae are suitable for human food, it is likely that some species of microscopic algae are also suitable.

In the second place, the results of the feeding of a mixture of algae to lepers are reported for the first time in chapter 14 of this monograph. Although these experiments lacked the careful control that one would like to see in a nutritional experiment, the available evidence indicates clearly that the daily ingestion of 20 grams of microorganisms including a large percentage of algae was not detrimental, inasmuch as an increase in weight and an improvement in general health were observed. It cannot be

ascertained whether these benefits resulted from the high vitamin content of the microorganisms or from their high protein content or from some other cause. Inasmuch as some of the patients were fed the algae daily for as long as two years, the possibility of toxic effects seems to have been eliminated.

In recent studies in Japan reported by Dr. Hiroshi Tamiya (private communication, May 1952), dry powdered Chlorella ellipsoidea has been described as tasting like a dried seaweed or powdered green tea, both popular in Japan. It can be added in amounts up to 20 per cent to powdered green tea without being noticed. When the powdered Chlorella was used with a mixture of buckwheat and wheat flours at 2 per cent of the dry weight, acceptable noodles were prepared. Hydrolysates of powdered algae showed promise as a substitute for soy sauce.

Acceptability as Human Food

Nutritional value is not all that is required of a human food. It must also be palatable, or capable of being made palatable, to be acceptable. The Flavor Panel at Arthur D. Little, Inc. has made preliminary appraisals of dry and fresh frozen Chlorella. The thawed, undried material alone showed vegetable-like flavor and aroma and was rated as "foodlike and food satisfying" but with some of the "notes" unpleasantly strong. There was, however, a noticeable tightening at the back of the throat ("gag factor") and a lingering, mildly unpleasant aftertaste. When the material was used to the extent of 15 per cent in a chicken-base soup, the stronger, less pleasant "notes" were much reduced and the "gag factor" was not noticeable. With freshly harvested Chlorella (not frozen), both raw and in soup, the strong flavor "notes" and "gag factor" were less evident. The conclusion was that the Chlorella and soups made from it were suggestive of vegetables and generally palatable and acceptable. The appearance, however, may not be so generally acceptable. It is interesting that the defatted product produced by the VioBin process ([245] and chapter 17) did not have vegetable-like or even foodlike properties, and showed quite different flavor "notes."

The flavor of dried Chlorella alone is too strong for one to enjoy eating large quantities of it. In this respect it might be compared to many of the herbs, which are pleasing as a garnish. If dried Chlorella could be stabilized against absorption of moisture, it might be marketed as a specialty item. Its bright green color would add interest if it were sprinkled on certain foods. Such a use, however, would be a rather far cry from its potential use as a large source of protein; and therefore it might be a mistake to promote it for this limited purpose.

It should be pointed out that all conclusions on flavor and acceptability must be considered tentative because the materials tested are not necessarily representative of the future products themselves or of the modifications or methods of preparing foods which can undoubtedly be developed to improve the appeal to human consumers.

Chapter 21

MICROSCOPIC ALGAE AS INDUSTRIAL RAW MATERIALS

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Though the most important potential use of algae grown on a large scale would without question be as human and animal food (see chapter 20), one of the aims of the over-all program has been to investigate the possibility that constituents of algae might find high-value uses which would justify operation on an intermediate scale. Nutritionally valuable by-products would remain after separation of many of the special materials considered.

Chapter 19 presents a summary of available information on the chemical composition of algae. The present chapter treats some of the possible uses that have been studied as well as some of those which remain to be considered. In addition to the groups working directly on the culture of algae, several industrial concerns have co-operated generously in the evaluation of the Chlorella produced in the pilot-plant program.

It is important to note that the investigations so far have been concerned almost entirely with Chlorella. As was shown in chapter 19, wide differences in types and proportions of constituents are indicated by the incomplete information available regarding the many species of algae which might be grown by mass-culture techniques. Furthermore, the work on chemical composition and possible use of algae is very limited and preliminary. Thus any conclusions which are drawn must be tentative and subject to revision.

A

Protein Fraction

Chlorella and some other unicellular algae contain 45 to 50 per cent protein when grown with an adequate nitrogen supply. The physical properties of the proteins have not been determined,[1] but if these proteins should prove to be similar to other vegetable types, they might find use as substitutes for some of the common proteins of industry in fibers^ sizing, adhesives, and the like.

Preliminary tests performed on frozen pilot-plant material by the Hercules Powder Company (private communication) did not show any great

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[1] Since this manuscript was written, Fowcten [221a] has published a thorough analysis—Sd,

promise for such applications of the protein. There was considerable difficulty in separating the proteins for testing, and those isolated showed a low molecular weight, making them much less effective than casein for adhesive use. The Virginia-Carolina Chemical Corporation (private communication) also reported that the protein fraction was difficult to separate and that the properties observed did not suggest that it would be valuable for fiber production.

Though future work might result in improved separation methods and applicability, the preliminary tests of the proteins do not indicate high enough quality or sufficient ease of separation to justify moderate-scale growth of algae with the primary object of industrial protein production.

So far as is known, the distribution of amino acids in algal protein is similar to that in most vegetable matter and, as is shown in chapter 20, to that in yeast. Therefore, algae appear to present no advantage over the many other sources of protein for hydrolysis as a source of mixed or individual amino acids. The glutamic acid content of some of the Chlorella from the pilot plant was determined by International Minerals and Chemical Company (private communication) at approximately 9 per cent of the protein (4 per cent of dry weight). This is not considered high enough to justify special interest in algae as a commercial source of monosodium glutamate, which at present is the largest-selling hydrolyzed protein product. Although mixed hydrolysates do not have a large volume of sales, preparations are being sold for use in special diets. All the essential amino acids have been identified in Chlorella. Whether the absence of sodium and calcium from the algae grown in mass-culture media such as have been employed in this study would be of particular interest in this field has not been determined. Quantitative data on both proteins and amino acids are almost nonexistent, but with increased knowledge new possibilities may be suggested.

B

Lipide Fraction

The early work of Spoehr and Milner [151] on mass-culture methods indicated that under conditions of nitrogen starvation, algae could be grown with a lipide content as high as 86 per cent, as compared with 20 to 25 per cent in normal Chlorella. Consideration of the data indicated, however, that the fat was not significantly different in composition from that grown under normal conditions and that the rate of fat production per unit area of culture was less. Thus the economics of growing algae for their lipide content appears to favor normal growth with extraction of the fat fraction, possibly by mild hydrolysis. The residual material would have value as animal feed.

Simultaneous defatting and dehydrating by the VioBin technique (chapter 17) gave a lipide extract of about 10 per cent of the dry weight, including some pigments (private communication). The composition of this

fraction has not been investigated. A considerable additional amount might be recovered if the dried algae received a mild chemical treatment. This process might provide for economical recovery of the fat fraction if the value of the dry solids remaining were great enough to justify culturing the algae on a moderate scale.

The main constituent of the lipide fraction is apparently a typical vegetable fat mixture, as reported in chapter 19. The oils are apparently slightly more unsaturated than many vegetable oils, and include some triply unsaturated acids of 16 and 18 carbon atoms. The possibility of using all or part of the oils as drying oil has not been clearly established, but is suggested by preliminary studies which have been carried out by General Mills, Inc., using Chlorella from the pilot plant (private communication).

The extraction of the frozen Chlorella was found to be difficult, but with saponification about 8 per cent of the dry weight was recovered as fatty acids showing the following characteristics: iodine number, 192.7; acid number, 201.4; saturated acids, 11 per cent; "linoleic" acid, 21 per cent; "linolenic" acid, 55 per cent. The linoleic and linolenic contents were estimated by spectral analysis of the whole acids. The investigation is being continued.

On the basis of apparent resemblance to linseed fatty acid composition, drying properties are indicated, but the low yield and difficulties in extraction are obstacles to economical use. Thus, unless combined with other uses for algae which require separation of all or part of the oil content, use of the lipide fraction does not appear to provide an unusual commercial possibility.

Chas. Pfizer & Company, Inc. (private communication) also separated the lipide fraction from some of the Chlorella grown in the pilot plant, and found it to be 26 per cent of the dry weight. They used a preliminary methanol extraction, followed by acetone treatment to achieve the separation. The unsaponifiable portion was 11.5 per cent of the fat fraction, which contained a low concentration, about 0.1 per cent of the total dry weight, of a sterol, probably ergosterol. Sterols are of interest as a source of pharmaceutical intermediates and are discussed separately in chapter 22 on the basis of a separate program on several algal species.

C

Carbohydrates

In general the carbohydrate fraction of algae has been studied very little. In the process of photosynthesis carbohydrates of low molecular weight are formed, but the extent to which they remain present in that form is not known. Over-all analyses indicate less than 20 per cent carlx>hydrate content. From the discussion in chapter 19, this fraction appears to be quite complex, containing several hexose and pentose types in various combinations. It seems probable that the typical carbohydrates in algae are not the same as those in the higher plants.

The cell walls and other structural parts in some algae are presumably complexes containing appreciable amounts of alginic acid as the fundamental molecule. Since many sources of alginic acid are available, it would appear to have interest only as a by-product of some such operation as separation of proteins and fats. Presumably the unicellular types of algae have relatively little cellulose, and though starch has been identified in some species, the majority of unicellular algae have fats and oils as food storage products, instead of starch and its analogues. In general it would appear that the carbohydrates of algae are not a likely source of high-value material, although much more information on this group of compounds is needed.

D

Pigments

The present high demand for chlorophyll as a deodorant is based on controversial evidence, but it may provide a continuing market for chlorophyll at high prices. The very high chlorophyll content, 4 to 6 per cent, of Chlorella and other green algae suggests that they are a potential future source of this material. By comparison, dehydrated alfalfa, which is commercially used for extracting chlorophyll, is reported to contain less than 0.2 per cent [273].

It is possible that the economics of mass culture would justify growing algae primarily as a source of chlorophyll, with the remaining portions used as by-product for feed. The simultaneous recovery of a portion of the lipide fraction might then be economical. The isolation of chlorophyll from Chlorella is currently under investigation. Should algae be grown on a large scale for other purposes, the extraction of chlorophyll would undoubtedly be attractive as a side line. Chas. Pfizer & Company, Inc. (private communication) report that the concentration of chlorophyll b was so high in their methanol lipide extract that it crystallized out. Mechanically, the very small size of the cells might present some difficulty in an extraction process, but this problem could be solved by a suitable development program.

Both carotene and xanthophyll are reported present in many of the green algae, including Chlorella, and there is some demand for these materials. The quantity of xanthophyll is not known, but beta carotene has been reported by Geoghegan (chapter 13) at 1200 ppm, as compared with about the same level in dry carrots and 200 to 300 ppm in dry alfalfa meal [273], The foregoing comments on chlorophyll apply also to the other pigment materials.

Whether whole algae could be treated to provide chlorophyll in a suitable chemical and physical form for the current use of this compound as a deodorant is unknown. Since the individual cells are so small, incorporation of whole dry treated algae into various products would be quite simple. The concentration of chlorophyll in Chlorella is about the same as in some

commercially available concentrates. The other components of the algae should not cause difficulty, if the materials were properly compounded.

E
Vitamins

Practically all the important vitamins are known to be present in Chlorella, and presumably also in similar algae, at reasonably high levels but probably not in sufficient proportion for economical extraction as concentrates. The available analyses, given in chapter 20, show that the vitamin content is comparable with that of yeast or a little lower. It is thought that the vitamins are sufficient to provide a premium value in the use of algae as human or animal food, but are not significant for the recovery of vitamin concentrates.

F
Antibiotics

There is some evidence in the literature that algae produce an antibiotic or bacteriostatic material. Evidence of such action on the part of several types of alga, including control of bacteria in sewage ponds by Chlorella, is reported by Pratt et aL [262]. An investigation by Spoehr et al. [152] also suggested some activity of this kind. The results of pilot-plant determinations of bacteria count (chapter 17) indicate that bacteria in the presence of healthy algal cultures survive but do not increase greatly in number and do not appear to attack the algae. It is not known whether the mechanism of this control is in the small amount of waste material available for bacterial growth, or whether a chemical control agent is excreted. Thus subject may be worth further investigation.

G
Future Possibilities

It is obvious from the foregoing discussion that there is much to be learned about the application of algae as a source of industrial raw materials. Much more work must be done on the chemical composition in order to provide leads for future investigations, both with applications suggested here and with others which may be indicated later. It is hoped that as additional material becomes available, more extensive studies can be made of the chemical nature of the compounds occurring in algae. It is possible that the production of interesting compounds may be increased by discovering "superior" strains in nature, by selecting these further, and by producing mutants which may accumulate even greater quantities of the desired product. The absence or low concentration of a potentially important compound in one particular culture should not be considered as necessarily indicating that the compound cannot be obtained from any other strain or species.

Chapter 22

GROWTH AND EVALUATION OF SPECIES OF ALGAE WITH REGARD TO STEROL CONTENT

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Treatment of the subject of mass culture of algae has dealt thus far with theoretical considerations of growth and with analysis of techniques which may give these organisms major economic importance. In the latter category, emphasis has been placed on utilization of the bulk products such as fat, carbohydrate, and protein. The possibility of using the synthetic capacity of algae for the production of valuable organic compounds difficult to construct in vitro has been less completely explored.

Facilities have recently been provided at the University of Maryland for growing large quantities of different species of algae for analytical purposes. Some of the analytical work is being done at the University of Maryland; additional study is being carried out by co-operating institutions elsewhere. Typical of the problems to be investigated is the determination of the quantity and nature of certain sterols produced by algae adaptable to mass culture. This part of the investigation is being carried out in co-operation with the Research Division of Merck and Company, Inc., Rahway, New Jersey. The present chapter is a preliminary report of the work thus far.

A

Precursors of Cortisone

In 1S48 Bergmann and Feeney [191] isolated a sterol from the alga **Scenedesmus obliquus (Turp.) Kutz.,** the characteristics of which match **chondrillasterol,** first isolated from a sponge, Chondrilla nucula, by Bergmann and McTigue [192]. The significance of this discovery can be better **understood** through a brief review of the problems encountered in synthesizing cortisone, a drug now of considerable value.

Since the discovery in 1929 that dogs from which the adrenal glands had been removed could be maintained alive longer if supplied with extracts of

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the gland, considerable attention has been directed toward isolation of the active principle in this extract [219]. The adrenal gland consists of a central part called the medulla and an outer mass called the cortex. The general term cortin has been applied to the natural secretion^emanating from this outer area. It is now clear that cortin is composed of a number of discrete compounds, many extremely active in their control of certain segments of animal metabolism. Of the thirty or more substances isolated from cortin, six show activity in extending the life of adrenalectomized animals.

These compounds all belong to a group called steroids, having in common the cyclopentanoperhydrophenanthrene ring structure shown in (a). The naturally occurring adrenal hormones, and related steroids occurring elsewhere, vary in the type and orientation of side chains and functional groups. All the active natural cortical hormones which have been isolated have in common the structure shown in (b). This appears to be the basic structure for the hormones active in adrenalectomized animals. In 1949, however, Hench et al. [229] observed the therapeutic effect of cortisone (c).
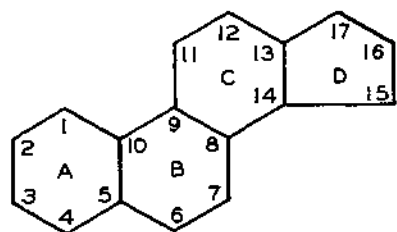
(a) Cyclopentanoperhydrophenanthrene
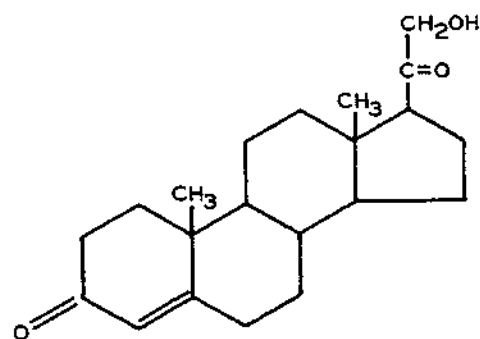
(b) Desoxycorticosterone
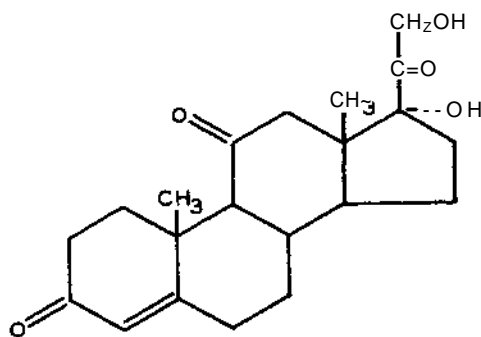
(c) Cortisone
(17-Hydbroxy-ll~dehydrocortieoaterone, Kendall's compound E)

It has since become clear that although the presence of oxygen or oxy-
gen functions at C J I and C17 does not condition the activity of the hor-
mones in maintaining adrenalectomized animals, it does exert additional
physiological control of considerable medical significance [260].   The in-
creasing demand for cortisone as a therapeutic agent has made its syn-
thesis a major problem in the pharmaceutical industry.   Whereas desoxy-
corticosterone can be synthesized from natural sterols or bile acids with
comparative ease, the addition of the oxygen and hydroxyl to $C\backslash\backslash$ and C17
is difficult and expensive.

The two major synthetic problems in converting the common sterols
to cortisone are: (1) the introduction of oxygen at **CJJ,** and (2) elaboration
of the cortical hormone side chain.

Sterols having unsaturation at $C\backslash\backslash$ and in the side chain at C22 offer
points of attack by which the above transformations may be accomplished.
Naturally occurring steroids with unsaturation at C^ are not known, but
reactions are known for synthetically introducing unsaturation at C J I from
sterols having a double bond between C7 and Cg.   Thus a sterol having
double bonds between C7 and Cg and between C22 and C23 would be a
promising starting material for the synthesis of cortisone.

Using this criterion, it is apparent that chondrillasterol (d) would
serve as an excellent starting material.   A sterol of fungi, ergosterol (e),
which also has double bonds at C7 and C22, has the disadvantage of an
additional double bond at C5 which must be reduced before the synthesis
can be carried on.   Stigmasterol (f), although having the requisite unsatu-
ration in the side chain, provides no functionality for readily introducing
a double bond at **CIJ.**

The most abundant sterols--sitosterol (g) (from plants) and choles-
terol (h) (from animals), as well as fucosterol (i) (from brown algae)--
have neither point of unsaturation and must be considered definitely
inferior starting materials.
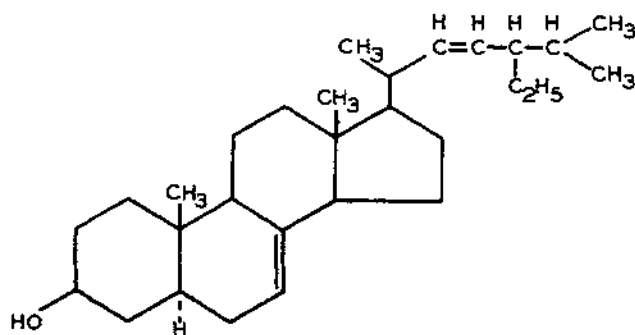
(d) Chondrillasterol
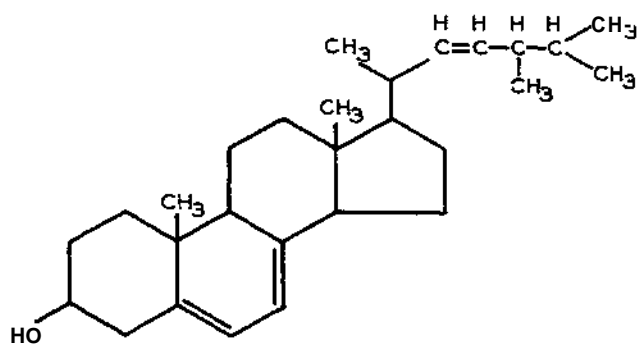
(e) Ergosterol
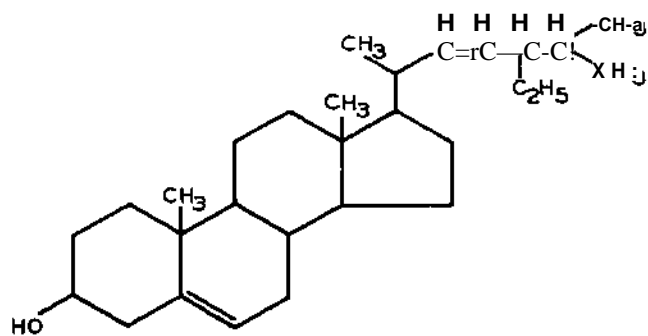
(f) Stigmasterol
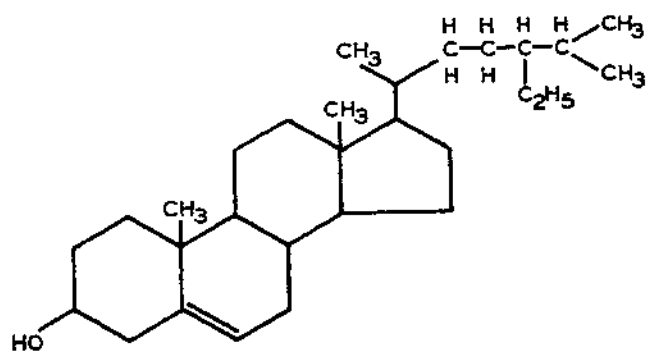
(g) 0 -Sitosterol
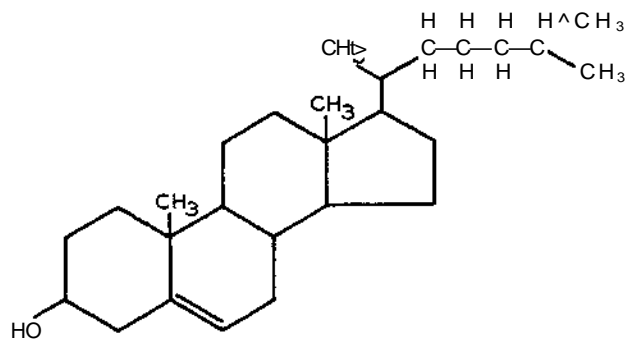
(h) Cholesterol
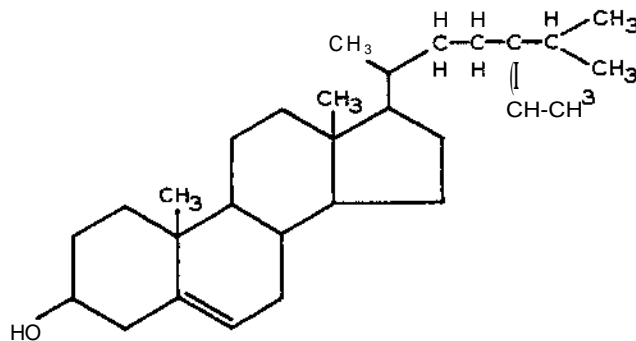
(i) Fucosterol

B

Sterols in Scenedesmus obliquus

Several hundred grams of Scenedesmus obliquus from previous cultures were on hand at the University of Maryland at the beginning of the present Investigation. The strain had been obtained at Woods Hole during 1950 and was designated WH-5Q. Extraction yielded 0.1 per cent crystalline sterol which melted at 111° C and gave a positive Tortelli-Jaffel reaction (see section D), suggesting a double bond at the Cg atom. The melting point, 111^0 C, differed too widely from that of chondrillasterol, 165-168° C, to suggest that the sterols from the two strains were identical. Sufficient material was not available for a complete characterization, but zymosterol (j), a minor component of sterol mixtures found in yeasts, was indicated.

As a starting material for cortisone synthesis, zymosterol is of equal interest with chondrillasterol. Before culturing the algae producing zymos-terol, however, it seemed essential to Investigate the strain of Scenedesmus
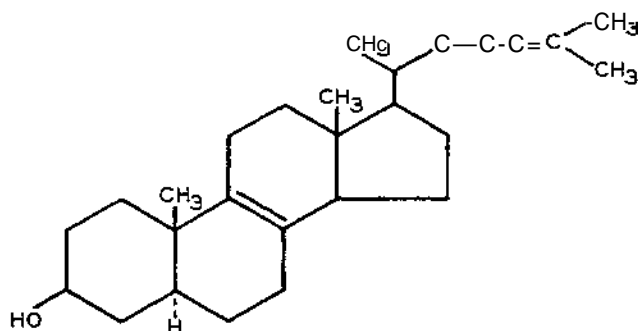
(j) Zymosterol

obliquus analyzed by Bergmann and Feeney [191], which had been reported to yield 0.23 per cent chondrillasterol. A slant of this strain designated D-3 was obtained from Dr. E. W. Fager of the University of Chicago. Subsequent analysis of several large samples of strain D-3 confirmed the report of Bergmann and Feeney that it contains chondrillasterol. Therefore the two strains of the same species apparently contain two different sterols.

C

Culture Technique

The equipment used for culturing Scenedesmus obliquus strain D-3 in the greenhouse during the last six months of 1951 is diagramed in figure 1. Two types of culture vessel were used: carboys containing 10 liters of culture medium and vats containing 400 liters. The carboys were used to produce samples of algae for analysis and also to grow a sufficient quantity of cells with which to inoculate the vats. The carboys were inoculated with pure cultures grown in 500-ml Erlenmeyer flasks under artificial illumination.

The medium for fresh-water Chlorococcales given in chapter 8 was employed, and was adjusted to pH 7. A 5 per cent CO_2-air mixture was supplied during daylight; at night, air alone was pumped into the cultures.

Control of temperature in the carboys was effected during the summer by means of a canvas hose encircling their tops. Water seeping out along the hose passed over the carboys in a film and flowed down the drainage trough. The trough had a gradual slope, and held approximately 0.5 inch of moving water at all times. The system was capable of holding the culture temperature below 85° F even though the maximum air temperature reached 110° F and was commonly in the nineties. Since operation of the vats was not begun until September, it was possible to omit cooling apparatus with them. On four of the hottest days, however, the temperature in the vats reached 91° F. Though this undoubtedly retarded the growth rate, no destruction of cells was observed.
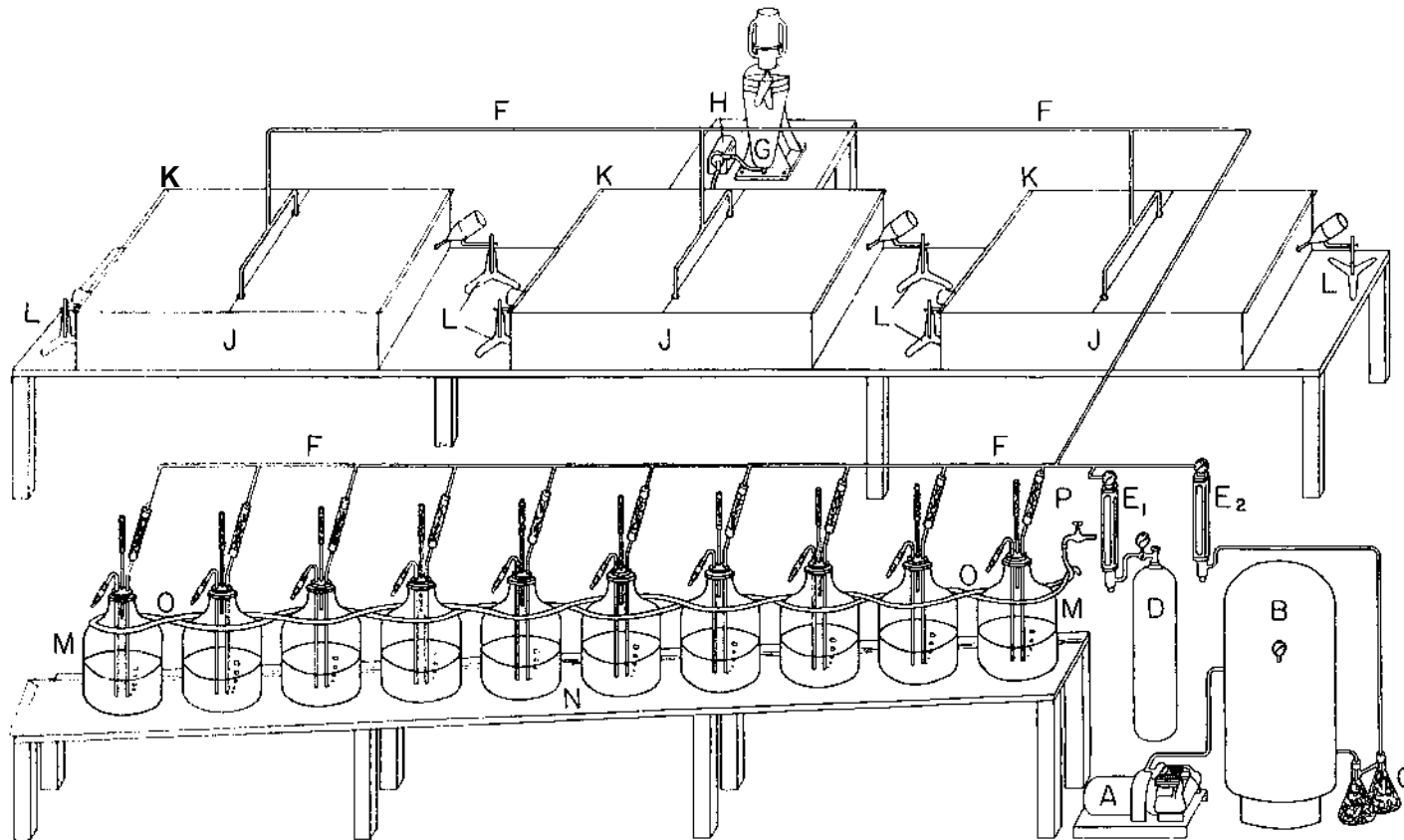
Fig. 1. Diagram of principal culture apparatus. A, compressor; B, receiver; C, glass-wool and cotton air filter; D, carbon dioxide cylinder; Ej, flow meter, carbon dioxìde; E2, flow meter, air; F, carbon dioxide-air manifold; G, Sharpies Super Centrifuge, type M-41-24 8 RY; H, rubber centrifugal pump; J, 500-liter vat (polyethylene-lined); K, plate-glass cover; L, stainless steel stirrer; M, 18-liter carboy; N, drainage trough; O, coarse-weave canvas hose; P, cold-water line.

Growth was determined by cell count. Centrifuged cell volume was also followed, using a 100-ml Goetz tube graduated in hundredths of a milliliter. The culture in the carboys was harvested by means of the Sharpies Super Centrifuge after reaching a cell count of 40-50x100 per milliliter. This corresponded to a centrifuged fresh weight of 2.0 g/L The carboys were immediately refilled with fresh medium and reinoculated. This occurred approximately once every 10 days, depending on light conditions.

The vats were handled differently, because they had less illuminated surface area for a given volume--the area was 20,000 cra^ gjid the depth was 20 cm. Once inoculated, they were maintained without replacement of the entire medium for several months. The pH was maintained near 7 by periodic additions of nitric acid. The other macronutrients were added less frequently in ratios commensurate with uptake. Iron and micronutrients were added in the original concentrations at the end of each harvest. Harvesting was accomplished by passing the culture through the Sharpies centrifuge whenever the cell concentration reached $20Xl06$ cells per milliliter (0.8 g/I fresh weight). The centrifuging was continued (the supernatant being returned to the vat) until the count had been reduced to $IQxlQ^$ cells per milliliter (0.4 g/1). Undoubtedly more favorable yields could have been obtained by more frequent but shorter centrifuging. Such a procedure is contemplated for the future.

Variation in temperature and light make determination of absolute growth rates under these conditions impossible. Production in the carboys, however, averaged 0.15 g/l/day fresh weight, and in the vats 0.08 g/l/day fresh weight.

D

Isolation of the Sterols

The harvested cells were frozen immediately after removal from the centrifuge and left frozen until just before drying. The method of isolation is based on that used by Heilbron (see [228]) in his earlier work on the extraction of algae. The cells are air-dried at approximately 60° C for 15 hours, and the dried ceils are pulverized to facilitate extraction. The dry powder is extracted in a Soxhlet apparatus for 43 hours with an organic solvent such as petroleum ether, acetone, or ethyl ether. Evaporation of the solvent gives a dark, viscous oil, which is the total lipid fraction of the algae.

This lipid fraction is saponified in methanolic potassium hydroxide (8%), the methanol evaporated off, and the aqueous residue extracted with ether. Concentration of the ether solution yields a crude sterol fraction contaminated with hydrocarbon oils and pigmented materials. Purified sterol is obtained from this fraction by repeated crystallization from methanol.

Characterization of the sterol is accomplished by determination of melting point, optical rotation, and response to either the Liebermann-Burchard reaction or the Tortelii-Jaffei reaction, or both. A positive

Liebermann-Burchard reaction is indicated when an intense green color results from treatment of a chloroform solution of the sterol with a mixture of concentrated sulfuric acid and acetic anhydride. Such a response indicates unsaturation in the steroid ring system, particularly a double bond at the C_5_g position. The Tortelli-Jaffei test is conducted by pouring a layer of a 5 per cent solution of bromine in chloroform onto a solution of the sterol in acetic acid; a green coloration appears at the interface. The reaction is characteristic of sterols that contain a ditertiary double bond between bridgeheads (exceptions: ergosterol and dehydroergosterol are positive) or that are easily isomerized to such substances.

The results of preliminary isolation and characterization of the sterols of four species or strains of algae obtained in quantity sufficient for exploratory analysis are given in table 1. The Chlorella and the Chlorococcum had been grown by Arthur D. Little, Inc. (see chapter 17). The strains

Table 1
Yield and characterization of sterols isolated from four algal sources

Source	$Y_j\wedge f$ (%)	Melting pt. (°C)	$[\alpha]_D^{20}$	Tortelli-Jaffei	Lieberraann-Burchard	Probable sterol
Chlorella pyrenoidosa (Emerson strain)	0.1			+	+	Ergosterol
Chlorococcum sp.	0.05			+	-	....
Scenedesmus obliquus (WH-50)	0.1	111.		+	+	Zymosterol?
Scenedesmus obliquus (D-3)	0.2	165-168	+1	+	+	Chondrillasterol

of Scenedesmus obliquus were from the University of Maryland. The kinds of sterol and the yields from both strains of Scenedesmus were promising, but neither Chlorella nor Chlorococcum appears to have a sterol with the desired structure.

The analysis of the D-3 strain of Scenedesmus obliquus cultured subsequently in bulk is given in table 2. Cells cultured in carboys are considered to be subject to greater light intensity than those in the vats. Such

Table 2
Yield of chondrillasterol from Scenedesmus obliquus (Fager's *D-3* strain)

Containers	^ a l fresh wt (g)	ChondriUastero^ yield (% dry wt.
Carboys	2066.8	0.35
Vats with NO_3	1696.1	0.11
Vats without NO3	1028.0	0.09

cells should be farther from the compensation point and more able to maintain reserves (of which the sterols may be one) than those grown in vats. The sterol content of other microorganisms is often directly proportional

to the fat content.   Therefore, to explore the possibility of increasing the sterol yield, cells harvested from a high-nitrogen medium in the vats were allowed to grow for two additional weeks in vats containing a fresh medium from which nitrogen was excluded.   Such cells would be expected to increase in fat content but to grow little [151].   The cell count increased from 13.0x 1()6 to 17.4X1Q6 in two weeks, and the weight of the centrifuged material increased from 833 to 1028 g in the 800-liter sample.   The gain in cell number was 34 per cent as compared with a gain in weight of 54 per cent, indicating an increase in individual cell weight.

The material produced under high light conditions in the carboys yielded the largest percentage of sterol.   This result was anticipated.   The yield from the cells held in the nitrogen-free medium, however, was unusually low.   There may be no significant difference between the sterol yield from high-nitrogen and that from low-nitrogen cells.   Increasing the fat content by reducing the nitrogen level clearly failed to increase the sterol content under the conditions of these experiments.

Cultures of a number of other species of Scenedesmus are now under way so that comparative analyses can be made.   It appears desirable to survey as many algae as possible before trying extensive variations in the physical and chemical environment designed to increase the sterol yield. Such experiments should be most productive if they are performed with an alga that has an unusually high yield of a suitable sterol when grown under the standard culture conditions.

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