A STUDY OF PHYCOPHYSIOLOGY IN CONTROLLED ENVIRONMENTS

TWENTY-FOURTH SEMIANNUAL STATUS REPORT AND FINAL REPORT
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
RESEARCH GRANT NO. NGR-21-002-003

TO:
THE UNIVERSITY OF MARYLAND

DEPARTMENT OF BOTANY
TECHNICAL REPORT
No. 1024

PREPARED BY DR. ROBERT W. KRAUSS
GRANT DIRECTOR AND PROFESSOR OF PLANT PHYSIOLOGY

COLLEGE PARK, MARYLAND
FEBRUARY 15, 1973

Twenty-fourth Semiannual Status Report and Final Report

National Aeronautics and Space Administration

A Study of Phycophysiology in Controlled Environments

Prepared by the Director

Dr. Robert W. Krauss

Head and Professor of Plant Physiology

Department of Botany

University of Maryland

College Park

and

Dean, College of Science

Oregon State University

Corvallis, Oregon

February 15, 1973

Introduction

The studies covered in this report are those conducted during the period between 15 December 1971 and 15 December 1972, plus an extension without additional funding to March 15, 1973. This research review constitutes the final report for NASA Research Grant No. NGR-21-002-003. Grant support under the above title was initiated on April 1, 1960 under NSG-70-60. Additional supplements carrying various numbers covered research to the current date. The results have been described in detail in a series of semiannual status reports of which this is the twenty-fourth. Each normally covered a six month period, however, permission was obtained to cover a full year's effort in the final report. Research during this final period has resulted in three papers submitted for publication. The manuscripts are included and give details of the work completed during this period. A Ph.D. thesis was also completed, the manuscript for which is included in the report. Investigations during this period can be summarized under four general headings: comparative studies on highly productive algae, photosynthetic ATP formation, carbon dioxide uptake, and lipid synthesis.

Personnel

Name	Title	Appointment
Dr. R. W. Krauss	Director and Professor	4/1/60 - 1/1/73
Dr. C. Sorokin	Research Professor	4/1/60 - 4/1/73
Dr. R. A. Galloway	Professor	4/1/60 - 1/1/73
Dr. G. W. Patterson	Associate Professor	7/1/60 - 1/1/73

Name	Appointment
Technical Assistant	
Erika Tanasijczuk	1/1/72 - 4/1/73
Part-time and Student Labor:	
Shirley Nishino	2/24/69 - 3/1/72
Valentino Russock	9/28/71 - 6/1/72
W. Basinger	3/10/71 - 6/30/72
Mary Reisinger	2/10/72 - 9/1/72
C. Maruszewski	2/23/72 - 8/1/72

Research Summary

From its inception, the support of research on phycophysiology in controlled environments has been directed toward the understanding of basic processes in the algae which would operate if algal life support systems were employed in long-term space flight. The problem for space vehicles has been one of miniaturizing and compacting a plant life support system into a reliable mechanism for the support of man. Many preliminary studies indicated that there was considerable cause for careful study of the physiology and biochemistry of unicellular green algae which seemed to offer the best hope for both high efficiencies of CO_2 absorption, oxygen evolution, and food production. The reasons for the interest in the unicellular algae were first that photosynthetic efficiencies were high per unit volume of cell material. No large volume of these plants are occupied by conductive tissue or in supportive tissue and the cellular components of the organisms can be varied to be primarily fat, protein or carbohydrate. Furthermore, the algae can be handled with little damage in most rigorously engineered circulating systems and therefore provide promise of ease of manipulation.

In spite of much continuing research on algal physiology and biochemistry, particularly with regard to their photosynthetic apparatus, much still needs to be learned about this group of microorganisms. Well known characteristics which qualify them as potential components of life support systems in space craft are paralleled by other characteristics which must be thoroughly understood if they are to be effectively utilized. Among the problem areas which have been addressed in the studies in this laboratory with unicellular algae have been the following:

(a) the variability found among the species and strains in the genus Chlorella and other unicellular green algae which appear the most likely candidates for life support systems.

(b) the analytical composition of green algae with special regard to those organic components that are of complicated structure and which may have an influence on mammalian growth if used as food supplements.

(c) the difficulty of culturing the algae in large scale systems in the absence of contaminents.

(d) the effect of nutrients not only on the growth rates of algae, but on their yields and chemical composition.

(e) the possibility that mutations will not be as useful in mass culture as the parent strain.

(f) the sources of illumination and maximal density for cultures for optimum yield.

(g) the temperature and light tolerances of algae.

(h) the way in which the algae differ in performance during the various phases of their life cycle.

(i) the effect of autoinhibitors in large scale culture.

(j) the evolution by the algae of potentially noxious volatile compounds into a recycled system.

(k) the susceptability of the algae to destruction during failure of one component or another in the culture system.

(l) the reliability of long-term cultures in terms of efficiency and productivity.

(m) the ease with which life support systems of microorganisms could be maintained by astronauts.

(n) the stability of a man-plant system with only one species in each part of the ecological loop.

The above problems required that as much information as possible would need to be accumulated about unicellular green algae before reasonable engineering could develop to accommodate their advantages and to manage possible disadvantages in a reliable system for space flight.

The development of a life support system based on a single plant is clearly a problem of enormous magnitude. Regrettably, all efforts to the solution of the problem have been attempted on a scale which is far too limited to ensure ultimate success. The basic questions that must be asked concerning the organisms, the engineering, and the efficiency of long-term cultures are so numerous that a very large research project would be necessary - perhaps something in the order of the Manhattan Project - to solve the problem effectively. In the absence of a truly large scale development program - a program which civilization may one day adopt with the utmost seriousness - it has been possible to achieve advances in our understanding of the growth characteristcs and of the biochemistry and nutrition of the algae to improve our appreciation of procedures if a truly operational

system were to be designed. The role of this laboratory has been to develop data in the context of the practical problems of ultimate long-term use. However, no effort has been made to engineer prototype designs for algal gas exchangers. The development of the Recyclostat to our laboratory did approximate a model of the kind of apparatus that ought to be designed prior to testing in actual space conditions. This apparatus is described thoroughly in earlier reports and has served as the basis for a number of studies of nutritional requirements in Chlorella.

In order to utilize most effectively the laboratory and facilities available, a team of researchers were assembled who were interested in algal culture, as well as basic questions concerning the biochemistry and physiology of algae. Prominent in this team were Dr. Constantine Sorokin, Dr. Raymond Galloway and Dr. Glenn W. Patterson. Each of them brought special competence and provided over the course of the years, significant additions to our understanding of algal physiology.

The approach to the problem in this laboratory was first to examine in some detail the strains and species of Chlorella which were available in culture collections and to attempt to characterize those strains in such a way that there would be a reasonable systematic base on which to build physiological studies. The identity of the unicellular green algae is at best difficult to establish, and an entirely new physiological key to the identification of the species of Chlorella was developed. This report was published in a book entitled CHLORELLA which is found in the list of references, published in 1965. At the same time, intensive studies of strains - were performed to determine their characteristics, expecially with regard to photosynthesis and respiration. To these studies were added

investigations on the life cycle of Chlorella. A series of papers reported the changes that took place in the metabolism of cells as they developed from autospores to mature adults. It was found that there was neither constant photosynthesis nor constant respiration during cell development, and that different stages of the life cycle of the cell were differently affected by physical parameters. These studies made it clear that care was needed in obtaining base-line data on gas exchange rates and sustained yields. It was shown that synchronized cultures of algae performed in quite a different fashion from nonsynchronized, randomly populated cultures. During these studies it also became clear that established growth rates of algae would bear careful scrutiny. The maximum growth rates of Chlorella were established at somewhat in excess of 12 doublings per day for high temperature strains. This was shown to be sustained only under ideal conditions of light and nutrition, and could be expected to be near maximum during the younger stages of synchronized cultures.

In an attempt to understand the responses of Chlorella to various quantities and qualities of light, studies were designed to give the maximum rates of growth as well as maximum yields at different densities of algae under different light intensities, and under light of different wave lengths. The results of studies on the effects of light on algal growth revealed that the effect was not only positive, as had been assumed in the case of photosynthesis, but that light had a negative action also. Light at the blue end of the spectrum was clearly inhibitory to cell division and vegetative reproduction. This was established by developing action spectra for colorless strains of Chlorella and correlating the the data with the performance of the pigmented strains. It is obvious

that in autotrophic growth there are competing reactions, those inhibitory and those supportive, of maximum growth rates in the algae. Any regimen of illumination for large scale cultures must take this antagonism into account.

With these facts available, it became obvious that determinations of population densities of cultures at varying illumination rates would be necessary in order to predict performance. Growth rate alone is an insufficient measure of performance inasmuch as rapidly growing organisms will not produce high yields unless the population base is large initially. In algal cultures, this is the classic problem - how to maintain a maximum growth rate and at the same time how to have a heavy enough population of algae so that a rapid growth rate will indeed result in a large amount of cellular production. In the last analysis, the rate of carbon reduction and oxygen evolution can best be measured by the amount of reduced carbon produced in growing cells. Curves showing the maximal yields for algae were developed, using the Recyclostat and were reported in 1968.

During the examination of the effects of light intensity on algal growth and yield, it became obvious that there was a shift in the chlorophyll a/chlorophyll b ratios in algal cells. Studies were initiated to establish the role of chlorophyll b. Inasmuch as the presence of chlorophyll b increased the absorption of available light to algal cultures, it was crucial to know whether chlorophyll b itself was involved in any of the reactions which supported photosynthesis. Studies showed that this pigment had comparatively little effect on the efficiency of cultures under high illumination. Therefore, it would be expected that algae with fairly low levels of chlorophyll b would be more efficient in grwoth. This may well account for the unexpectedly high yields of algae grown under high light intensities where the shift to lower chlorophyll b levels was obvious.

Parallel studies were conducted dealing with the production by the algae of reduced organic compounds. Special attention was paid to lipid synthesis and to the development of sterols and allied compounds which could conceivably have special growth effects on organisms utilizing the algae for food. A series of studies, especially by Dr. Patterson, has developed new insight into the biosynthetic capabilities of the algae and has produced for the first time a logical sequence of sterol biosynthesis. This has been compared with hydrocarbon biosynthesis and lipid biosynthesis, as well. At no time were any sterols found which could be specifically damaging to the growth or metabolism of humans.

Second only to light in limiting algal growth is carbon dioxide. Inasmuch as the carbon dioxide levels in algal cultures can be maintained artificially and can be expected to reach fairly high levels in space craft, it seemed desirable to establish those levels which were optimum for photosynthesis. Studies indicated that 1% CO_2-in-air mixtures saturating cultures of algae were near optimum for maximal growth. Furthermore, a clear-cut inhibition of CO_2 on cell division was established in Chlorella and confirmed in a colorless yeast Saccharomyces. The latter studies coincided with the development of a flight experiment for Saccharomyces which was designed to measure the possible effects of a gravity-free environment and/or radiation on the growth of microorganisms maintained in a liquid culture. Inasmuch as 1% CO_2-in-air seems near the optimal level for space craft environments, there is a fortunate agreement between the levels which are optimal for algal growth and those which are at the limit of acceptability for human well being.

The Recyclostat has been previously mentioned as a culture device aimed at providing data on the long-term recycling of nutrient media used to support algal growth. The Recyclostat is essentially a device which allows for the bacteria-free culture of algae and the automatic introduction to the medium of an inorganic nutrient supplement to sustain the algal cultures in the presence of progressive removal of nutrients. The device essentially allows for automatic dilution and removal of algae above a pre-set population density, and the recycling of the nutrient media. In this respect, the instrument is different from the usual automatic algal culture devices where media is run through the culture rather than being recycled. It is obvious that recycling puts a very severe test on the nutrient replacement regime. In the course of experiments with the Recyclostat in long-term culture, it became obvious that some of the nitrogen supplied to the medium was being lost by the algae. The loss was not detected as ammonia or as any of the other normally expected metabolic by-products of growth. However, near the end of the report period, a study was completed which showed that nitrous oxide was being produced by the algae, especially under periods of high illumination. The nitrogen compounds supplied for the nutrition of the algae were in part converted to nitrous oxide and lost in the gas train. This information suggests a re-study of the balance sheets for nitrogen replacement to algal cultures and also accounts for a significant leak to the atmosphere of a gas not suspected as a by-product.

The information summarized briefly above is contained in detail in the publications listed after this section. The data included in some 85 publications from this laboratory, which are now available in the

literature, consittute a significant addition to knowledge of the growth of the unicellular green algae. The data support the view that the utilization of unicellular algae as potential partners of man in space craft requires a profound knowledge of their growth characteristics, their biochemistry, and their physiology. In a closed ecological system - with only one species of plant supporting one species of animal - the situation is inevitably unstable. Stability can be bought by ingenious engineering and by adequate utilization of energy sources. In the last analysis, this is precisely the way in which man has been able to exist beneath the oceans, in space craft, and in other difficult environments. The presence of another organism in the system which supports man introduces an added element of ecological stability. At the same time, it requires a sophisticated body of knowledge to create the engineering to accommodate another species in the system while at the same time skipping most of the normal trophic sequences that man is accustomed to on earth. There is no apparent theoretical reason to believe that the necessary trade-offs cannot be made which will permit the engineering of a life support system utilizing a biological component. There is nothing that we have seen in the study of Chlorella that lead us to suspect that there are insurmountable obstacles to employing it or similar species in such a system. The main existing obstacle is lack of total knowledge about the requirements of the plant species. In spite of the great mass of medical knowledge about human beings, there is still much unknown about the requirements of man. The magnitude of the literature concerning man far exceeds that concerning the unicellular green algae. Nevertheless, in order for critical reliance to be placed upon the plant component of the balanced system, a mass of knowledge about the plant approaching in completeness that of the human

must be developed. Certainly some such approach is essential if the current work is to be utilized in the engineering essential for biological life support systems which are truly regenerative, and if the goal is to sustain man reliably away from earth at a great distance for a very long period of time.

PUBLICATIONS DURING TENURE OF NASA RESEARCH GRANT NGR21-002-003 AND PREDECESSOR NASA GRANTS

It may be of interest to review here the publications during research under the NASA supported program dealing with phycophysiology. Appended herewith is a list of 85 publications that have come specifically from projects supported by NASA funds. It should be pointed out, also, that a total of 11 Ph.D. theses have been completed in this laboratory under NASA support. Six of them, under the direction of the Project Director, three under Dr. Patterson, and two under Dr. Galloway. Furthermore, during this period, two senior post-doctoral fellows spent time in the laboratory and contributed to our understanding of the field - Dr. Carl Soeder from Germany and Dr. Torre Levering from Sweden.

1. Krauss, R. W., C. Sorokin, and R. A. Galloway. 1961. Metabolic pathways and growth of algae. Recent Advances in Botany, p. 257-260. University of Toronto Press.

2. Galloway, R. A. and R. W. Krauss. 1961. The effect of CO_2 on pH in culture media for algae. Plant and Cell Physiol. 2: 331-337.

3. Krauss, R. W. 1961. Fundamental characteristics of algal physiology. In: Transactions of the Seminar on Algae and Metropolitan Wastes, SEC Tr W61-3, U. S. Department of Health, Education and Welfare. Public Health Service. pp. 40-47.

4. Krauss, R. W. and A. Osretkar. 1961. Minimum and maximum tolerances of algae to temperature and light intensity: In: P. A. Campbell (ed.), The Medical and Biological Aspects of the Energies of Space. Columbia Univ. Press, New York. pp. 253-273.

5. Sorokin, C. 1961. Effects of illuminance near and below light saturation of time course of photosynthesis in synchronized cultures of algae. Journ. Experimental Bot. 12: 56-64.

6. Sorokin, C. 1961. Time course of oxygen evolution during photosynthesis in synchronized cultures of algae. Plant Physiol. 36: 232-239.

7. Sorokin, C. 1961. Time course of photosynthesis in bicarbonate buffer in synchronized cultures of algae. Archiv. fur Mikrobiol. 40: 418-429.

8. Sorokin, C. and R. W. Krauss. 1961. Relative efficiency of photosynthesis in the course of cell development. Biochim. Biophys. Acta 48: 314-319.

9. Karlander, E. P. and R. W. Krauss. 1962. Effects of monochromatic light on the growth of Chlorella vulgaris. Plant Physiol. 37: lxiv.

10. Krauss, R. W. 1962. Mass culture of algae for food and other organic compounds. Am. J. Bot. 49: 425-435.

11. Sorokin, C. and R. W. Krauss. 1962. Effects of temperature and illuminance on Chlorella growth uncoupled from cell division. Plant Physiol. 37: 34-42.

12. Sorokin, C. 1962. Carbon dioxide and bicarbonate in cell division. Arch. fur Mikrobiol. 44: 219-227.

13. Sorokin, C. 1962. Inhibition of cell division by carbon dioxide. Nature 194 (4827): 496-497.

14. Sorokin, C. 1962. Effects of acidity on cell division. Experimental Cell Research 27: 583-584.

15. Galloway, R. A. and R. W. Krauss. 1963. Utilization of phosphorus sources by Chlorella. In: Studies on Microalgae and Photosynthetic Bacteria. Plant and Cell Physiol. Special Issue.

16. Krauss, R. W. 1963. Inhibitors. In: R. Lewin (ed.), The Physiology and Biochemistry of Algae. Academic Press, N. Y. P. 673-686.

17. Krauss, R. W. and R. A. Galloway. 1963. Endogenous metabolism in Chlorella. Ann. New York Acad. Sci. 102: 707-716.

18. Sorokin, C. 1963. Injury and recovery of photosynthesis in cells of successive developmental stages: temperature effects. Pp. 99-109 in: Studies on Microalgae and Photosynthetic Bacteria. Japanese Society of Plant Physiologists, University of Tokyo Press.

19. Sorokin, C. 1963. Characteristics of the process of aging in algal cells (Abstract). Science 26 (April).

20. Sorokin, C. 1963. On the variability in the activity of the photosynthetic mechanisms. Symposium on "Photosynthetic Mechanisms in Green Plants," pp. 742-750. National Academy of Sciences - National Research Council.

21. Sorokin, C. 1963. The capacity for organic synthesis in cells of successive developmental stages. Arch. fur Mikrobiol. 46: 29-43.

22. Karlander, Edward P. and Robert W. Krauss. 1964. The mechanism of the light requirement for heterotrophic growth in Chlorella vulgaris Beyerinck. Plant Physiol. 39: Suppl. lii.

23. Patterson, Glenn W. and Robert W. Krauss. 1964. Comparative studies of sterols in Chlorella. Plant Physiol. 39: Suppl. lii.

24. Sorokin, C. 1964. Buffering activity of algal cells and its effect on cell division. Exptl. Cell Res. 33: 508-515.

25. Sorokin, C. 1964. Aging at the cellular level. Experientia 20: 353-362.

26. Sorokin, C. 1964. Organic synthesis in cells separated into age groups by fractional centrifugation. Arch. fur Mikrobiol. 49: 193-208.

27. Sorokin, C. 1964. Temperature tolerance: algae. In: P.L. Altman and D.S. Dittmer (eds.) Biology Data Book. Federation of American Societies for Experimental Biology.

28. Sorokin, C. 1964. Carbon dioxide and cell division. Nature 203(4951): 1252-1254.

29. Sorokin, C. 1965. Photosynthesis in cell development. Biochim. Biophys. Acta 94: 42-52.

30. Bowen, John E., Hugh G. Gauch, Robert W. Krauss, and Raymond A. Galloway. 1965. The non-essentiality of boron for Chlorella. J. Phycology 1: 151-154.

31. Osretkar, Anthony and R. W. Krauss. 1965. Growth and metabolism of Chlorella pyrenoidosa Chick during substitution of Rb for K. J. Phycology 1: 23-24.

32. Patterson, G. W. and R. W. Krauss. 1965. Hydrocarbons and sterols from Chlorella. Plant Physiol. 40: (Suppl. xviii).

33. Patterson, Glenn and Robert W. Krauss. 1965. Sterols of Chlorella. I. The naturally-occurring sterols of Chlorella vulgaris, C. ellipsoidea, and C. saccharophila. Plant and Cell Physiol. 6: 211-220.

34. Shihira, Ikuko and R. W. Krauss. 1965. Chlorella, Physiology and Taxonomy of Forty-one Isolates. Port City Press, Baltimore, Md. 97 p.

35. Sorokin, C. and Robert W. Krauss. 1965. The dependence of cell division in Chlorella on temperature and light intensity. Am. J. Botany 52(4): 331-339.

36. Sorokin, C. 1965. Carbon dioxide and cell division. Nature 206: 35-37.

37. Sorokin, C. 1965. Van Slyke's buffer values for cell secretions. Protoplasma 60: 79-85.

38. Epel, Bernard and Robert W. Kraus. 1966. The inhibitory effect of light on growth of Prototheca zopfil Kruger. Biochim. Biophys. Acta 120: 73-83.

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

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

41. Krauss, Robert W. 1966. The physiology and biochemistry of algae, with special reference to continuous-culture techniques for Chlorella. Pp. 97-109. In: Bioregenerative Systems, National Aeronautics and Space Administration Publ. SP-165.

42. Krauss, Robert W. 1966. Contributions to light as a controlling factor in natural phytoplankton societies. Pp. 54-56, 270-271 In: Marine Biology II. Proc. Second International Interdisciplinary Conference, Carl. H. Oppenheimer (ed.). The New York Academy of Sciences Interdisciplinary Communications Program, New York, N. Y.

43. Sorokin, C. and L. Mitrofanov. 1966. The secretory activity of algal cells in buffered media. Protoplasma 62: 201-209.

44. Patterson, G. W. 1966. Comparative lipid biochemistry of Chlorella. J. Phycol. Suppl. 3.

45. Soeder, Carl J., Heinrich Strotmann, and Raymond A. Galloway. 1966. Carbon Dioxide-Induced Delay of Cellular Development in two Chlorella species. J. Phycol. 2, 117-120.

46. Sorokin, C. 1967. New High-temperature Chlorella. Science 158: 1204-1205.

47. Patterson, G. W. 1967. The effect of culture conditions on the hydrocarbon content of Chlorella vulgaris. J. Phycol. 3: 22-23.

48. Patterson, G. W. 1967. Sterols of Chlorella. II. The occurrence of an unusual sterol mixture in Chlorella vulgaris. Plant Physiol. 42: 1457-1459.

49. Patterson, G. W. 1967. Fucosterol reduction to clionasterol in vivo by Chlorella ellipsoidea. Plant Physiol. 42: 1651-1652. (with E. P. Karlander).

50. Devlin, Robert M. and Raymond A. Galloway. 1968. Oxidative Enzymes and Pathways of Hexose and Triose Metabolism in Chlorella. Physiologia Plantarum Vol. 21, 11-25.

51. Krauss, Robert W. 1968. Contributions to bioregeneration. Pp. 188-195. In: Human Ecology in Space Flight III. Proc. Third International Interdisciplinary Conference, Doris H. Galloway (ed.). The New York Academy of Scienes Interdisciplinary Communications Program, New York, N. Y.

52. Karlander, E. P. and Robert W. Krauss. 1968. The laser as a light source for the photosynthesis and growth of Chlorella vannielii. Biochim. Biophys. Acta 153: 312-314.

53. Patterson, G. W. and E. P. Karlander. 1968. Chlorophyll bleaching fluorescence and absorption shifts in Chlorella sorokiniana promoted by NaCl. Plant Physiol. Suppl. S-21.

54. Patterson, G. W., L. G. Dickson and R.A. Galloway. 1968. Environmental effects on the fatty acids of Chlorella fusca. Plant Physiol. Suppl. S-46.

55. Patterson, G. W. and E. P. Karlander. 1968. Terminal steps in the biosynthesis of plant sterols. Plant Physiol. Suppl. S-46.

56. Patterson, G. W. and E. P. Karlander. 1969. Lipid variations in Chlorella sorokiniana induced by darkness, light and NaCl. Plant Physiol. Suppl. P-41.

57. Marcus, Lillian S. and Robert W. Krauss. 1969. Factors affecting the rate of calcification in Halimeda opuntia (L.) Lamouroux and Halimeda discoidea Decaisne. J. Phycology 5(4): 305-312.

58. Galloway, Raymond A. 1969. The Use of Isotopes in Measuring Primary Productivity. Chesapeake Science Vol. 10, No. 3 & 4, P. 331-335.

59. Drown, David and Raymond A. Galloway. 1969. A Study of the Mechanism of Action of Streptomycin in Euglena gracilis. Arch. Mikrobiol. 68, 377-386.

60. Sorokin, C. 1969. Growth rate measurements on phytoplanktonic organisms. Chesapeake Science 10: 313-321.

61. Patterson, G. W. 1969. Effects of light and temperature on lipid biosynthesis in Chlorella. Plant Physiol. Suppl. P-42.

62. Patterson, G. W. 1969. Sterols of Chlorella III Species containing ergosterol Comp. Biochem. Physiol. 31, 391-395.

63. Patterson, G. W., L. G. Dickson and R. A. Galloway. 1969. Environmentally induced changes in the fatty acids of Chlorella. Plant Physiol. 44, 1413-1416.

64. Patterson, G. W. 1970. Effect of culture temperature on fatty acid composition of Chlorella sorokiniana Lipids 5, 597-600.

65. Grunberg, Helmut and Raymond A. Galloway. 1970. Some Effects of High Carbon Dioxide content on Autospore Release and Number in Chlorella, Journ. Phycology. Vol. 6, No. 2, pp. 224-224

66. Patterson, G. W. 1970. The distribution of Sterols in algae. J. Am. Oil Chem. Soc. 47, 90A.

67. Patterson, G. W. and P. J. Doyle. 1970. Triparanol inhibition of sterol biosynthesis in Chlorella emersonii. Plant Physiol. 46. Suppl. P-28.

68. Norton, Jane S. and Robert W. Krauss. 1972. The Inhibition of Cell Division in Saccharomyces cerevisiae (Meyen) by Carbon Dioxide. Plant and Cell Physiol. 13: 139-149.

69. Reger, Bonnie J. and Robert W. Krauss. 1970. The Photosynthetic Response to a Shift in the Chlorophyll a to Chlorophyll b Ratio of Chlorella. Plant Physiol. 46: 568-575.

70. Talbert, David M. and Constantine Sorokin. 1971. Respiration in cell development. Arch. Mikrobiol. 78, 281-294.

71. Evans, William Kirk and Constantine Sorokin. 1971. Studies of the effect of gibberellic acid on algal growth. Life Science 10 (Part II) 1227-1235.

72. Sorokin, Constantine. 1971. Calefaction and phytoplankton, BioScience 21:1153-1159.

73. Sorokin, Constantine. 1971. The capacity of growing algal cells to affect the pH and the buffering properties of the medium. Plant & Cell Physiol. 12:979-987.

74. Patterson, G. W. 1971. The distribution of sterols in algae. Lipids 6, 120-127.

75. Doyle, P. J., G. W. Patterson, S. R. Dutky and C. F. Cohen. 1971. 14-α-methyl-5α-ergost-8-en-3β-ol and 14α-Methyl-5α-ergosta-8, 24(28)-dien-3β-ol from triparanol-treated Chlorella emersonii. Phytochemistry 10, 2093-2098.

76. Patterson, G. W. 1971. Relation between structure and retention time of sterols in gas chromatography. Anal. Chem. 43, 1165-1170.

77. Dickson, L. G. and G. W. Patterson. 1971. Inhibition of growth and sterol biosynthesis of Chlorella ellipsoidea with AY-9944. Plant Physiol. 47, suppl. p.22.

78. MacCarthy, J. J. and G. W. Patterson, 1971. The effect of nutrient medium cation levels on the biochemistry of Chlorella. Plant Physiol. 47, suppl. p.7.

79. Doyle, P. J., G. W. Patterson, S. R. Dutky and M. J. Thompson. 1972. Triparanol inhibition of sterol biosynthesis in Chlorella emersonii, Phytochemistry 11, 1951-1960.

80. Patterson, G. W. The sterols of Nitella flexilis and Chara vulgaris. Phytochemistry (in press).

81. Dickson, L. G., G. W. Patterson, C. F. Cohen and S. R. Dutky. Isolation and identification of two novel sterols from Chlorella ellipsoidea. Phytochemistry (in press).

82. Dickson, L. G. and G. W. Patterson. 1972. Inhibition of sterol biosynthesis in Chlorella ellipsoidea by AY-9944. Lipids 7, 635-643.

83. Chan, J. T. and G. W. Patterson. 1972. Effect of triparanol on sterol biosynthesis in Chlorella ellipsoidea and Chlorella sorokiniana Plant Physiol. Suppl. 50, 36.

84. Sorokin, Constantine, M. K. Corbett, and Shirley Nishino. 1972. Helical Chloroplasts in a Stichococcus-type alga. J. Phycol 8: 152-156.

85. Sorokin, Constantine. 1972. Dry weight, packed cell volume and optical density measurements. Chapter in J. R. Stein's (ed.) Handbook of Phycological Methods. Cambridge University Press (in press).

Summary of Research Completed During the Final Report Period

Comparative Studies on Highly Productive Algae

During the course of isolation of various strains of algae which demonstrated high growth rates, one organism attracted our attention. It is a Stichococcus type green alga which nevertheless has a helical chloroplast characteristic of other genera. In attempting to identify this organism, it became clear that the rotation of the chloroplast was considered to be a generic characteristic of major diagnostic value. However, the rotation of the chloroplast in the Stichococcus type in our collection changes, depending upon the culture conditions. This matter has been examined in some detail in characterizing this organism and the report included in this review discusses the observation that the rotation of the chloroplast is a phenomenon which is determined by the developmental process of the cell. Rotation is obviously a normal developmental phenonmenon that can vary. Consequently, it should not be used as a diagnostic character for green algae with any degree of confidence. Further studies with this organism have indicated that it is a highly variable species which can change its cell shape, size and reproductive characteristics depending upon the culture conditions. This kind of variability has been observed before in the monograph published on the genus Chlorella. However, rarely has an organism bee isolated which so clearly exemplifies the tremendous variability and plasticity of a species. It further demonstrates the observation that the systematic identification of unicellular algae that are most productive for long-term space flight will require a more physiological and chemical approach than has been utilized for the other algae.

When grown autotrophically in basal inorganic medium, the cells of our organism are rod-shaped with the widths ranging from 1.8 to 2.5 μ. Under conditions of slow growth, the length of the cells is from 2 to 6 μ. Each cell has a single, parietal, plate-like plastid encircling from 1/2 to almost the entire cell lumen and extending from 3/4 to nearly the whole cell length. Pyrenoids are absent. Under these conditions, cell characteristics generally fit the description given to the genus Stichococcus of the order Ulotrichales. Under conditions more favorable for cell growth, the length of the cells may reach up to 10 to 12 μ although the width generally remains nearly the same as in shorter cells. With the extension of the cell, the chloroplast by expansion, attenuation, and twisting takes a helical form.

Longitudinal growth of cells can be repressed, if not completely stopped, by transferring autotrophically grown cells into some organic media Then, a segment of the cell begins to increase in width. The swelling of the segment of the cell progresses with time eventually producing a spherical or, if less perfect, spheroidal expansion. The percentage of the spherical cells in the suspension of the originally rod-shaped cells and the degree of perfection of the spherical shape of the individual cells varies with the strain, the physiological condition of the original cells (preculture), and the experimental conditions during transformation. Observations on the significance of the preculture of the original rod-shaped cells generally indicate that healthy cells, capable of vigorous growth, make better material for obtaining spheres.

Of all the environmental conditions necessary for the transformation of the rod-shaped cells into spheres, the most essential proved to be composition of the medium. Tryptic soy broth was found to influence both the

percentage of the cells transformed and the degree to which perfect spheres were formed . Concentrations of the tryptic soy broth were tested beginning with 1.25 g/l and up to 40 g/l. Sphere formation was observed to be parallel to the growth of cultures: minimal at 1.25 g/l, increasing at intermediate concentrations, highest at 10 to 20 g/l and decreasing at 40 g/l. Therefore, 10 to 20 g/l is an optimal concentration of the tryptic soy broth for sphere production. The addition of yeast extract as well as of the ingredients of the basal inorganic medium (TSB-BI) to the tryptic soy broth had no discernable effect on sphere formation. Preliminary observations indicated that of the ingredients of the tryptic soy broth, tryptone and/or soytone were essential for sphere formation. They were, however, effective only in the presence of glucose which assured good cell growth.

Of other media, only cultures on tryptic soy agar (TSA) and on basal organic agar (BOA) produced spheres, although the percentage of the rod-shaped cells turned into spheres was generally not as large on these two media as in the tryptic soy broth. Remarkably, the liquid peptone, glucose, yeast extract medium (PGY) failed to induce sphere formation. Bacto nutrient broth (BNB), bacto liver infusion broth (BLIB) and trypticase broth (TB) were also ineffective in conversion of rods into spheres.

The effects of environmental factors on the tryptic soy broth cultures generally paralleled the effects of these factors on heterotrophic and mixotrophic growth. Thus, growth and sphere formation were favored by the increase in temperature from 25 C to 32 and 38 C, by light (400 lumen/m^2) compared to darkness, and by bubbling 5% CO_2-in-air mixture compared to no bubbling or bubbling with atmospheric air. Bubbling with pure nitrogen produced no growth and no spheres. However, bubbling with 5% CO_2 plux 95% N_2 caused good growth but no sphere formation.

The conversion of rod-shaped cells into spheres usually takes from several days up to one to two weeks. Then, by regular transfers into fresh TSB medium at 7 - 10 day intervals, the culture of spherical cells can be maintained without degeneration for several months. In one experiment, a culture of spherical cells was maintained without degeneration for 10 months.

Several species of the rod-shaped and filamentous algae (3 species of Stichococcus, 2 of Hormidium and one of Ulotrix) obtained from the Indiana Culture Collection were examined for their capacity to form spherical cells. All organisms grew well on BOA and TSA and with varying degrees of success in TSB. Of the six tested organisms, only a few cells of Stichococcus mirabilis showed slight swelling when grown on organic agar; other species being entirely unresponsive.

Eventually, reversion of the spherical cells into the rod-shaped cells takes place even if the culture is maintained by regular transfers to a fresh tryptic soy broth. The process is opposite to the conversion of rods into spheres. A cell gradually elongates with a concomitant decrease in width. After the rod shape is attained, the cells can be maintained as rods indefinately by regular transfers into tryptic soy broth. One such culture has been maintained in our laboratory for two and one half years without any signs of further deterioration. Thus, for these regenerated rods, tryptic soy broth proves to be ineffective in transforming them back into spheres.

The process of reversion of spheres into rods can be hastened by transferring spherical cells from tryptic soy broth into inorganic (BI) medium and by growing cultures autotrophically in light with CO_2 supplied in the gas mixture. Within 5 days to 3 weeks, the change is complete, there being no detectible morphological differences between the rods transformed from the spheres and the rods which never underwent the transformation to spheres.

The rod-shaped cell cultures obtained by reversion from spheres retain physiological characteristics different from those of the cultures of the rod cells which in their history did not experience the conversion into spheres. One difference is the lower capacity of the retransformed rods to again form spheres. When after a period of autotrophic growth, the regenerated rod cells are again inoculated into tryptic soy broth, the percentage of shperes formed is notably lower an the spheres on an average are less perfect than in cultures of spherical cells obtained by inoculation into tryptic soy broth of rods having no prehistory of conversion into spheres.

Another difference in the characteristics of the original and regenerated rod cells is in the rate of their autotrophic and heterotrophic growth. Growth rates of rod-shaped cells with four different prehistories are given in Table 1. The original cells were continuously maintained under autotrophic conditions and thus never experienced a conversion into spheres. The 153 day cells were obtained by transferring spherical cells from TSB into BI and keeping them in BI 153 days before taking them for growth measurements. The 46 and 11 day cells were obtained by similar reversion from the spheres after inoculation from TSB cultures into BI and keeping them in BI correspondingly for 46 and 11 days.

TABLE 1. Growth rates as doublings per 24 hrs of the original and regenerated rod-shaped cells of a Stichococcus-like organism.

Kinds of cells	Autotrophic conditions	Heterotrophic conditions
Original cells	5.24	2.24
153 day cells	4.56	2.40
46 day cells	3.63	2.76
11 day cells	4.02	2.84

As seen in Table 1, rates of growth (expressed as the number of doublings of cell material per 24 hour period) were generally lower for heterotrophic than for autotrophic growth. The rate of autotrophic growth for the original cells was higher and the rate of heterotrophic growth was lower than the corresponding rates for the rod-shaped cells obtained by regeneration from the spherical cells. Within the regenerated cells, the rate of autotrophic growth increased and the rate of heterotrophic growth decreased with the length of the period during which the regenerated cells were kept in inorganic medium. However, even after 153 days of keeping regenerated rods in inorganic medium, these cells remained markedly distinct in their growth characteristics from rod cells which in their history had never experienced transformation into spheres.

One of the most important conditions for the transformation of a rod into a sphere in our work proved to be the retention of growth activity during transformation. Since in unicellular rod-shaped (and filamentous) algae, the width of the cell changes comparatively little from one cell to another and for the same cell during its developmental cycle, one must conclude that the cell wall growth in these organisms is largely directed to the two opposite poles of the rod-shaped cell. The cell wall growth, in the process of transformation of a rod-shaped cell into a sphere, is redirected toward the middle of the cell and after the sphere is formed it is evenly distributed over the whole sphere. The apparent loss of, or decline in, the polarity of the cell wall growth resulting from the redirection of growth is, therefore, a salient characteristic of the process of transformation of our rods into spheres.

The loss of polarity in the cell wall growth of the spherical cells is not complete or final. The presence in some cultures of transformed

cells of an admixture of sheroidal cells indicates that in some cells the polarity is not completely overcome. In spheroidal cells, forces of polarization are in obvious competition with the forces directing an even distribution of the cell wall growth over the entire sphere. The latent capacity for polarity is also present in perfectly spherical cells since after their transfer into inorganic medium these cells quickly and completely revert into rod-shaped cells. Even if continuously kept in trypticase soy broth, the transformed spheres eventually revert to the rod shape.

Morphological alterations in the process of conversion of rod cells into spherical cells and of spheres back into rods are obviously accompanied by profound biochemical changes which persist well after the changes in morphological characteristics are over. The long lasting changes in the properties of the transformed cells are indicated by observations that rod cells obtained by reversion from the spherical cells differ in their capacity to again form spheres and also differ in rates of autotrophic and heterotrophic growth from the rods which in their prehistory did not experience a transformation into spheres.

A great deal of basic work needs to be done in order to carefully lay the ground for a reasonable system of classification of the small unicellular coccoid algae which are obviously of polyphyletic origin and which nevertheless are those organisms which are not only critical for potential algal life support systems, but which also provide a major portion of the food supply in the oceans of the world.

Photosynthetic ATP Formation

The conversion of the electromagnetic energy in the form of light to chemical energy by green algae is the crucial reaction that impels consideration of these organisms as potential segments of life support in

regenerative systems. The normal way of measuring conversion of electromagnetic energy to chemical energy is by monitoring absorbed carbon dioxide and/or evolved oxygen. Recent studies in many laboratories have made it clear that there is a direct conversion of electromagnetic energy by the photosynthetic mechanism to chemical energy as adenosinetriphosphate (ATP). The photosynthetic mechanism therefore is not only concerned with the reduction of carbon, but is also involved in the direct synthesis ATP which is the source of chemical bond energy for many of the synthetic conversions of the plant. Photophosphorylation is a difficult problem to study, but nevertheless is an important consideration in determining the efficiencies of photosynthetic reactions. It may suggest some revisions in our understanding of the quantum yield of photosynthesis. Consequently, a series of studies was aimed at determining the amount of photophosphorylation that takes place in the algal cell, the differences in the rate under low and high light intensities, and the potential quantum yield of the photophosphorylation mechanism. Initially, studies were begun on Chlorella, but because of the interferring effects of chlorophyll b, Anacystis nidulans, a blue-green unicellular alga, which also has considerable potential for photosynthetic gas exchange systems, was subject.

A method for the determination of enzymatically produced NADPH from ATP was utilized as a monitor of ATP formation. This method involved flourometry and proved to be extremely sensitive. It was found that the ATP level in the light was 0.15 to 0.25 micromoles per milligram of chlorophyll. In the darkness, the ATP level was only 70% of that in light. In both darkness and anaerobosis the level was 20%. When the cells were returned to light conditions, the ATP level was restored.

Dark, anaerobic cells were exposed to measured irradiancies of 710 nm and 620 nm. The rate of ATP formation was measured within the first

few seconds and found to be directly proportional to absorbed intensity. Saturation of the rates occurred at an intensity one-tenth the optimum for oxygen production. Quantum requirements of 6-8 were similar for each of the two wavelengths. The system II inhibitor DCMU had a greater effect of 620 nm than at 710 nm indicating an involvement of system II in photophosphorylation only at 620 nm.

At low intensities and over long time periods white light failed to produce a saturating steady-state level of ATP indicating a simultaneous consumption of ATP. Measurements in short dark periods following marginal illumination showed consumption of ATP to be 2 to 4 times greater than production in weak light. Thus, the quantum requirement can be calculated to be 2.

The evidence is that the production of ATP is not a limiting factor in the carbon dioxide reduction system. This photochemical conversion is obviously operating at an optimal rate in algal cells and is an extremely efficient mechanism for converting electromagnetic radiation to chemical energy. Any rate limiting series of reactions affecting optimal photosynthetic yield in the algae, therefore, must be sought in some other segment of cellular metabolism. The identification of the truly rate limiting enzymatic sequence would be extremely helpful because it would make possible a theoretical basis for the search for more highly efficient mechanisms for photochemical energy conversion.

Dark Fixation of CO_2

In any continuous culture device for algae there are periods in which the cells are in darkness. This is true if the engineering design involves a pumping system in which the algae actually are taken away from

the source of illumination, or if the algae are grown in dense cultures and consequently are unavailable for absorption of light during part of the time. The metabolism of the algae while they are in darkness is a source of considerable interest. It is well known that during this period they are able to absorb glucose and other organic carbon sources and in most strains, can utilize these sources effectively for energy and for protein and carbohydrate synthesis. Considerable interest has also been paid the possibility of significant amounts of fixation of carbon dioxide in the dark. Dark-fixation of carbon dioxide has been established for some microorganisms and is known to take place in higher plants. Experiments were run to determine whether there was significant dark-fixation of carbon and what state this carbon might be in when the only source of supply was CO_2. The details are given in the enclosed paper on the fate of glucose and bicarbonate in Chlorella. The work can be summarized by comparing the incorporation of CO_2-carbon and glucose-carbon into dark grown Chlorella cultures. The technique was the employment of carbon-14 tracers for detecting the movement of $^{14}CO_2$ into the cell. The carbon-14 from glucose at 12 minutes was distributed as follows: sugars, 43%; insoluble residue (primarily polysaccharides), 37%; lipids, 12%; amino acids, 5%; and organic acids, 3%. The ^{14}C from CO_2 was primarily recovered as organic acids (43%) and amino acids, (38%), followed by insoluble residue fraction, (12%). Lipids essentially did not change after a rapid incorporation in the first two minutes until a raise at ten minutes and accounted for 6% of the total radioactivity at 12 minutes. The least amount of ^{14}C (1%) was incorporated into sugars. The kinetics of the incorporation of ^{14}C into the lipids and the sugars was similar.

a Differences in the distribution of ^{14}C assimilated from glucose-^{14}C and $^{14}CO_2$ made it clear that there are different roles for the two carbon sources in heterotrophy. Glucose is considered the major source of carbon and energy as evidenced by its far greater incorporation.

The large incorporation of CO_2 into the organic acid fraction is consistent with its envisioned role in the replenishment of intermediates drained from the Krebs cycle for synthetic purposes. Furthermore, entry of labeled carbon into the sugar and lipid fractions lends substance to the concept of the dark operation of the Calvin cycle.

It is therefore clear that in the design of large scale culture devices, allowances should be made for the fact that the algae are capable of absorbing significant amounts of carbon dioxide in the dark and fixing the carbon into organic components of the cell. The availability of CO_2 during the dark phase will provide a substrate for the algae to employ during the photosynthetic process that takes place during the illumination phase of the culture.

Lipid Synthesis

An additional step in the study of the biosynthesis of sterols in Chlorella has been completed. The capability of Chlorella elipsoidia to synthesize its normal sterols was measured in the presence of triparanol. Triparanol has been shown to affect sterol biosynthesis by blocking the reduction of desmosterol to cholesterol in mammals. Studies were done on Chlorella emersonii because this organism is different from other species in that it is possible to see the effect of triparanol on the second alkylation reaction, the removal of 14 alpha-methyl and the reduction of the

7(8) double bond. Triparanol does inhibit the removal of the 41 alpha-methyl group, the second alkylation at C-24, the delta 7-reductase, and the delta 8-delta 7 isomerases. Examination of the cells indicated that Chlorella is susceptable to triparanol in a much more diversified fashion than the specific function assigned to it in animals. It has, nevertheless, been useful in the ellucidation of the biosynthetic pathway in Chlorella which now is very well understood.

The details of the inhibition of triparanol on sterol biosynthesis are given in the paper included with this report. A definitive review of the distribution of sterols in algae has been prepared by Dr. Patterson and published in 1971. There does not appear to be any serious problem with regard to sterol synthesis in algae as it might affect human metabolism or reproduction. None of the growth regulating steroids which are so powerful in human growth and development are present in Chlorella. The data do not suggest that this will not be an area of concern if the organisms are used for nutrition of man in long-term space flight.

Nitrogen Metabolism

Since the completion of the work initiated in the Recyclostat which identified the evolution of nitrous oxide, efforts have been made to determine whether or not nitrous oxide is found evolving from natural sources. Studies have been carried on in the field and it does appear from preliminary investigation that algae in the natural environment are capable of producing nitrous oxide. The difficulties in analytical determination of nitrous oxide levels require that this exploratory work be repeated. It is hoped that soon it will be possible to make definitive statements with regard to the amount of nitrous oxide in the atmosphere that can be attributed to its production in natural aquatic environments

by algae. The development of this work will require more intensified research on natural aquatic systems in the years ahead. The identification of the evolution of nitrous oxide by algae during the process of photoysnthesis is considered to be a significant breakthrough in understanding the gas exchange system of plants not only for space craft, but on the planet Earth. Nitrous oxide has recently been identified at above expected levels in various parts of the ocean where rapid primary productivity is high. The work in our laboratory correlates well with field studies. It is to be expected that the evolution of nitrous oxide from algal cultures in balanced gas exchange systems will be a manageable phenomenon. The oxidation or reduction of nitrous oxide is not considered to be a difficult step and gaseous mixtures coming from algal cultures can be readily screened from contamination by this compound.

Plans for the Future

Because of the move of the Director, Dr. Robert W. Krauss, to the position of Dean of the College of Science at Oregon State University, this phase of the study of the basic phenomena associated with those algae which are potentially capable of life support in space is concluded. It is anticipated that when laboratory operations are resumed at Oregon State University, a proposal will be prepared for NASA for the continuation of research. The first area which appears at this point to be most promising, and represents a significant scientific advance, deals with the study of nitrous oxide evolution which adds to our understanding of the Earth's nitrogen cycle and is important in management of regenerating systems in spacecraft. The second is the continuation of the investigation of the mechanism of calcification in Halimeda. This study which was a special study suggested

by the concern for the reduction in calcium levels in the bone of astronauts has significant promise of future usefulness. Comparatively little is known of the mechanism by which calcium is removed from blood and synthesized into calcium phosphate and calcium carbonate in the bone. Advances have been made in our laboratories in the study of a similar mechanism, essentially the conversion of calcium into calcium carbonate in the marine alga Halimeda. There is a possibility that the mechanism of concentration of calcium in marine algae can be understood. It is almost inevitable that this will be an analagous system to that in bone and may lead to further insight into the reasons for the loss of calcium in human bone during weightlessness. In the meantime, steps are being taken to move critical laboratory equipment associated with the NASA grant to Oregon State University for the initiation of a continuing research program.

THE KINETICS AND QUANTUM YIELD OF PHOTOPHOSPHORYLATION

IN

ANACYSTIS NIDULANS (RICHT.) DROUET

by
Olga v.H. Owens

Dissertation submitted to the Faculty of the Graduate School
of the University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1972

ABSTRACT

Title of Thesis: The Kinetics and Quantum Yield of Photophosphorylation in Anacystis nidulans (Richt.) Drouet.

Olga v. H. Owens: Doctor of Philosophy, 1972

Thesis directed by: Dr. Robert W. Krauss, Head and Professor of Plant Physiology

The active metabolite, ATP, serves not only as a high energy intermediate but also as a controller of some enzymatic reactions. In plant cells, the larger part of the ATP is formed by photophosphorylation. In this paper the rates, the quantum yields, and the wavelength dependencies of photophosphorylation in the blue-green alga Anacystis nidulans are reported.

A fluorometric method for determination of enzymatically produced NADPH from ATP was adapted for use on cell extracts.

In the light, the ATP level was 0.15 to 0.25 μmoles/mg chl. In the dark, the ATP level was 70% of that in light. In both darkness and anaerobosis, the level was 20%. A return to the light restored the ATP level from both conditions.

Dark, anaerobic cells were exposed to measured irradiancies of 710 nm and 620 nm. The rate of ATP formation was measured within the first few seconds and found to be directly proportional to absorbed intensity. Saturation of the rates occurred at an intensity one-tenth the optimum for oxygen production. Quantum requirements of 6-8 were similar for each of the two wavelengths. The system II inhibitor DCMU, had a greater effect at 620 nm than at 710 nm indicating an involvement of system II in photophosphorylation only at 620 nm.

At low intensities and over long time periods white light failed to produce a saturating steady-state level of ATP indicating a simultaneous consumption of ATP. Measurements in short dark periods following marginal illumination showed consumption of ATP to be 2 to 4 times greater than production in weak light. Thus, the quantum requirement can be calculated to be 2.

ATP formation, therefore, is not the limiting factor in CO_2 fixation. The evidence is the high quantum yield of photophosphorylation and the unsaturation of CO_2 fixation at intensities at which ATP synthesis is saturated.

INTRODUCTION

In cells, the level of ATP is determined by the rates of synthesis and utilization which occur simultaneously. The ATP level, in relation to the levels of ADP and AMP, controls the rates of some key enzymatic reactions (1). In photosynthetic cells in the light, ATP is produced by both photophosphorylation and dark phosphorylation. When consumption of ATP equals production, a characteristic steady-state level results. In the dark no photophosphorylation occurs and, for obligate autotrophs, no growth occurs. Thus there is a simultaneous decrease in both production and utilization.

Several investigators have examined ATP levels in algae. Strehler (19) showed that in Chlorella, with an anaerobic pretreatment, the rate of ATP formation increased with increasing light intensity. The maximum level of ATP occurred in weak light. He also found that in aerobic cells after a period of light the ATP level increased in the dark immediately after the light. Pedersen et al. (12), on the other hand, have shown that Chlorella cells, after a period of steady-state photosynthesis, show a brief drop in ATP during the first few minutes of darkness. A gradual recovery in darkness to a level similar to the initial light level occurred. When the light was turned on again, there was a brief, small rise in ATP followed by a return to the initial steady-state level in two minutes. Syrett (20) examined ATP levels in Chlorella in the dark and found differences associated with respiratory changes. The presence of uncouplers was associated with a drop in ATP as was the addition of glucose. Biggins (3) showed that in Anacystis a drop in ATP level accompanied anaerobiosis.

The rates of ATP formation have been examined by Simonis (17), and Urbach and Simonis (24). They used light dependent ^{32}p uptake into organic trichloroacetic acid-soluble fractions of phosphate depleted cells of Ankistrodesmus as an indication of photophosphorylation. By using suitable inhibitors they concluded that cyclic photophosphorylation occurs and that oxidative phosphorylation is suppressed in light. Tanner et al. (23) have used anaerobic photoassimilation of glucose by Chlorella as an indicator of photophosphorylation. A linear relationship of glucose uptake to weak light intensity and a quantum requirement of 4 at 712 nm and 6 at 658 nm were established.

Direct measurement of rates of ATP formation in whole cells of non-photosynthetic micro-organisms and photosynthetic bacteria have led to more substantial data because of the larger difference in ATP levels obtained by darkness and anaerobiosis. Welsh and Smith (25) have shown that in Rhodospirillum rubrum, preconditioned by anaerobiosis and darkness, both light and oxygen produce ATP but at different rates--the oxygen stimulated rate being about one-half that in light. The maximum levels attained by the two phosphorylating conditions are also different. Light produces a level twice that of oxygen. This and additional evidence suggested that there are two phosphorylating systems in these cells.

The possibility of ATP compartmentation in photosynthesizing cells of higher plants was examined by Santarius et al. (15) and by Santarius and Heber (14). The latter authors separated chloroplasts from cytoplasm by a non-aqueous procedure and determined adenylate concentrations in the two fractions obtained from Elodea. They concluded that ATP passes freely through the chloroplast membrane and therefore is immediately available for reaction in other parts of the cell. There is

some evidence from the work of Gibson and Morita (5) using Chromatium and Welsh and Smith (25) using R. rubrum that compartmentation of ATP, nevertheless does exist in prokaryotic cells.

The efficiency of photophosphorylation has not been determined for whole algal cells by direct methods. The experimental isolation of photophosphorylation and the measurement of its contribution to the ATP budget of the cell has not been achieved. In order to do this, it proved necessary to have a simple means for determining ATP, an accurate determination of light absorption, a fast killing method, and an organism with characteristics suited to the required measurement techniques. Anacystis nidulans, a blue-green alga was selected because it has a high ratio of photosynthesis to respiration (3,9) and lacks the mechanism for heterotrophy (18). Furthermore, its auxillary pigment, phycocyanin, has an absorption maximum at 620 nm producing a good wavelength separation of the two photosystems. By making the cells dark and anaerobic the ATP level can be lowered significantly, then with short light periods measurable changes in ATP levels occur which can be related to time and light intensity. This paper will show that ATP is formed with high quantum efficiency.

MATERIALS AND METHODS

Cells

Anacystis nidulans (Richt.) Drouet was grown in 500 ml of the medium of Kratz and Myers (8) in one liter Roux bottles before a bank of cool-white fluorescent bulbs. Incident intensity was 900 to 1000 ft-c and the temperature was 26 to 28 C. The cultures were aerated with 2.5% CO_2 in air. Cells were harvested after 16 to 24 hrs at a cell density of 0.2 to 0.8 µl packed cells/ml measured with a modified hematocrit tube centrifuged for 20 min at 1000 g.

To prepare for experiments, the cell suspension was centrifuged at 10,000 g for 10 min at room temp. The medium was decanted and the cells were resuspended in sufficient medium to give a final concentration of 5 to 6 µl packed cells/ml. The suspension was put into a 60 ml "lollipop"-shaped vessel with a 1 cm light path and exposed to 100 ft-c at 24 C for the several hours required to complete the experiments. During this time, the cells were bubbled with 5% CO_2 in air. The pH remained at 7 and the increase in packed cell volume and chlorophyll did not exceed 15%

Chlorophyll Concentration

A 2 ml sample of the final cell suspension was centrifuged at 10,000 g for 10 min and the medium decanted. The pellet was resuspended in 5 ml of 80% acetone which extracted the chlorophyll and precipitated the phycocyanin-protein complex. The extract was filtered into a cuvette and the OD at the chlorophyll peak, 663 nm, was determined in a spectrophotometer. The specific absorption coefficient, 82,000 (11) and appropriate factors for dilution were applied to calculate the chlorophyll content of the

cell suspension used in the experiment. The chlorophyll content was variable and ranged from 3.0 to 4.5 μg/μl packed cells.

Light Source and Polarograph

Light from a 750W incandescent bulb was focused through a 30 cm water bath then through an electrically operated shutter onto a 1 cm^2 area of a 1 ml polarograph vessel. Narrow band interference filters provided the specific wavelengths and wire screens were used for intensity variations. The vessel was equipped with a platinum-silver polarograph covered with a 1 mil teflon membrane. A voltage of 0.65 was applied and the current produced by oxygen was amplified and recorded when necessary. A magnetic stirring bar provided vigorous and stable stirring. All experiments were carried out at 24 C.

For each wavelength, the light falling on the 1 cm^2 surface area of a "solar cell", temporarily replacing the position of the vessel, was measured with a sensitive ammeter. The solar cell had been previously calibrated against a standard thermopile. The values were converted to μ E/cm^2/sec.

Preparation of Cell Extract

The cell suspension was killed in an estimated time of 0.1 sec after exposure to the experimental conditions by rapidly squirting 0.1 ml of N_2-bubbled 60% perchloric acid into the polarograph vessel. An aliquot, 0.85 ml, of the killed and denatured suspension was removed and immediately put into an iced 5 ml beaker. Five minutes were allowed for cooling and extraction, then the suspension was brought to pH 6.8 to 7.0 with 2.5 M KOH containing 0.5 M Hepes buffer. The amount required was about 0.4 ml but varied slightly with the amount of acid actually present. The neutralized sample was again cooled to 0 C then filtered

through a 1.2 μ millipore filter. The filtrate was clear and free of pigment, cell debris, and $KClO_4$. This was the cell extract used in the assay described below.

Time in Darkness

The time in darkness following a light period was estimated by using a storage oscilloscope with a two second sweep. The sweep was triggered when the shutter was closed. A small battery was connected to the oscilloscope from the barrel and plunger of the acid syringe so that when the plunger was pushed past the half-way point a "blip" was put into the oscilloscope trace. Times as short as 0.2 seconds were recorded.

ATP Assay

The method for ATP assay was essentially that of Estabrook et al. (7) with some minor modifications. It is a biochemical assay using the coupled hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) system to produce NADPH. The amount of NADPH formed is stoichiometrically related to the amount of ATP available for glucose phosphorylation and is determined fluorometrically.

In our assays, the NADPH fluorescence was measured in a double beam fluorometer containing as a reference a 10^{-5} M solution of quinine in 0.01 M H_2SO_4. Fluorescence was excited at 340 nm and measured at 420 nm.

Anacystis contains pteridines which have a fluorescence emission near that of NADPH and are carried over in the extract. A slow increase in fluorescence, probably due to oxidation of the pteridines was seen as a continuous increase in the baseline, therefore samples were analysed immediately. The baseline drift was linear for the 3 to 5 min

necessary to complete the reaction. Added pyrophosphate had no effect on the reaction, thus this compound, possibly present in the extract (2) did not appear in the assay.

Table I shows the components of the assay reaction in the order in which they were added. The cell extract contained glucose-6-phosphate as well as ATP, so it was essential to add glucose-6-phosphate dehydrogenase first and allow the reaction to be completed before adding the hexokinase. Extensive precautions were necessary to prevent spurious signals from fluorescent impurities.

To check for linearity of response, standard ATP solutions in conjunction with the coupled enzyme system were used to compare the response to added, standard NADPH solutions. Figure 1 shows a typical calibration curve. Routinely, the instrument was calibrated for each experiment by addition of standard NADPH because the concentration could be easily determined spectrophotometrically.

After completion of the assay reaction the ATP in the original cell suspension was calculated from the NADPH signal and expressed as n moles/μl packed cells or μmoles/mg chlorophyll. A series of ten identical determinations made from a single cell suspension gave a standard deviation that was 7% of the mean and the amount of ATP in the extract was directly proportional to packed cell volumes of 1 to 10 μl/ml

Table I. Components of the ATP Assay Reaction

Nos. 1 to 5 were added first. When the raction was complete, No. 6 was added. Bracketed components were added together. Water was added in sufficient quantity for 3 ml.

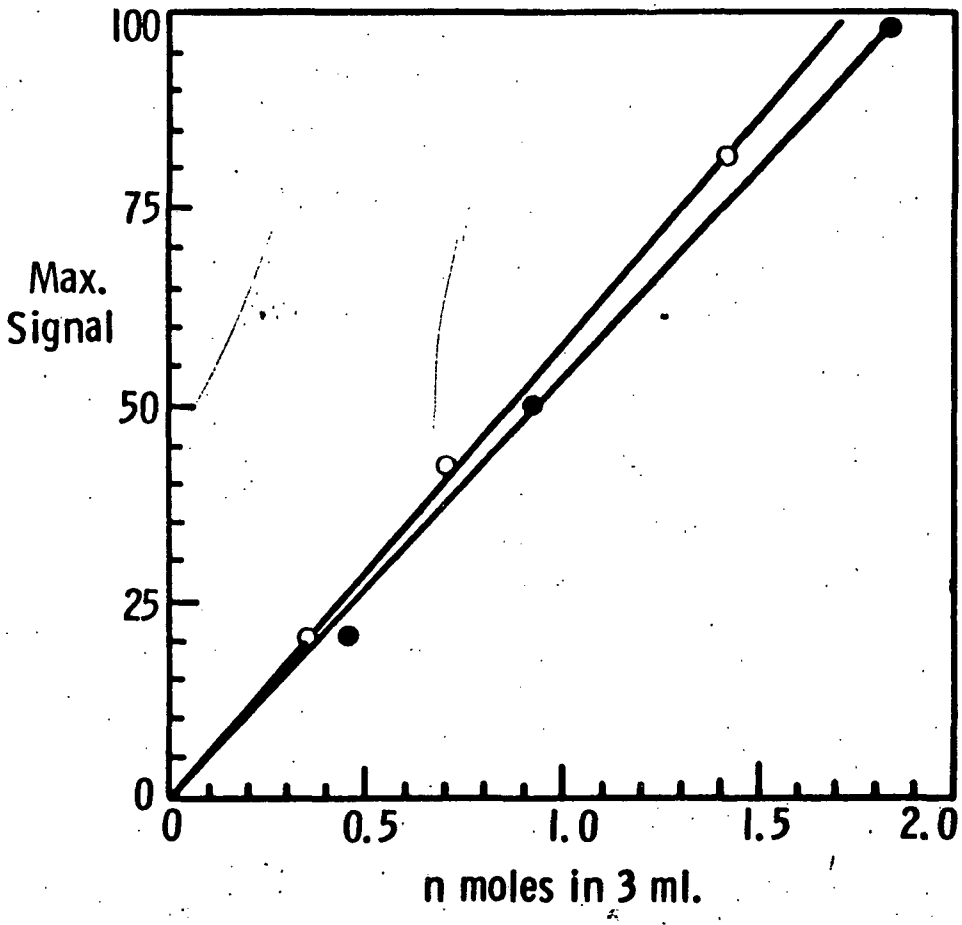
No.	Component	Final Conc.
1	Water	...
2	Cell Extract, 0.2 ml	...
3[1]	KCl	0.01M
	$MgCl_2$	0.01M
	EDTA	0.005M
	Hepes Buffer, pH 7.4	0.05M
4	NADP	2×10^{-5}M
5	Glucose-6-phosphate dehydrogenase[2]	0.7 units/ml
6[1]	Hexokinase[3]	0.7 units/ml
	Glucose	5×10^{-4}M

1. Filtered through a 1.2 μ millipore filter just prior to use.

2. Sigma, Type XI. Received as crystalline suspension in $(NH_4)_2SO_4$. Dialyzed against water overnight.

3. Sigma, Type F-300, crystalline. Dissolved in 0.05 M Tricine, pH 7.4.

FIGURE 1. The fluorescence signal of NADPH formed from added, standard ATP, solution (solid circles) compared to the signal from standard NADPH solution (open circles). The reaction mixture adjusted to pH 7.4 included Hepes buffer, KCl, $MgCl_2$, EDTA, NADP, glucose-6-phosphate dehydrogenase, hexokinase, and cell extract.


100
75
Max.
Signal
50
25
0
0
0.5
1.0
1.5
2.0
n moles in 3 ml.

RESULTS AND CONCLUSIONS

ATP Pool Sizes

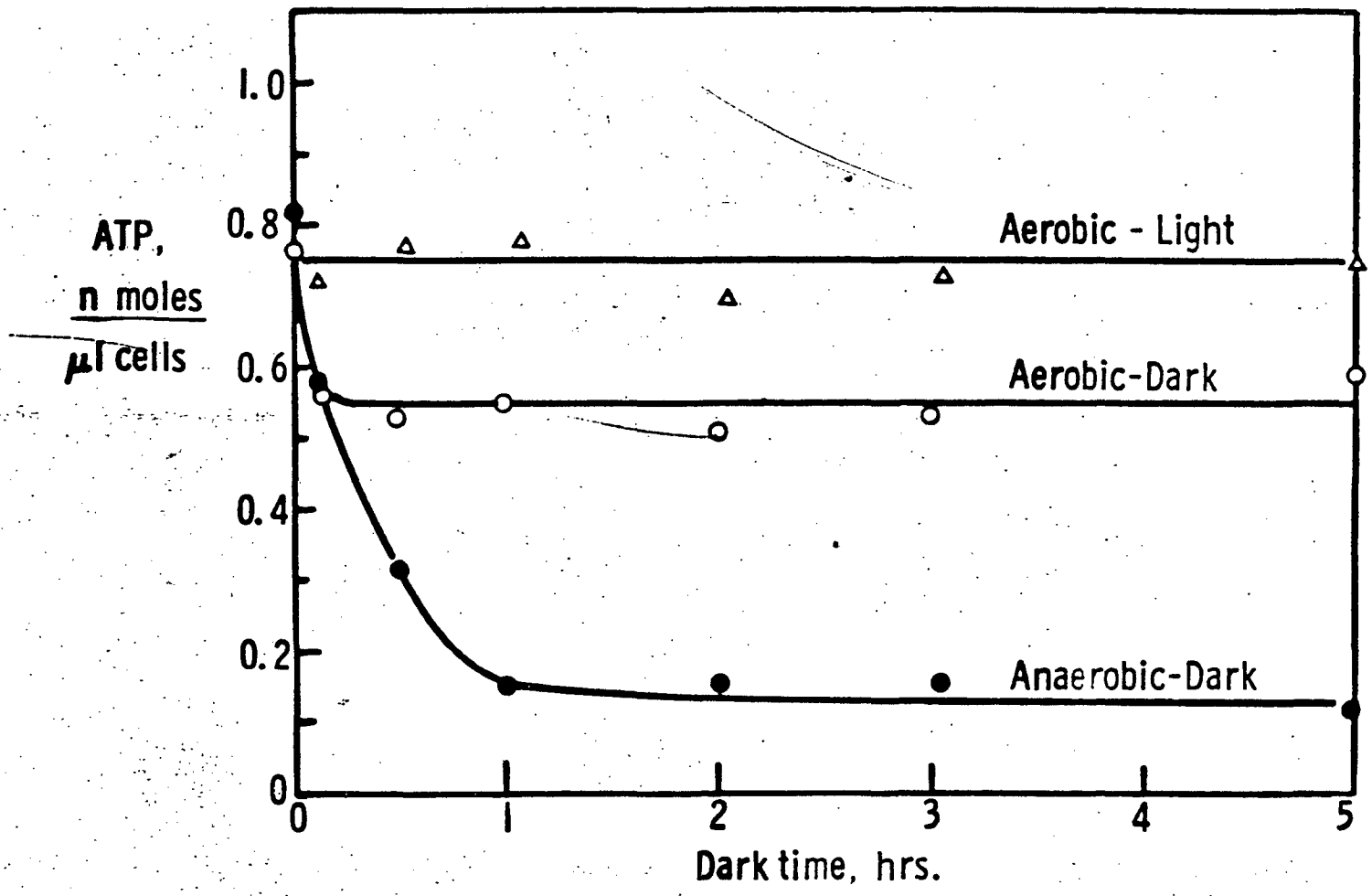
The ATP pool size in saturating light at the optimum growth rate of the alga is the resultant of the maximum rates of production and utilization. This value is equal to approximately 3.5 n moles/mg dry wt or 0.15 to 0.25 μmoles/mg chl. Since the chlorophyll *a* concentration in these cells varied considerably, the ATP level per mg chl also varied within this range.

To determine the effect of darkness, tubes containing aerated cell suspensions were maintained in darkness for five hours. At the time intervals shown in Figure 2, a pair of tubes was removed; one culture was killed and the other was exposed to two minutes of saturating white light, then killed. ATP determinations were made of both cultures.

In darkness the ATP level of the aerobic cultures dropped to 70% of the light level. The new dark, steady-state level was maintained for at least 5 hr. The cells did not grow in darkness in spite of the relatively high level of ATP. The transition from light to dark evidently involved a brief imbalance of consumption over production followed by equalization of these two processes to produce the new steady-state level. During the 5 hr of dark, the cells did not lose the ability to produce ATP. The upper curve shows the level of ATP after 2 min of saturating white light following the dark period.

Under N_2 maintained anaerobic conditions and darkness there was a progressive lowering of the ATP level to about 20% of the light level followed by a further, slow decrease as shown in the lower curve of Figure 2. The rate of ATP decrease during the first hour depended somewhat on the stirring and N_2 bubbling rate. These anaerobic cells were

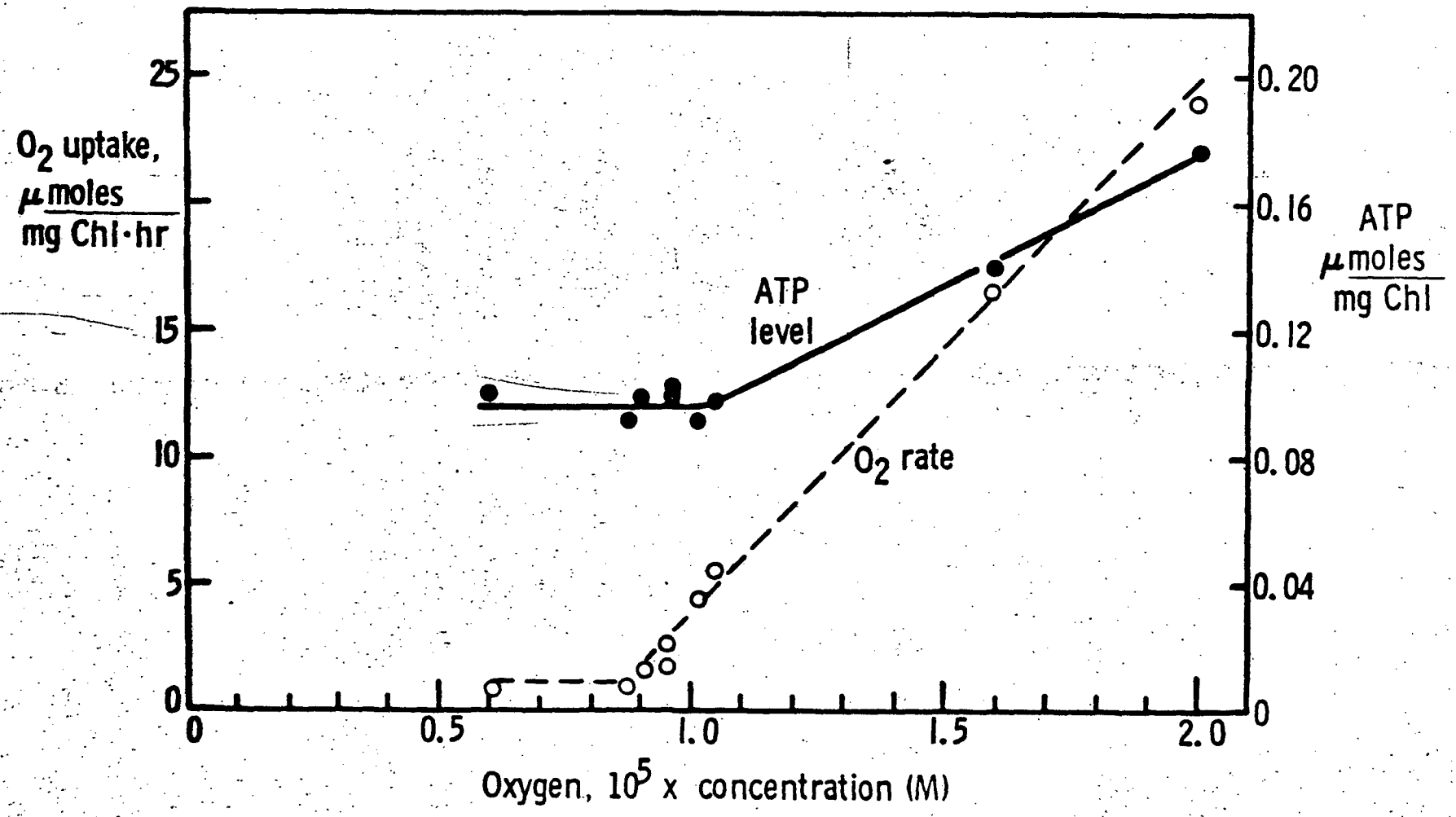
Figure 2. ATP levels in cells of Anacystis vs. time in darkness: Aerobic (open circles); anaerobic (closed circles); two min saturating white light following aerobic darkness (triangles). Aerobic cultures were bubbled with 5% CO_2 in air, anaerobic ones with nitrogen. Anaerobic cultures, given two min saturating white light following the dark, gave the same ATP level as aerobic cultures in the light.


ATP,
n moles
µl cells
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Aerobic - Light
Aerobic-Dark
Anaerobic-Dark
Dark time, hrs.

still able to achieve the 2 min light level of ATP at any time during the period of anaerobiosis. It should be pointed out that the oxygen produced in photosynthesis precluded the maintenance of anaerobiosis in the light. In another experiment in which the cells were kept anaerobic for 6 hr, the level of ATP fell to zero, but these cells were then unable to make new ATP in the light.

Another experiment was designed to show the effect of anerobiosis on the ATP level in more detail. The cells respired normally in darkness at air saturation (2.6×10^{-4} M oxygen at 25 C). When the oxygen concentration was lowered to about 5×10^{-5}M, the rate of respiration began to decrease and became progressively lower as the oxygen concentration decreased. Figure 3 shows the decreasing rate of respiration at low oxygen concentrations. The values in the figure were obtained as follows: samples of cell suspension were put in the polarograph vessel after a preliminary bubbling with nitrogen to lower partially the oxygen concentration. The vessel was closed and darkened and the oxygen monitored while the cells respired, then the cells were killed. The ATP level was determined and compared to the rate of oxygen uptake just before killing. A series of such experiments was run and each succeeding one was allowed to reach a lower oxygen concentration. The results were in substantial agreement with those of Biggins (3). Figure 3 shows that the ATP level continued to decrease as the oxygen uptake rate decreased indicating that the rate of oxidative phosphorylation could no longer maintain a constant ATP level--consumption of ATP exceeded production. When the oxygen concentration was less than the K_m of cytochrome oxidase, about 10^{-5} M (26), the rate of respiration diminished to a new quasi steady-state level. In these cells the ATP level was quite variable depending on the condition of the cell suspension,

Figure 3. The respiration rate (open circles) and ATP level (closed circles) of Anacystis in lowered oxygen concentrations. The respiration rate was that found just prior to killing with perchloric acid.


25
15
10
5
0
O_2 uptake, $\frac{\mu moles}{mg\ Chl \cdot hr}$
0.20
0.16
0.12
0.08
0.04
0
ATP $\frac{\mu moles}{mg\ Chl}$
ATP level
O_2 rate
0
0.5
1.0
1.5
2.0
Oxygen, 10^5 x concentration (M)

but was generally from 0.04 to 0.1 μ moles/mg chl. These experiments showed that ATP is produced in the dark by a respiratory process, probably involving cytochrome oxidase.

Tate of Photophosphorylation

In order to determine the rate of formation of ATP, it was necessary to have a relatively large difference between dark and light levels. As shown in Figure 2, aerobic dark conditions did not produce a sufficiently low level of ATP. In addition, changes observed on darkening the cells after a period of light were extremely variable as noted already in reference to other investigators. This variability might have been expected in view of the large fluctuations in respiratory rates noted by Reid (13) for this and other algae during the first few seconds of darkness. Therefore, the anaerobic, dark pretreatment was adopted.

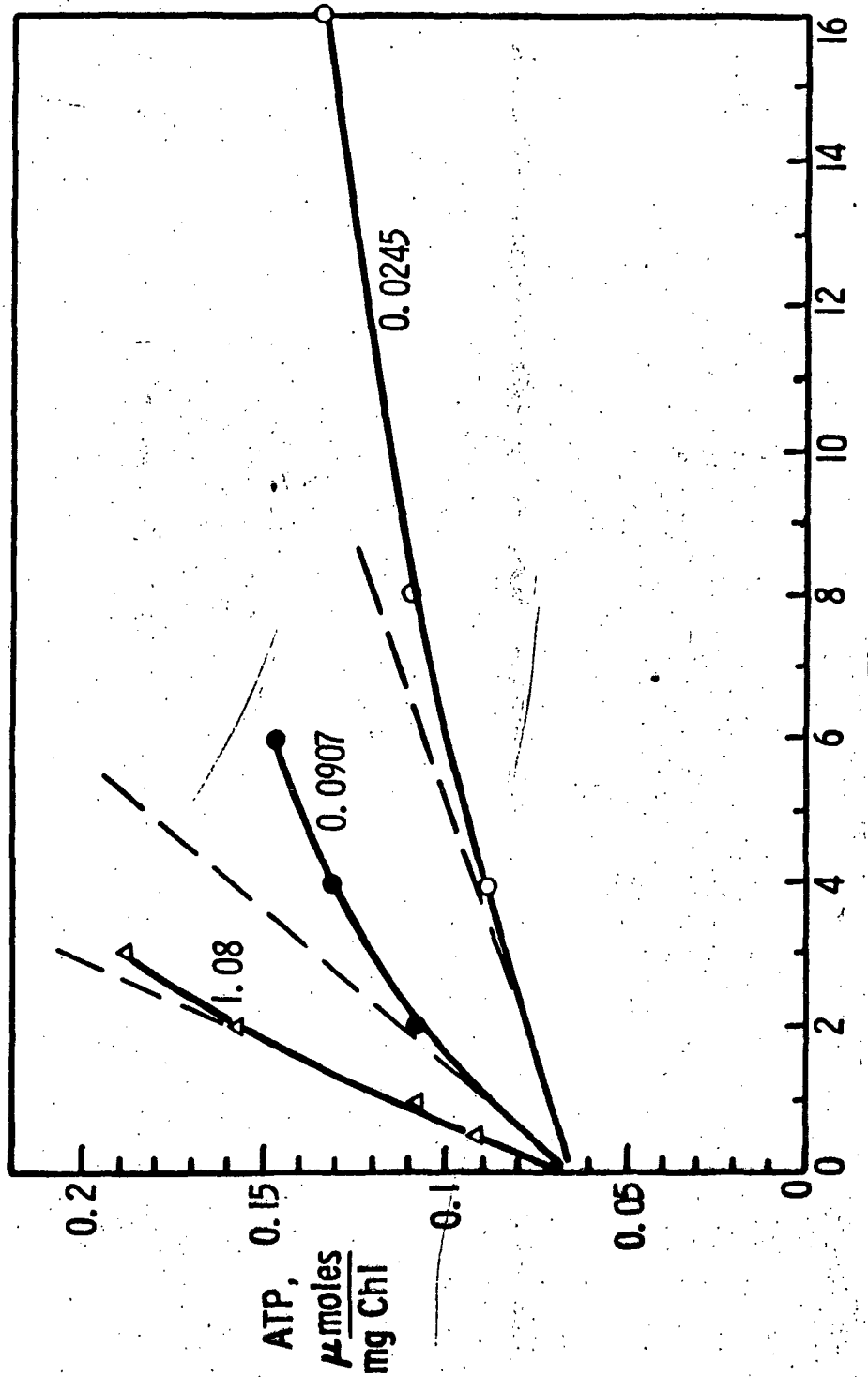
It is well established that for photosynthetic cells, after a period of anaerobosis, oxygen production will not occur immediately on exposure to light. The time of the induction period depends on the duration of anaerobiosis. This induction period was present in these cells, but, as shown below, no induction period in ATP formation occurred.

Having established a condition which produced a low ATP level in the cells and no oxygen production, samples of cell suspensions, each pretreated as described in the methods section, were exposed to short times in the light. Figure 4 shows the results of three intensities of 710 nm. The initial rates are the slopes of the broken lines.

The lowest curve has an initial rate of 0.007 μ moles/mg chl/sec and the middle curve has an initial rate of 0.024 μ moles/mg chl/sec.

These rates are proportional to the absorbed light intensities shown below each curve as μ E/mg chl/sec. The upper curve shows the

Figure 4. The time course of ATP formation of anaerobic Anacystis in 710 nm light of three intensities. The figures below each curve are absorbed μE/mg chl/sec/cm^2. The broken lines are the initial slopes from which rates were calculated. The two lower initial slopes are proportional to the light intensity. The upper initial slope is above the saturating intensity.


0.2
0.15
0.1
0.05
0
2
4
6
8
10
12
14
16
ATP, μmoles / mg Chl
Time, secs.
1.08
0.0907
0.0245

response to a higher intensity and an initial rate of 0.048 μ moles/mg chl/sec. At higher intensities there was no further increase in rates. Thus the saturating rate was about 173 μ moles/mg chl/hr. In other experiments, the saturating rate approached 200 μ moles/mg chl/hr. Several experiments such as these were done and the initial rates vs. intensity were plotted (Fig. 5). The curve, giving data uncorrected for utilization, represents an average quantum requirement of eight, i. e. eight quanta of 710 nm light were required to make one molecule of ATP. The figure shows that at 710 nm, where system I absorption far exceeds system II, ATP formation is carried out relatively efficiently in spite of the lack of cooperation from system II.

Figure 6, obtained in the same way as Figure 5 shows the initial rate of ATP production in 620 nm. The slope of the line gives a quantum requirement of 6, not significantly different from that at 710 nm considering the scatter in the data.

Photophosphorylation Compared to Oxygen Production

To compare the data for the initial rate of ATP production to steady-state oxygen production, samples of the cell suspension before being made anaerobic were put in the polarograph vessel. In light conditions identical to those used for ATP production, oxygen exchange was measured. Figure 7 shows the rates for 710 nm and 620 nm. The rates were corrected for the respiration rate observed immediately following the light period. The slope of the broken line represents the maximum quantum yield at 620 nm under the conditions of these experiments--12 to 15 quanta were required to make one oxygen molecule. As expected, the quantum yield for oxygen at 710 nm was very low.

Figure 5. The rate of ATP formation in Anacystis vs. absorbed intensity of 710 nm light. The line was drawn by inspection. The slope of the line equals the quantum yield.

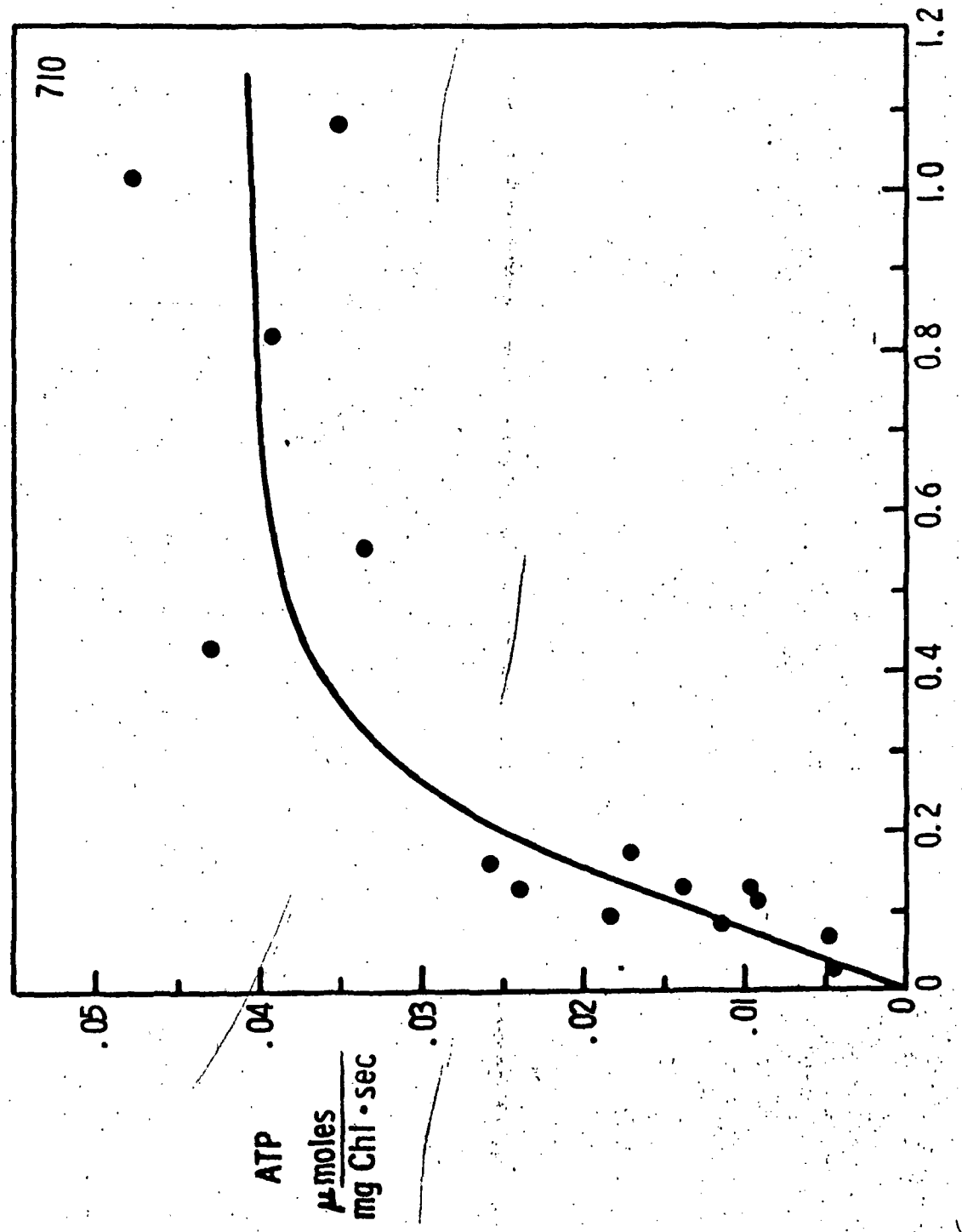

710
ATP
μmoles / mg Chl · sec
.05
.04
.03
.02
.01
0
0
0.2
0.4
0.6
0.8
1.0
1.2
μE/mg Chl/sec/cm²

Figure 6. The rate of ATP formation in Anacystis vs. absorbed intensity of 620 nm light. The line was drawn by inspection. The slope of the line equals the quantum yield.

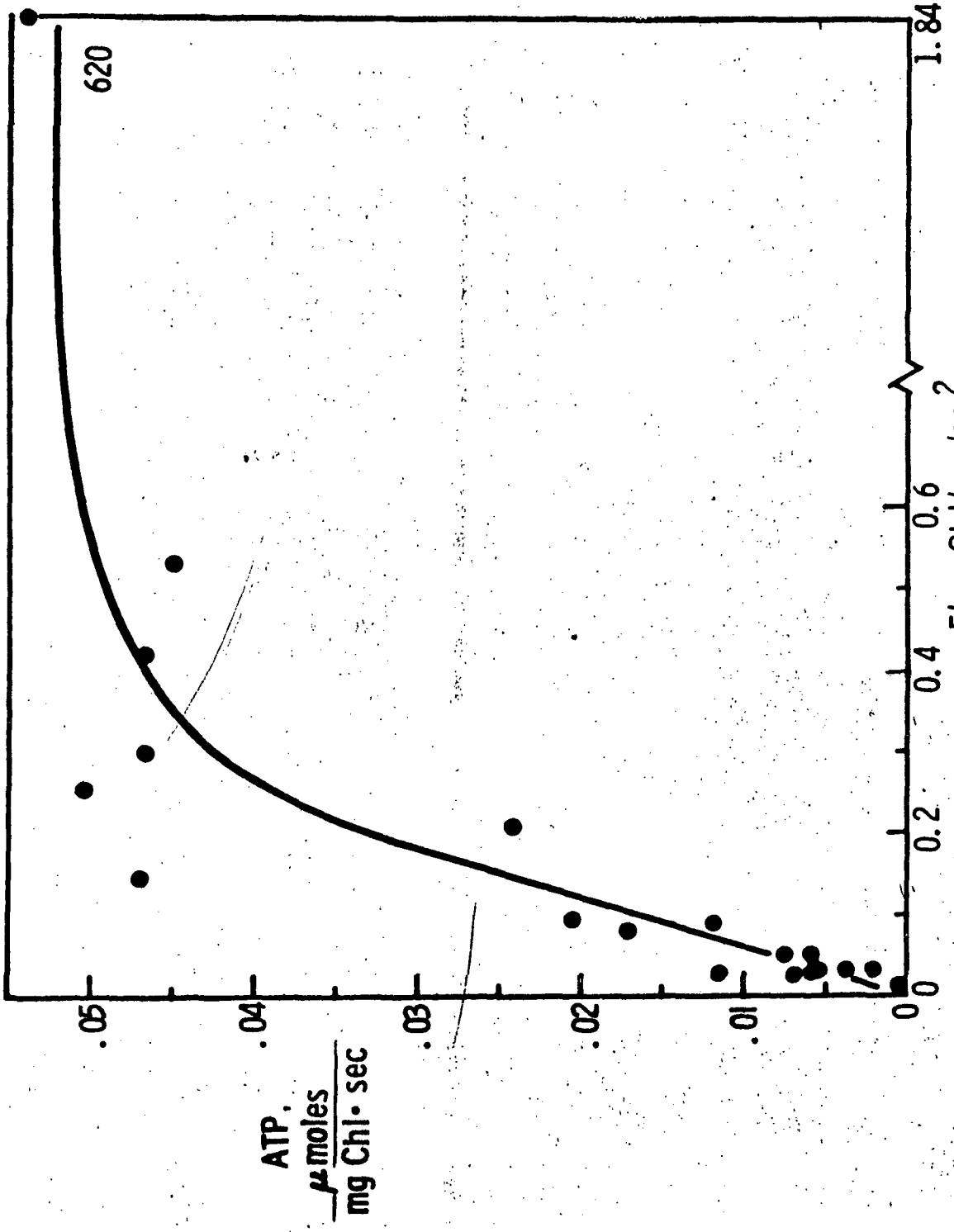
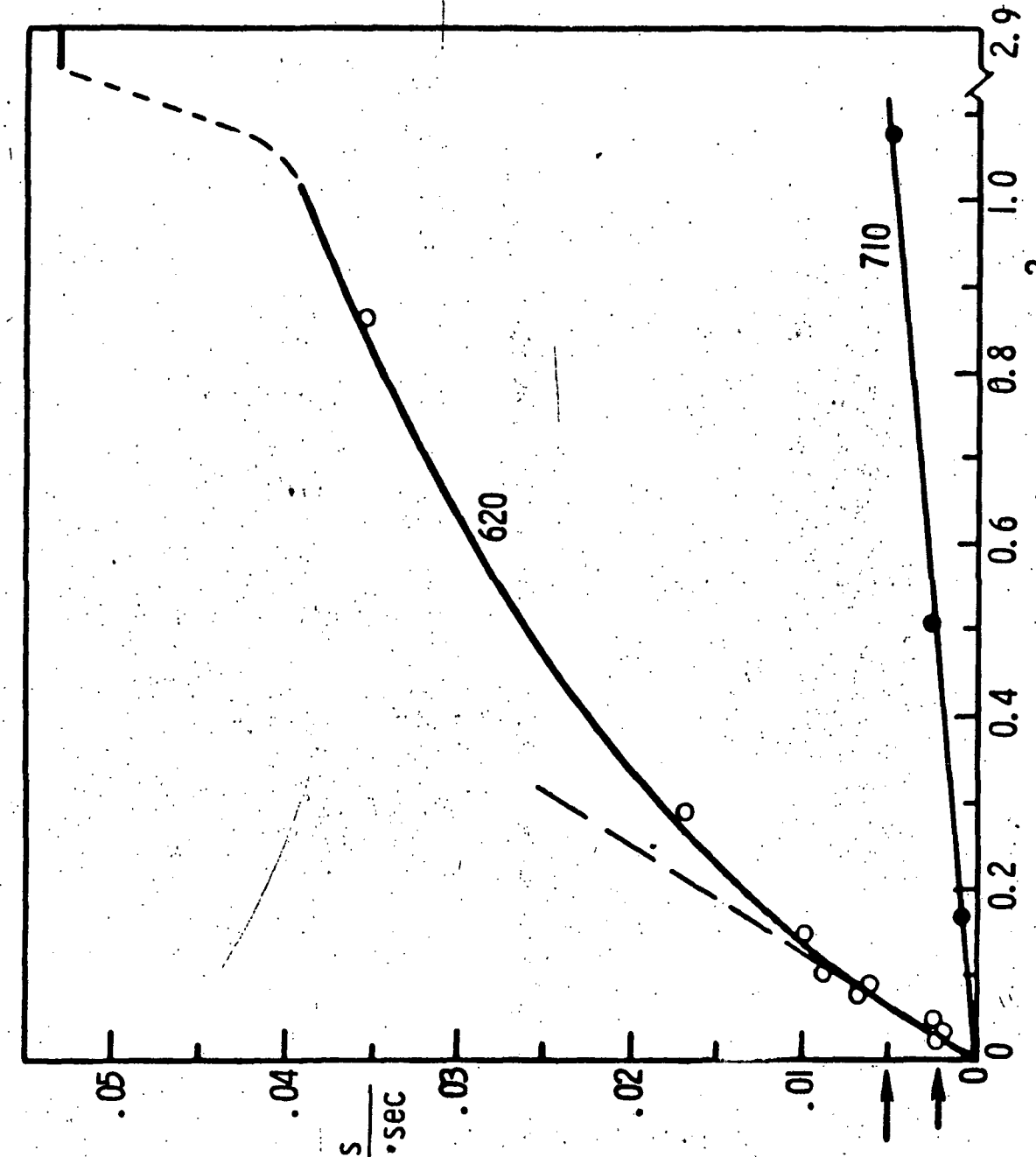

620
1.84
0.6
0.4
0.2
0
.05
.04
.03
.02
.01
0
μE/mg Chl/sec/cm²
ATP, μmoles / mg Chl • sec

Figure 7. The rate of oxygen evolution in Anacystis vs. absorbed intensity of 710 nm light (solid circles), and 620 nm light (open circles). Rates were corrected for respiration measured immediately after the light. The saturating rate in 620 nm light is shown at the upper right. The small arrows on the lower left indicate compensating rate for 620 nm (upper) and 710 nm (lower). They differ because of the inhibition of respiration in 710 nm light. The slope of the broken line represents the quantum yield for oxygen in 620 nm light.


.05
.04
.03
.02
.01
0
0
0.2
0.4
0.6
0.8
1.0
2.9
620
710
O_2, $\frac{\mu moles}{mg\ Chl \cdot sec}$
$\mu E/mg\ Chl/sec/cm^2$

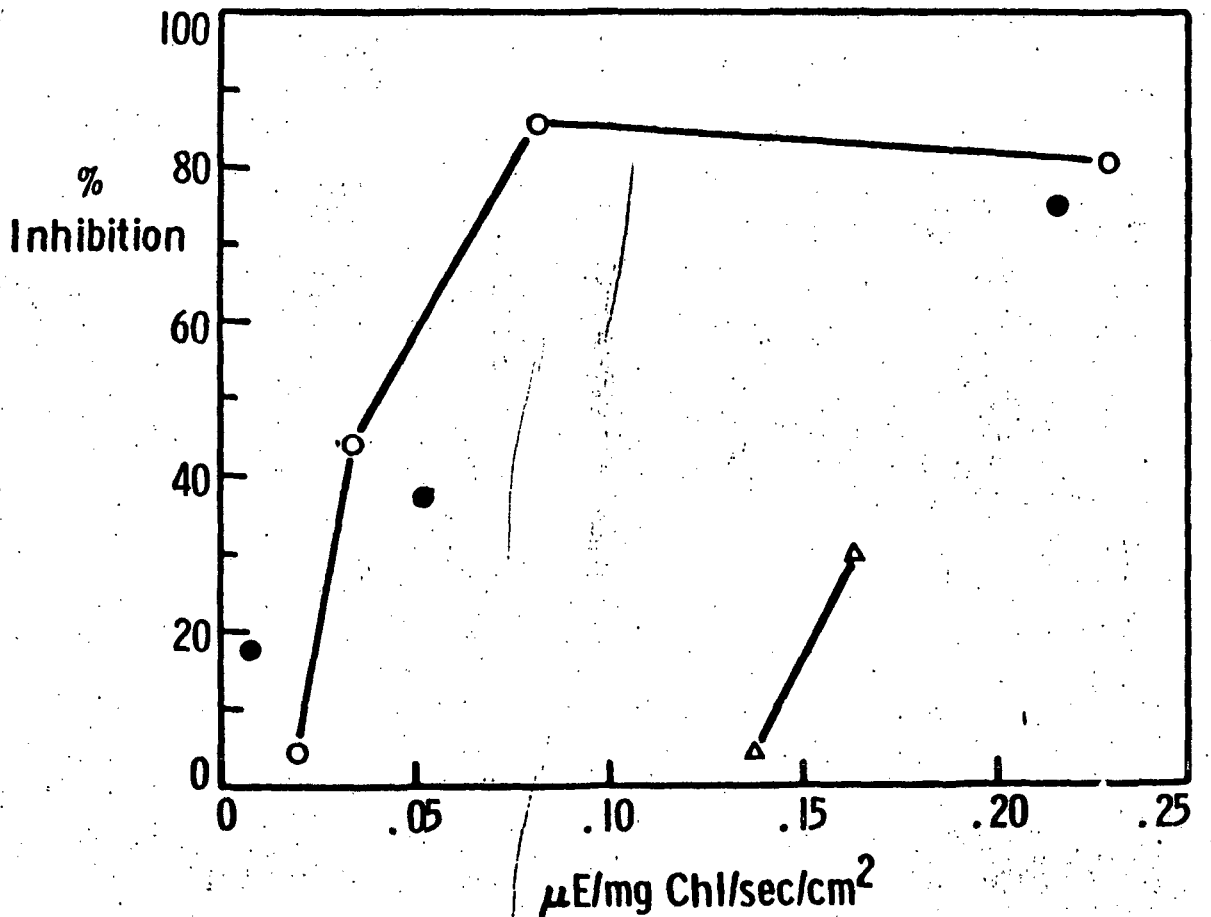
A comparison of ATP production at 620 nm (Fig. 6) with oxygen production at the same wavelength and intensities (Fig. 7) indicates that the cells were capable of making two ATP molecules under anaerobic conditions for each oxygen molecule evolved under aerobic conditions.

However, if under anaerobic conditions, no oxygen evolution occurred and no non-cyclic electron transport occurred either, then phosphorylation at 620 nm must have been due to complete spillover of excitation energy to system I, a phosphorylation mechanism associated with system II, or a combination of the two. On the other hand, if electrons from excitation of system II were transported to some unknown acceptor, in spite of anaerobiosis, then the ability of the cells to make ATP can be related to their ability to transport these electrons. In these cells the rate of ATP formation at low intensity is twice the observed rate of oxygen production at the same intensity. If four electrons are necessary to produce one oxygen molecule, the ratio of the rates of formation indicates a P/e_2 of one. Assuming that, as in isolated chloroplasts, one ATP is formed for each pair of electrons transported, then the value of one indicates that only one phosphorylation site is involved in photophosphorylation at 620 nm.

Effect of DCMU

To prove that ATP formation in 710 nm light is strictly a system I reaction, DCMU, an inhibitor of system II was added at a relatively high concentration. Figure 8 shows that ATP formation was inhibited by only 4 to 30% at intensities which were on the ascending part of the rate vs. intensity curve. Thus we can conclude that phosphorylation at 710 nm does not require electrons from system II. In contrast, DCMU inhibition at 620 nm was greater and increased with increasing intensity

Figure 8. The effect of absorbed light intensity on the inhibition of photophosphorylation rate by DCMU in Anacystis. Closed circles, 620 nm, 10^{-5}M DCMU; open circles, 620 nm, 2 x 10^{-5}M DCMU: triangles, 710 nm, 10^{-5}M DCMU. Each point is an average of two determinations.


100
80
60
40
20
0
% Inhibition
0
.05
.10
.15
.20
.25
μE/mg Chl/sec/cm²

and concentrations of DCMU. More inhibition at higher intensity means that the inhibitor acts on a thermal step rather than a photochemical step. Thus ATP formation occurs beyond a thermal step in the electron transport system. The fact that the extent of DCMU inhibition is different at the two wavelengths further suggests that complete spillover of excitation energy does not occur.

Maximum Level of ATP

The data reported so far were all derived from initial rates of ATP formation, mostly in the first one to ten seconds. Examination of Figure 4 shows that the rate decreases with time. In order to determine how steady-state level is related to intensity, anaerobic cells were exposed to five minutes of 710 nm light of various intensities and the ATP level determined. Figure 9 shows the results. At very low intensities, the rate of ATP formation was not sufficient to reach the normal level in five minutes, although at the initial rate, the time should have been sufficient even at the lowest intensity used in this experiment. Therefore, one must conclude that another factor, increasing during the light period, prevents the cells from maintaining their initial rate of ATP production. The most obvious candidate for this factor is a consumption of the ATP.

To determine the rate of consumption the cells were exposed to a moderate intensity of 710 nm, 0.12 μ E/mg chl/sec. Then short dark times, following the standard 6 sec light exposure, were given and ATP determined.

Figure 10 shows the results of two experiments. The lower curve shows the dark consumption of ATP immediately following a period of light. It resembles a first order decay reaction with a half-time of 0.6 sec. The upper curve shows another experiment under the same conditions but with shorter times in order to determine more accurately the initial slope.

Figure 9. ATP levels in Anacystis after 5 min vs. absorbed intensity of 710 nm light.

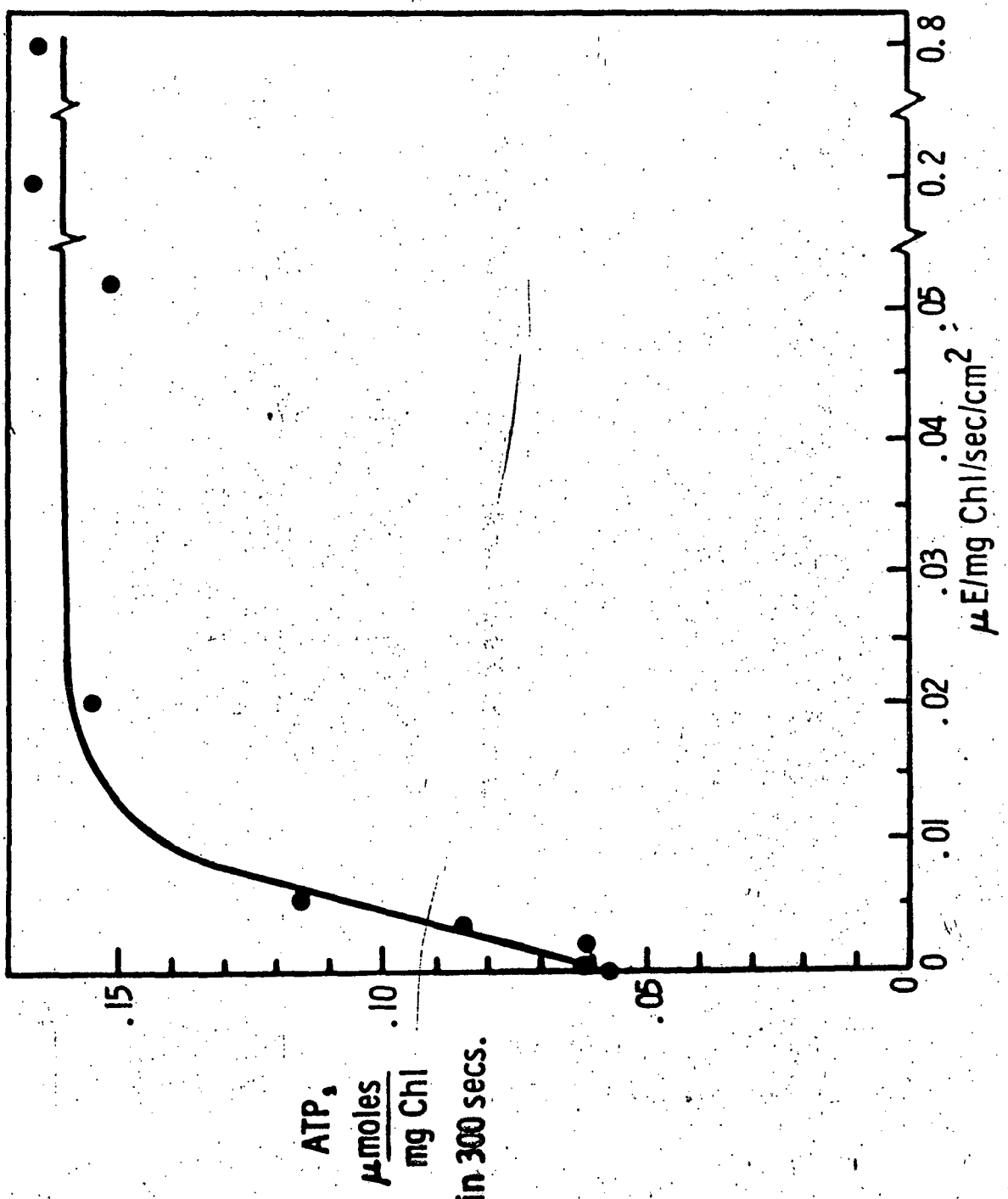
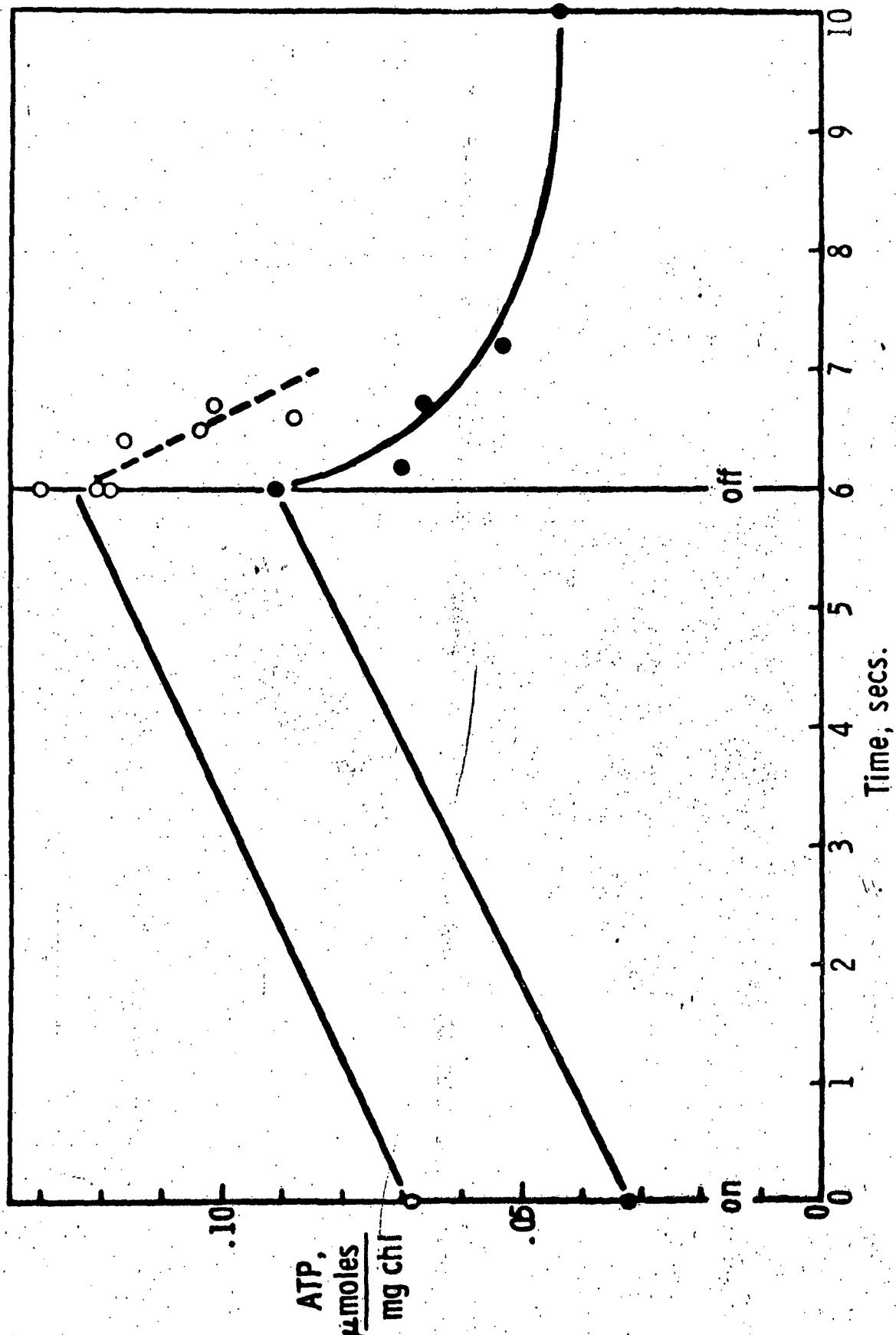

ATP, μmoles / mg Chl in 300 secs.
.15
.10
.05
0
.01
.02
.03
.04
.05
0.2
0.8
μE/mg Chl/sec/cm²

Figure 10. Two experiments with Anacystis showing the production of ATP when irradiated with 710 nm, and the consumption of ATP in subsequent darkness. The first experiment (lower curve) shows the decay in 4 sec darkness. The second experiment (upper curve) shows the average initial slope in the first sec of darkness. Absorbed light intensity was 0.12 μ E/mg chl/sec/cm^2.


ATP, μmoles / mg chl
.10
.05
0
on
off
0
1
2
3
4
5
6
7
8
9
10
Time, secs.

The broken line is the calculated average slope for the points shown. An initial photophosphorylation rate of 0.015 μ moles ATP/mg chl/sec was expected from the light intensity used although in this experiment it actually was, 0.009 μ moles/mg chl/sec. The average dark rate, shown in the figure, was 0.038 μ moles/mg chl/sec. The fact that the dark rate was two to four times the light rate indicated a dark consumption which is not dependent on the amount of ATP made in the light. One might suppose that there is a dark consumption rate dependent on the total amount of ATP present, but the lower curve of Figure 10 does not show a decay to zero.

How can the initial "dark" rate be greater than the initial "light" rate? This would occur if there were a rate of consumption in the light which was dependent on the light intensity. The rate of consumption in the light would depress the observed rate. When the light is turned off production stops immediately, but a delay of several seconds occurs before consumption stops, so a relatively high negative rate is observed as in Figure 10. To obtain the true total rate of production the initial "dark" rate should be added to the observed "light" rate. In the experiment being discussed here, the observed light rate 0.009, plus the rate of initial dark consumption, 0.038, equals 0.047 μ moles/mg chl/sec. Thus the quantum requirement is lowered from eight to about two.

Any time the rate of consumption begins to exceed the rate of production, the observed rate will become negative. This has been seen on several occasions, especially in 620 nm light after ten seconds exposure. Such fluctuations in rate having a regular periodicity have recently been shown to occur in Chlorella fusca (10).

DISCUSSION

Early Saturation of Photophosphorylation

The rate of photophosphorylation vs. intensity curves at the two wavelengths examined here show a saturation at an intensity considerably below the saturation of photosynthesis, several synthetic processes in algae which require light have shown a similar, early saturation. These processes are either known to require ATP or ATP has been implicated as a requirement. For example Syrett (21) recorded for Chlorella an early saturation in both isocitrate lyase formation, a protein synthesizing process, and in glucose conversion to polysaccharides. Hoch, Owens, and Kok (6) reported early saturation in respiratory inhibition by light in A. nidulans and Tanner et al. (22) using anaerobic glucose assimulation have seen the same thing.

The discussion above suggests that ATP requiring processes should show saturation at low intensities but the prime ATP requiring process, CO_2 fixation, does not show it, nor does photoreduction of CO_2 by hydrogen adapted Ankistrodesmus (4). A continuing increase in the rate of CO_2 reduction beyond the saturation rate of ATP formation indicates that ATP is not the limiting factor.

Lack of Time Lag

Several investigators using isolated chloroplasts have reported both a time and intensity lag in the formation of ATP. These lags have been equated with the formation of a proton gradient whose energy is necessary for synthesis of ATP (16). No time lag has been observed in the present study. An obvious intensity lag has not been observed either but the data are too scattered to rule out the possibility of its occurrence at very low intensities. It is possible that if a proton gradient

exists in whole cells it is a much faster phenomenon and not observable in these experiments.

Quantum Efficiency

It is well established that in low light, where oxygen production shows maximal efficiency, a minimum of eight quanta are required to produce one O_2 molecule, or, assuming a photosynthetic quotient of one, to fix one CO_2. If in the Calvin cycle three ATP's are required to fix one CO_2, then three ATP's must be made from eight quanta or about 0.4 ATP/quantum. We report about 0.5 ATP/quantum which is enough to maintain CO_2 fixation at the maximal rate.

It is clear that photophosphorylation can be quantitatively related to light absorption in algal cells. A direct relationship holds and the process is efficient when compared to oxygen evolution. There is a distinct system I activated phosphorylation and another phosphorylation site between the photoacts or possibly associated with system II.

The ability to isolate experimentally this fundamental metabolic reaction makes it now possible to determine the relationship of photophosphorylation to other energy requiring and producing reactions in the whole cell.

REFERENCES

1. Atkinson, D. E. 1969. Regulation of enzyme function. Annu. Rev. Microbiol. 23:47-68.

2. Baltscheffsky, M. 1967. Inorganic pyrophosphate as an energy donor in photosynthetic and respiratory electron transport phosphorylation systems. Biochem. Biophys. Res. Commun. 28:270-276.

3. Biggins, J. 1969. Respiration in blue-green algae. J. Bacteriol. 99:570-575.

4. Bishop, N. I. 1961. The photometabolism of glucose by an hydrogen-adapted alga. Biochim. Biophys. Acta 51:323-332.

5. Gibson, J. and S. Morita. 1967. Changes in adenine nucleotides of intact Chromatium D produced by illumination. J. Bacteriol. 93:1544-1550.

6. Hoch, G., O.v.H. Owens, B. Kok. 1963. Photosynthesis and Respiration, Arch. Biochem. Biophys. 101:171-180.

7. Estabrook, R. W., J. R. Williamson, R. Frenkel, P. K. Maitra. 1967. The fluorometric determination of mitrochondrial adenine and pyridine nucleotides. In: R. W. Estabrook and M. E. Pullman, eds., Methods in Enzymology, Vol. X. Academic Press Inc., New York, pp. 474-482.

8. Kratz, W. A. and J. Myers. 1955a. Nutrition and growth of several blue-green algae. Amer. J. Bot. 42:282-287.

9. Kratz, W. A. and J. Myers. 1955b. Photosynthesis and respiration of three blue-green algae. Plant Physiol. 30:275-280.

10. Lewenstein, A. and R. Bachofen. 1972. Transient induced oscillations in the level of ATP in Chlorella fusca. Biochim. Biophys. Acta 267:80-85.

11. MacKinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140:315-322.

12. Pedersen, T. A., M. Kirk, J. A. Bassham. 1966. Light-dark transients in levels of intermediate compounds during photosyntesis in air-adapted Chlorella. Physiol. Plant. 19:219-231.

13. Reid, A. 1970. Energetic aspects of the interaction between photosynthesis and respiration. In: F. Setlik, ed., Prediction and measurement of photosynthetic productivity. Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands, pp. 231-245.

14. Santarius, K. A. and U. Heber. 1965. Chages in the intracellular levels of ATP, ADP, AMP and Pi and regulatory function of the adenylate system in leaf cells during photosynthesis. Biochim. Biophys. Acta 102:39-54.

15. Santarius, K. A., U. Heber, W. Ullrich, W. Urbach. 1964. Intracellular translocation of ATP, ADP and inorganic phosphate in leaf cells of Elodea densa in relation to photosynthesis. Biochem. Biophys. Res. Commun. 15: 139-146.

16. Schwartz, M. 1968. Light induced proton gradient links electron transport and phosphorylation. Nature 219:915-919.

17. Simonis, W. 1966. Problems of photosynthetic phosphorylation in vivo by unicellular algae (Ankistrodesmus). In: J. B. Thomas and J. C. Goedheer, eds., Currents in Photosynthesis. Donker, Rotterdam. pp. 217-225.

18. Smith, A. J., J. London, R. Y. Stanier. 1967. Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. 94:972-983.

19. Strehler, B. L. 1953. Firefly luminescence in the study of energy transfer mechanisms. II. Adenosine triphosphate and photosynthesis. Arch. Biochem. Biophys. 43:67-69.

20. Syrett, P. J. 1958. Respiration rate and internal adenosine triphosphate concentration in Chlorella. Arch. Biochem. Biophys. 75:117-124.

21. Syrett, P. J. 1966. The kinetics of isocitrate lyase formation in Chlorella: evidence for the promotion of enzyme synthesis by photophosphorylation, J. Exp. Bot. 17:641-654.

22. Tanner, W., L. Dächsel, and O. Kandler. 1965. Effects of DCMU and antimycin A on photoassimilation of glucose in Chlorella. Plant Physiol. 40:1151-1156.

23. Tanner, W., E. Loos, W. Klob, O. Kandler. 1968. The quantum requirement for light dependent anaerobic glucose assimilation by Chlorella vulgaris. Z. Pflanzenphysiol. 59:301-303.

24. Urbach, W. and W. Simonis. 1967. Further evidence for the existence of cyclic and non-cyclic photophosphorylation in vivo by means of desaspidin and DCMU. Z. Naturforsch. 22b:537-540.

25. Welsch, F. and L. Smith. 1969. Kinetics of synthesis and utilization of adenosine triphosphate by intact cells of Rhodospirillum rubrum. Biochemistry 8:3403-3408.

26. Yonetani, T. 1963. Cytochrome oxidase. In: E. C. Slater, ed., Proceedings of the Fifth International Congress of Biochemistry. Vol. V. Pergamon Press, Amsterdam. p. 403, Fig. 3, from B. Chance

TRIPARANOL INHIBITION OF STEROL BIOSYNTHESIS IN *CHLORELLA ELLIPSOIDEA**

J. T. CHAN and G. W. Patterson

Department of Botany
University of Maryland
College Park, Maryland 20742

SUMMARY

The sterol composition of *C. ellipsoidea* was markedly changed when this alga was grown in the presence of 1 ppm triparanol. Triparanol appears to inhibit the removal of 14α-methyl group, the second alkylation at C-24, Δ^7-reductase, and $\Delta^8 \rightarrow \Delta^7$ - isomerase. The effect of triparanol in *Chlorella* is much more diversified than the specific effect originally assigned to it in animals.

Triparanol has been found to affect sterol biosynthesis in rat skin by blocking the reduction of desmosterol to cholesterol which causes the accumulation of Δ^{24}-sterols (1,2,3). Triparanol has also been noted to inhibit the reduction of the Δ^7-bond causing an accumulation of $\Delta^{5,7}$- sterols (4,5).

In plants, sterol biosynthesis prceeds by a different pathway involving cycloartenol, and, alkylation rather than reduction normally occurs at the 24(25) double bond. Earlier in our laboratory, it was found that in *Chlorella emersonii*, triparanol inhibited the second transmethylation at C-24 and blocked the removal of the 14α-methyl group, resulting in an accumulation of 24-methylene sterols and 14α-methyl sterols, respectively (6). A related organism, *Chlorella ellipsoidea*, synthesizes Δ^5-sterols in contrast to the Δ^7 sterols of *Chlorella emersonii*. This organism is of interest because in it we can study the effect of triparanol on the second alkylation reaction, the removal of the 14α-methyl, and the reduction of the 7(8) double bond.

MATERIALS AND METHODS

Chlorella ellipsoidea was grown in a glucose-inorganic salts medium in

*Scientific Article No. A1839 Contribution No. 4747 of the Maryland Agricultural Experimental Station.

carboys as described elsewhere (7). Triparanol-treated cells were grown under identical conditions except for the addition of 1 ppm triparanol to the culture medium. Cells obtained were freeze-dried and extracted with $CHCl_3$-CH_3OH (2:1, v/v). After saponification, sterols were precipitated from the nonsaponifiable fraction with digitonin and sterols were recovered by the method of Issidorides et al. (8). Total free sterols were separated into dimethyl-, methyl- and desmethyl sterols on a Woelm Grade II neutral alumina column. After acetylation, sterol acetates were further separated by $AgNO_3$-silica gel column chromatography (9). GLC analyses were made on Glowall Chromalab, Model A-110 and Model A-310 gas chromatographs equipped with argon ionization detectors and a Honeywell 12-in. recorder with Disc integrator. Sterols were identified using GLC data on four columns and by GC-mass spectroscopy. MS were recorded on an LKB Model 9000 gas chromatograph-mass spectrometer. The compounds were introduced into the ion chamber through a 0.75% SE-30 column.

RESULTS AND DISCUSSION

A large qualitative and quantitative difference between the control and inhibited cultures was revealed when their sterol mixtures were separated by $AgNO_3$-silica gel column chromatography and analysed on an SE-30 column (Fig. 1). Relative retention times of the peaks obtained from control cultures indicated that the major sterols are identical to those described previously (7,10). The chromatogram of the inhibited culture showed four major peaks as opposed to three major peaks on that of the control culture. In addition, some of the peaks obtained from the inhibited culture were broader than those from the control culture, indicating the occurrence of more than one sterol per peak.

Sterols observed in inhibited cultures are listed in Table I. They were identified by their movement on the Al_2O_3 column and $AgNO_3$-silica

gel column, their relative retention times on four GLC columns (11) and by GC-MS. The chromatographic characteristics of all these sterols were identical to those of authentic compounds. A quantitative comparison of control sterols versus inhibited sterols was performed, using a gas chromatograph equipped with a Disc integrator. There was a reduction in total sterol concentration of approximately 68% in the inhibited culture. This seems to indicate that triparanol blocks certain steps of sterol biosynthesis before the cyclization of squalene, as previously postulated in rats (12) and in Ochromonas (13).

Production of the three major sterols of the control culture was reduced from near 100% of the total sterol to 23% of the total sterol in inhibited cultures. Poriferasterol was reduced by the greatest amount (89%). Significant accumulations of ergosta-5,7-dien-3β-ol, Δ^7-chondrillastenol, 5α-(24S)-stigmasta-8, 14-dien-3β-ol, obtusifoliol, 5α-ergost-7-en-3β-ol, and 5α-ergosta-8,14-dien-3β-ol were found. These sterols were not detected in control cultures. Ten of the sterols identified in inhibited cultures were also present in the AY-9944-treated cultures of the same alga (10). The effect of triparanol in C. ellipsoidea seems to be similar in some respects to that postulated for C. emersonii (6). The accumulation of 14α-methyl sterols (12% of total sterol) suggests that triparanol also prevents the removal of the 14α-methyl group in C. ellipsoidea. The ratio of sterols with 10-carbon side chains to those with 9-carbon side chains is 2:1 in the control by 2:5 in inhibited cultures. This indicates that triparanol is inhibiting alkylation that leads to sterols with 10-carbon side chains. The accumulation of 24-methylene cycloartanol and obtusifoliol provide further evidence for the postulated inhibition, since 24-methylene sterols are usually considered as precursors for the second alkylation (14,15). However,

this study did not detect 24-methylene pollinastanol or 14α-methyl-5α-ergosta-8(9), 24(28)-dien-3β-ol which accumulated to a great extent in triparanol-treated C. emersonii (6). The increase of $\Delta^{5,7}$-sterols from unnoticeable amounts in the control (less than 0.1% of total sterol) to 31% of the total sterol in inhibited cultures, reveals the inhibitory action of triparanol on Δ^7-reductase (16). This was not found in C. emersonii, since that organism apparently does not contain a Δ^7-reductase (17). There is also an increase in ratio of Δ^8/Δ^7 sterols in the inhibited cultures. Similar results were obtained with triparanol-treated C. emersonii and with AY-9944 treated cultures of C. ellipsoidea in this laboratory (6,10). This effect was reported earlier with sterols in rat skin (18). This suggests an inhibitory effect of triparanol on Δ^8—Δ^7-isomerase.

From preceding observations, triparanol seems to have a much more diversified effect than has been anticipated. Some of these effects could possibly account for its adverse effects on human beings as a hypocholesterolemic drug (19,20,21).

ACKNOWLEDGEMENTS

The authors wish to thank S. R. Dutky for the mass spectra and T. R. Blohm for the triparanol. This work was supported in part by a grant from the National Aeronautics and Space Administration.

REFERENCES

(1) Avigan, J., Steinberg, D., Vroman, H. E., Thompson, M. J., and Mosettig, E. J. Biol. Chem., 235, 3123 (1960).

(2) Steinberg, D., and Avigan, J., J. Biol. Chem., 235, 3127 (1960).

(3) Horlick, L., and Avigan, J., J. Lipid Res., 4, 160 (1963).

(4) Dempsey, M. E., Proc. 6th Intern. Congr. Biochem. VII:570 (1964).

(5) Dempsey, M. E., J. Biol. Chem. 240, 4176 (1965).

(6) Doyle, P. J., Patterson, G. W., Dutky, S. R., and Thompson, M. J., Phytochemistry 11, 1951 (1972).

(7) Patterson, G. W., and Krauss, R. W., Plant and Cell Physiol. 6, 211 (1965).

(8) Issidorides, C. H., Kitagawa, I., and Mosettig, E., J. Org. Chem. 27, 4693 (1962).

(9) Vroman, H. E., and Cohen, C. F., J. Lipid Res. 8, 150 (1967).

(10) Dickson, L. and Patterson, G. W. Lipids (In Press).

(11) Patterson, G. W., Anal. Chem. 43, 1165 (1971).

(12) Holmes, W. L., and Ditullio, N. W., Am. J. Clin. Nutr. 10, 310 (1962).

(13) Aaronson, S., and Bensky, B., J. Protozool. 12, 236 (1965).

(14) Castle, M., Blondin, G., and Nes, W. R., J. Am. Chem. Soc., 85, 3306 (1963).

(15) Patterson, G. W., and Karlander, E. P., Plant Physiol. 42, 1651 (1967).

(16) Dempsey, M. E., Ann. N. Y. Acad. Sci., 148, 631 (1968).

(17) Patterson, G. W., Plant Physiol. 42, 1457 (1967).

(18) Clayton, R. B., Nelson, A. N., and Frantz, I. D., J. Lipid Res. 4, 166 (1963).

(19) Achor, R. W P., Christensen, N. A., Berge, K. G. and Mason, H. L., Proc. of Stf. Mtg. Mayo Clinic 38, 32 (1963).

(20) Otto, H. F., Beitr. Pathol. 142, 177 (1971).

(21) Kirby, T. J., Trans. Amer. Opthalmol. Soc. 65, 493 (1967).

TABLE I

A Quantitative Comparison of Sterols from Cpntrol and Triparanol-Treated Cultures of *Chlorella ellipsoidea*

Sterols	Control		Triparanol-Treated	
	% of Sample	µg/g DW[a]	% of Sample	µg/g DW
4α,14α-dimethyl-5α-(24S)-stigmast-8-en-3β-ol			1.2	12
cycloartenol			0.1	1
24-methylene cycloartanol			0.7	7
obtusifoliol			7.5	76
24-dihydroobtusifoliol			1.3	13
14α-methyl-5α-(24S)-stigmast-8-en-3β-ol			0.1	1
14α-methyl-5α-ergost-8-en-3β-ol			0.9	9
5α-ergosta-8,14-dien-3β-ol			7.8	79
5α-ergost-8(9)-en-3β-ol			2.7	28
5α-ergost-7-en-3β-ol			7.1	72
ergosta-5,7-dien-3β-ol			29.3	297
5α-(24S)-stigmasta-8,14-dien-3β-ol			7.5	76
5α-(24S)-stigmast-8(9)-en-3β-ol			1.7	17
24S-stigmasta-5,7,22-trien-3β-ol			2.0	21
Δ^7-chondrillastenol			6.8	69
ergost-5-en-3β-ol	33.2	1062	13.8	140
poriferasterol	62.3	1993	6.7	68
clionasterol	4.5	144	2.8	29
	100.0	3199	100.0	1015

[a]DW=Dry Weight

The Reversion of Rotation of Helical Chloroplasts in a Green Rod-Shaped Alga

Constantine Sorokin

Department of Botany, University of Maryland
College Park, Maryland 20742

Shape of the chloroplast has been considered a stable characteristic of cells of a certain organism (Smith 1950). In the four genera of green algae--Spirogyra, Sirogonium, Spirotenia and Geniculatia--known to have helical chloroplasts, the direction of the rotation of the chloroplast--either clockwise (right-handed) or counter-clockwise (left-handed)--is maintained (Fritsch 1965) to be inherently characteristic of the particular genus. Butterfass (1957), however, observed that in Spirogyra majuscula, which normally has left-handed chloroplasts, some cells in laboratory cultures possessed chloroplasts rotated in the opposite, that is right-handed direction. No explanation has been given for such a change in the supposedly stable taxonomic characteristic except for the suggestion that "culture conditions apparently have an effect on the direction of spiraling of the plastids in Spirogyra" (Hoshaw 1968). In describing another case of the reversion of the rotation of helical chloroplasts, we are able to propose a mechanism by which the reversion may take place.

The rod-shaped cells of the organism in which the reversion was observed are 1.8 to 2.5 μ in width and, under the conditions of slow growth, 2 to 4 μ in length. Each cell has a single parietal girdle-like chloroplast. In the absence of pyrenoids, the organism can be considered as a Stichococcus-type green alga of the order Ulotrichales. Under more favorable growth conditions, the cells elongate reaching up to 10-12 μ in length. The chloroplast by extension, attenuation and twisting assumes a helical shape (Sorokin et al., 1972).

As seen in Fig. 1, in each of the three photographs there is a mixture of cells with chloroplasts rotating in the opposite directions: either to the right or to the left. The attention is drawn to the fact that each culture, represented by a separate photograph in Fig. 1, is of clonal origin obtained by two successive platings and isolations of single colonies from the surface of the nutrient agar.

We suggest that the change in the direction of rotation of the chloroplasts in individual cells occurs during their growth-division cycle. The direction of the rotation of the chloroplast in our organism is not genetically controlled but is determined by chance in the course of cell development. In regard to the development of the chloroplast, the growth-division cycle in our organism consists of two phases. During one phase, the cell, under favorable for growth conditions, rapidly elongates and the chloroplast by extension and twisting assumes a helical shape. The building of the body of the chloroplast during this developmental stage falls behind the extension process and the chioroplast due to process and the chloroplast due to stretching and attenuation occupies a progressively smaller portion of the lumen of the cell. Sometimes before or during cell division, this stage is superseded by another one during which the cell extension is minimal or absent. The building of the body of the chloroplast catches up and the chloroplast attains the form characteristic of that in short cells in a slow growing culture. (Sorokin et al., 1972) and of the shortest cells in Fig. 1B. It now occupies the larger portion of the lumen of the cell. The compression of the chloroplast makes its twisting minimal or absent (Sorokin et al., 1972). With the advent of the stage of rapid extension, the chloroplast again undergoes attenuation and twisting with the 50 to 50 chance that the rotation is in one or another direction.

Naturally, the proposed mechanism can account for the change in the rotation of the helical chloroplasts only in organisms and under conditions which favor the existence of the two described above phases of cell development: the phase of

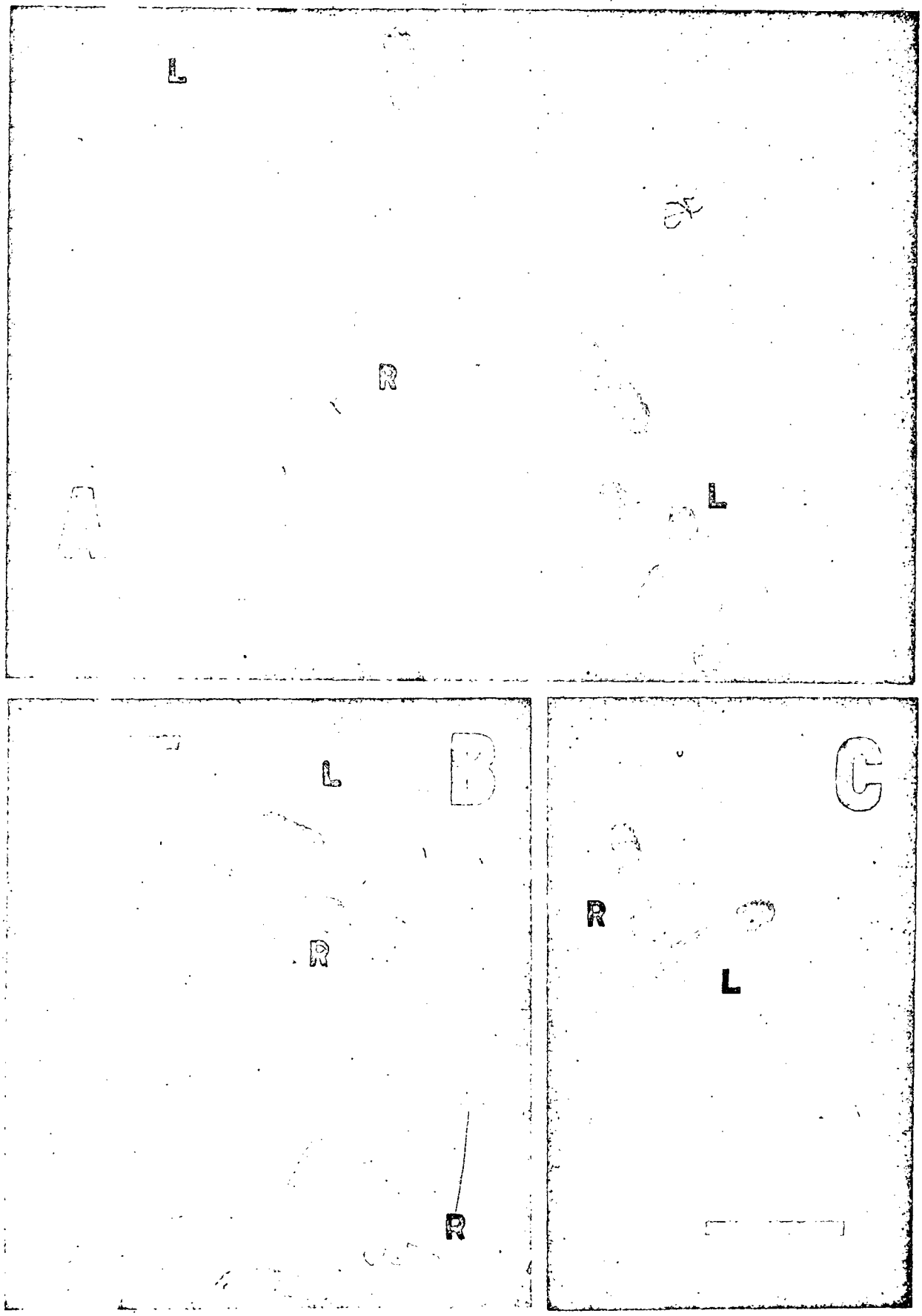
rapid cell elongation with the accompanying twisting of the chloroplast and the phase of slow or no extension during which the chloroplast is straightened into a direct rod or plate. It seems unreasonable to expect that under any circumstances a well developed coiled up chloroplast in a mature cell can unwind itself and then wind up again in the opposite direction without going through the cell developmental stage which makes such a change in the chloroplast rotation possible.

References

1. Butterfass, T. 1957. Über das Vorkommen umgekert gewundener Chromatophoren bey Spirogyra. Protoplasma 48:419-421.
2. Fritsch, F. E. 1965. The Structure and Reproduction of the Algae. Cambridge Univ. Press, Vol. 1, 791 pp.
3. Hoshaw, R. W. 1968. Biology of the filamentous conjugating algae. Pp. 135-184 in Jackson D. F. (ed.) "Algae, Man and Environment" University Press, Syracuse.
4. Smith, G. M. 1950. The Fresh-Water Algae of the United States. 2nd ed., McGraw-Hill, New York, 719 pp.
5. Sorokin, C., Corbett, M. K., and Nishino, S. 1972. Helical chloroplasts in a Stichococcus-type alga. J. Phycol. 8:152-156.

Legend

Figure 1. A-C. Cells with the right-and left-handed rotation of the chloroplasts in three clonal cultures each culture depicted separately in one of the three photographs. Length of the bars -- 10 μ.


L
R
L
B
L
R
R
C
R
L

The Fate of Glucose and Bicarbonate in Heterotrophically Grown *Chlorella Fusca*

Larry B. Tsai and Raymond A. Galloway

Department of Botany

University of Maryland

College Park, Maryland

20742

INTRODUCTION

Carbon dioxide and glucose have long been employed as carbon sources in the heterotrophic growth of unicellular algae. Numerous data have been provided concerning the pathways of glucose utilization in the dark (8, 11, 21). Several pathways for the fixation of CO_2 in the dark have also been proposed in a few unicellular algae (13, 15, 16, 17, 18, 19). However, most experiments were carried out after preillumination (4, 20, 28). In this paper, we provide the results of experiments performed with no preillumination. Chlorella was grown in the dark for several generations. Using short intervals of exposure to radioactive carbon sources, the analysis of the distribution of radiation in amino acids, sugars, organic acids, lipids and cell residues provides a general comparison between the fate of the two carbon sources of dark grown Chlorella cells.

MATERIALS AND METHODS

Culture Techniques

Chlorella fusca (Shihira and Krauss) was cultured at 29° in tubes 20 cm in length and 2 cm in diameter and containing 30 ml of either inorganic medium (27), or the same medium with the addition of 0.5% glucose. The autotrophic cultures were bubbled with a 1% CO_2-in-air mixture (made in our laboratory), which served to keep the cells in suspension. Two banks of cool white fluorescent lamps provided a light intensity of 800 foot candles at each surface of the culture bath. These cultures supplied the inocula for the heterotrophic cultures used in the experiments.

Heterotrophic culture conditions were as follows: cells were grown in the dark on the heterotrophic (glucose) medium and bubbled with 3% CO_2-in-air mixture supplied by the Air Products Division of Southern Oxygen Corporation.

Cells were standardly grown in the light for two generations and then transferred to the heterotrophic medium. (An adaption to glucose seems to be required as revealed by a long lag in growth after the first transfer to glucose medium. Cells grow at a faster rate on subsequent transfers, but growth is never as rapid as in autotrophic conditions). Heterotrophic cultures were transferred several times in the dark and finally grown to an optical density of 1.5.

Labeling of the cells

The cells were harvested by centrifugation at 1600 g for 10 minutes, re-suspended in 60 ml of inorganic medium supplemented with 0.01% of glucose and kept bubbling with 3% CO_2-in-air mixture.

Uniformily labeled D-glucose ^{14}C (3 m ci/m mole) and sodium bicarbonate-^{14}C (57 m ci/m mole) were obtained from the Radiochemical Center Amersham/Searle Corporation. Each solution was diluted with distilled water to contain 20 μci/ml.

Six 5-ml aliquots of cell suspension were transferred into 10 ml Erlenmeyer flasks for each experiment. Twenty μci of either radioactive glucose or sodium bicarbonate was added to the flasks which were then tightly sealed with rubber stoppers. The flasks were shaken throughout the entire sampling period. Samples were taken at two minute intervals up to twelve minutes, filtered as described below, and washed with 10 ml of inorganic medium supplemented with 0.01% glucose. The filters were then dropped immediately into approximately 80% ml of boiling ethanol to kill and extract the cells (1). Sampling was done in very weak green light with an emission peak at 515 nm. No photosynthetic carbon fixation is accomplished under this light (Galloway and Soeder, unpublished data).

The filtration mentioned above was with millipore filters (white plain filters with a 1.2 μci pore size). The flask into which the medium was pulled.

was attached to a second suction flask which was attached to a third, which in turn was attached to the vacuum pump. The second and third flasks (30 ml capacity and containing 15 ml of 1 M KOH) served to trap any $^{14}CO_2$ released from the medium.

Extraction

The 5 ml aliquots yielded cells with an average dry weight of 5 milligrams. The extractions (at 60° for ten minutes each) were first with 6 ml of 80% ethanol, followed by 6 ml of 20% ethanol, 6 ml of water, and finally 6 ml of 80% ethanol (5). The cells were removed after each extraction by centrifugation at 1600 g for ten minutes. Finally, the cell residues were resuspended in 5 ml of distilled water and assayed for radioactivity as described below.

The combined extracts were dried under nitrogen at room temperature. This dry residue was washed 5 times with 10 ml of ethyl ether to remove lipids. The combined ethyl ether extracts were dried under nitrogen at room temperature, then redissolved in 10 ml of toluene and assayed for ^{14}C. The lipid-free residue was dried and dissolved in water. The resulting water-soluble fraction was passed through a 6 x 1 cm column of AG 50W-X8 (H^+) and washed with 60 ml of water. The effluent was then passed through a 6 x 1 cm column of AG 1-X10 (Formate), and washed with 50 ml of water. The effluent from this column, which constituted the sugar fraction (7), was dried at 40° under nitrogen. The dry residue was dissolved in 10 ml of water and its radioactivity assayed.

The soluble amino acid fraction absorbed on AG 50W-X8 cation exchange resin was eluted with 60 ml of 2 N NH_4OH. The elutant was dried at 40° under air (7). The dry residue was dissolved in 10 ml of hot water and assayed for ^{14}C.

The organic acid fraction absorbed on AG-1 x 10 was eluted with 60 ml of 4 N formic acid (7). It was dried at 40° under air, and the dry residues were

dissolved in ten ml of water and assayed for ^{14}C.

Assay of Radioactivity

Fifty µl aliquots of the various extracts were added to 10 ml of scintillation fluid (24) in low back-ground glass vials (from Nuclear Experiment Chemical Corporation). Counts were determined with a Nuclear Chicago Scintillation Spectrometer model 500 B.

Fifty µl of cell residue suspensions were also placed in scintillation fluid as described above. Thixotropic Gel Powder was added until the scintillation solution became gelatinous and kept cell residues in suspension and counts were assayed with the Scintillation Spectrometer.

RESULTS AND DISCUSSION

Glucose Incorporation

Glucose is readily utilized by Chlorella fusca (Fig. 1) (22,23). At the end of 12 minutes, about 8.2% of the glucose had been metabolized. As seen in Figure (2) and Table (1), the major portion of the label was recovered in the sugar fraction and the cell residue fraction. In view of the fact that approximately 70% of the latter is comprised of carbohydrate (6, 26), the sugars obviously account for most of the product of incorporated glucose. The radioactivity of both the cell residue fraction and the sugar fraction increased linearly without any apparent lag. Organic acids were found to be the third most heavily labeled component at the first two minutes. After that, the rate of increase of radioactivity in the organic acid fraction was slower and at the end of the experiment, this fraction was the least labeled. ^{14}C recovered in the lipids and in the amino acids was relatively low at the earliest sampling time. The ^{14}C in the lipids continued to rise, however, reaching the level of that in the organic acid and amino acid fractions at four minutes, and

Figure 1 The uptake of glucose or bicarbonate by heterotrophically grown *Chlorella fusca* (5 mg dry weight) at 29° in dark, under 3% CO_2-in-air

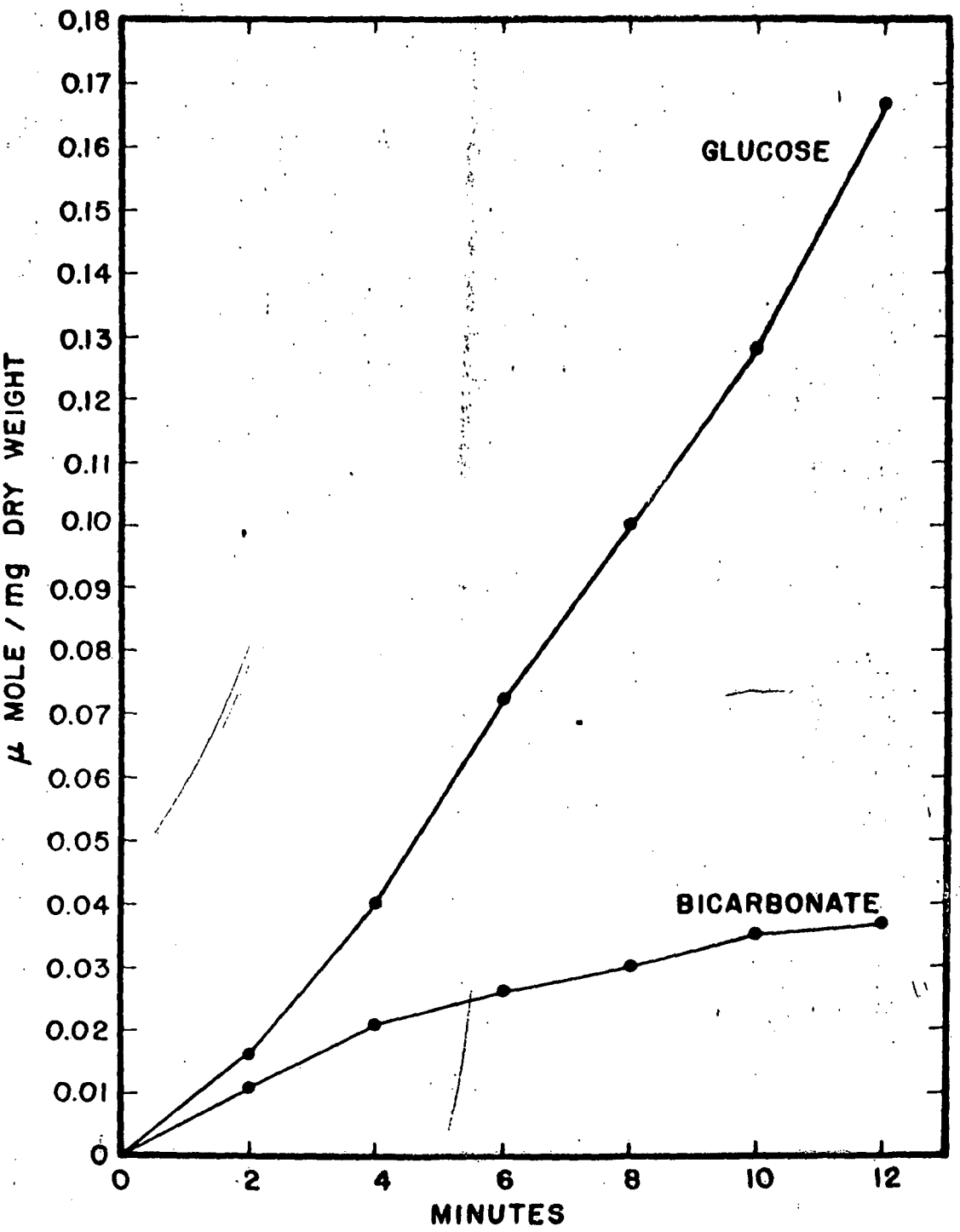
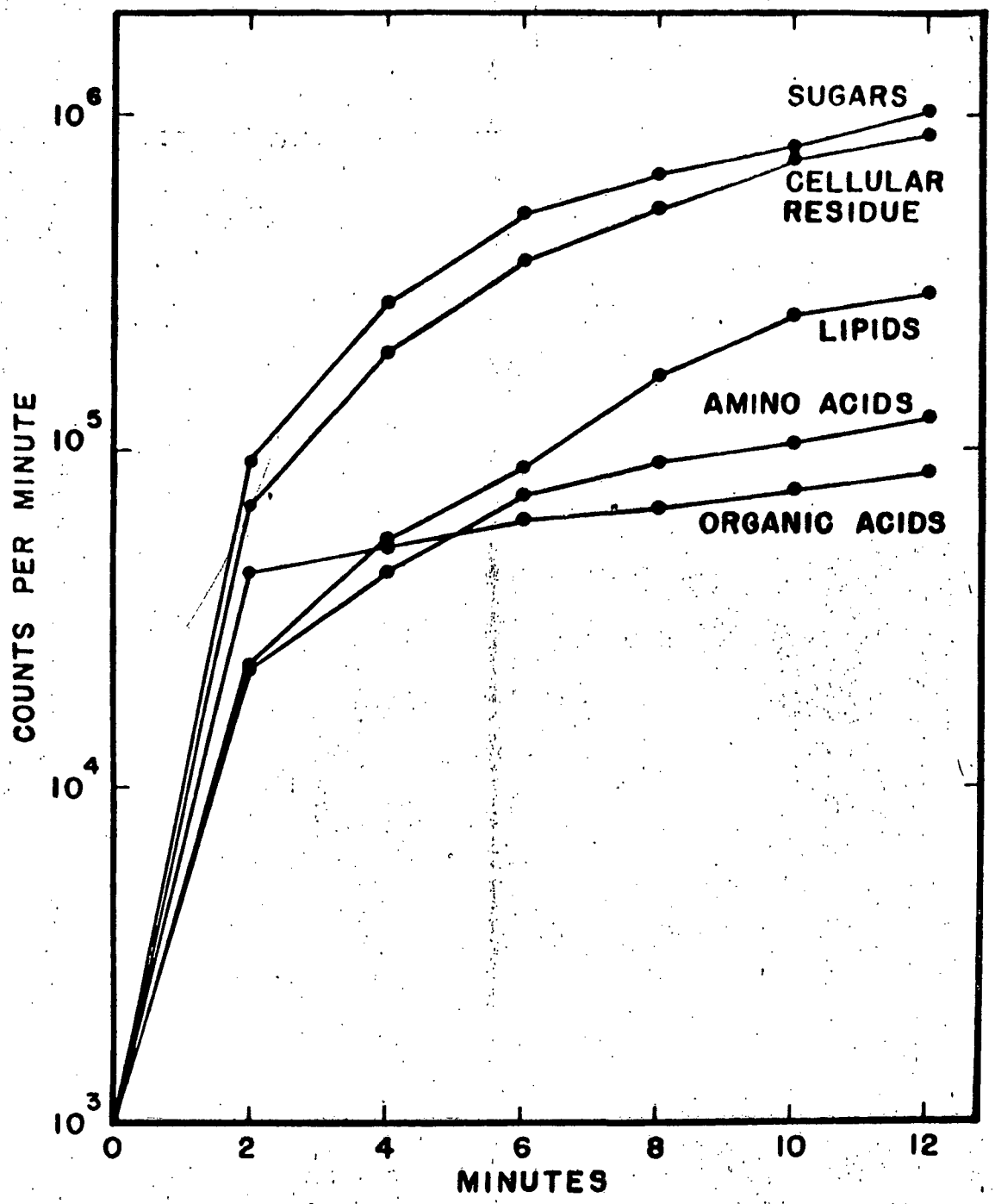

0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
μ MOLE / mg DRY WEIGHT
GLUCOSE
BICARBONATE
0
2
4
6
8
10
12
MINUTES

Figure 2 Incorporation of ^{14}C into various fractions of heterotrophically grown Chlorella fusca (5 mg dry weight) during continuous exposure to uniformly labeled glucose-^{14}C (20 µci) (Culture was at 29° and 3% CO_2-in-air).


SUGARS
CELLULAR RESIDUE
LIPIDS
AMINO ACIDS
ORGANIC ACIDS
COUNTS PER MINUTE
10^6
10^5
10^4
10^3
0
2
4
6
8
10
12
MINUTES

eventually exceeded both. After the low initial reading, the incorporation of activity into the amino acid fraction exceeded that of organic acids at six minutes and subsequently increased in a similar, somewhat higher, manner.

Bicarbonate incorporation

Relative to the incorporation of exogenous glucose, the $^{14}CO_2$ uptake by Chlorella fusca was very slow (see Fig. 1), (18) only 0.225% of the ^{14}C-bicarbonate had been incorporated at the end of twelve minutes. As seen in Fig. (3) and Table (2), the organic acids were the heaviest labeled metabolites throughout the entire time under study. A rapid increase in label in the organic acid fraction in the first few minutes was followed by a leveling off. Although radioactivity in the amino acid fraction increased more slowly, it was at the same level as the organic acid fraction at the end. ^{14}C recovered in the rapid, early incorporation, until the end of the experiment when it appeared to be increasing somewhat. The shape of the curve of ^{14}C recovered in the cell residue was similar to that of the amino acids, although the amount of incorporation was lower throughout.

The sugar fraction always contained the least of the carbon from $^{14}CO_2$. A loss after two minutes was followed by a leveling at six, a rise at eight, and a plateau at 10 minutes. Thus, organic acids and amino acids were the major labeled constituents at the end of the experiment.

The differences of ^{14}C distribution between the two carbon sources reflects the different pathways by which CO_2 and glucose are assimilated.

Comparison of the sugar fraction of cells exposed to $^{14}CO_2$ or glucose-^{14}C

Figure (4) shows that considerably less radioactivity was found in the sugars of Chlorella after $^{14}CO_2$ uptake than when glucose-^{14}C is supplied. It should be noted that the presence of the exogenous glucose would assure the formation of relatively large pools of sugar phosphates. This may be expected

Figure 3 Incorporation of ^{14}C into various fractions of heterotrophically grown Chlorella fusca (5 mg dry weight) during continuous exposure to ^{14}C-bicarbonate (20 μci). (Culture was at 29° and 3% CO_2-in-air.

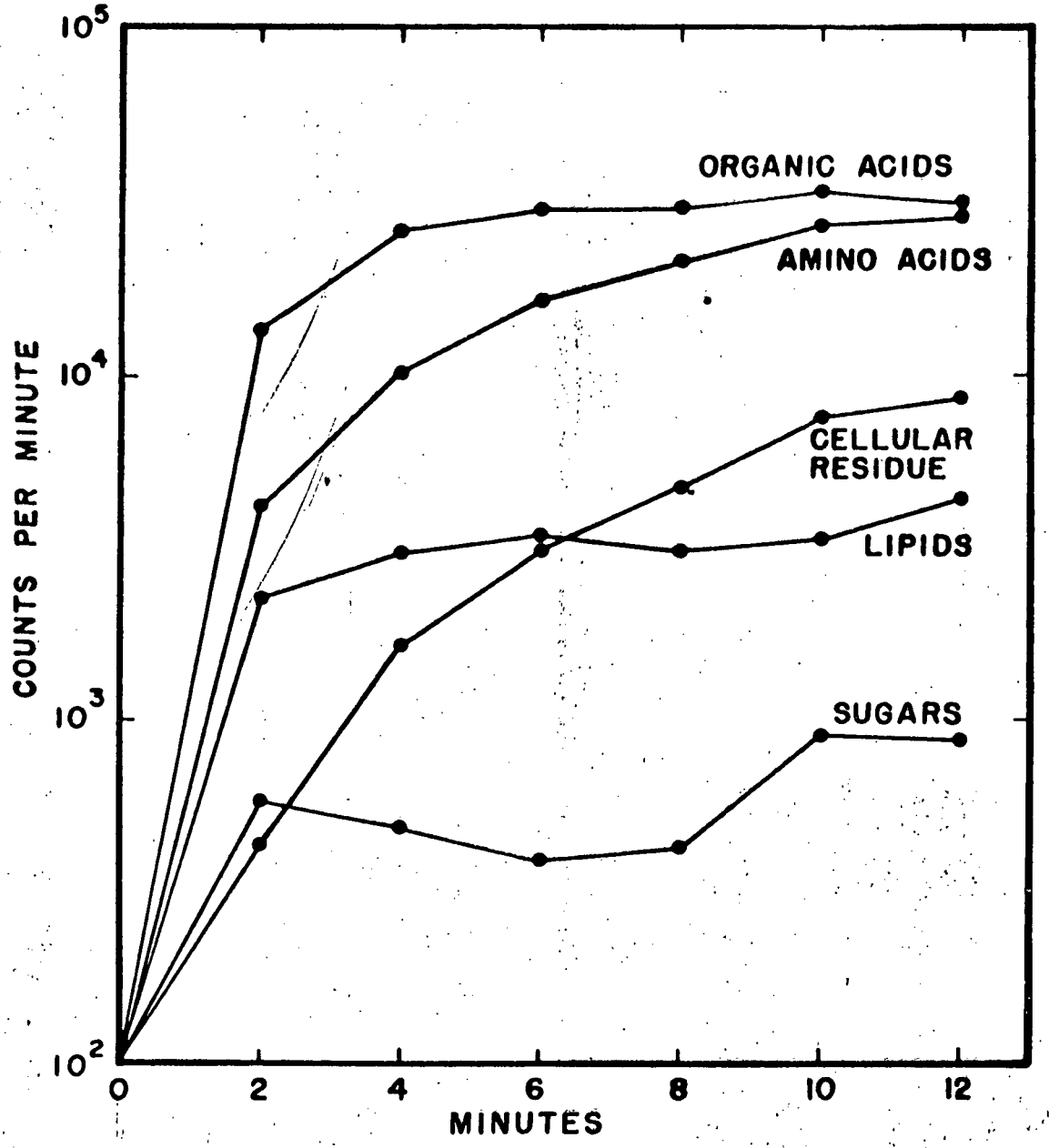
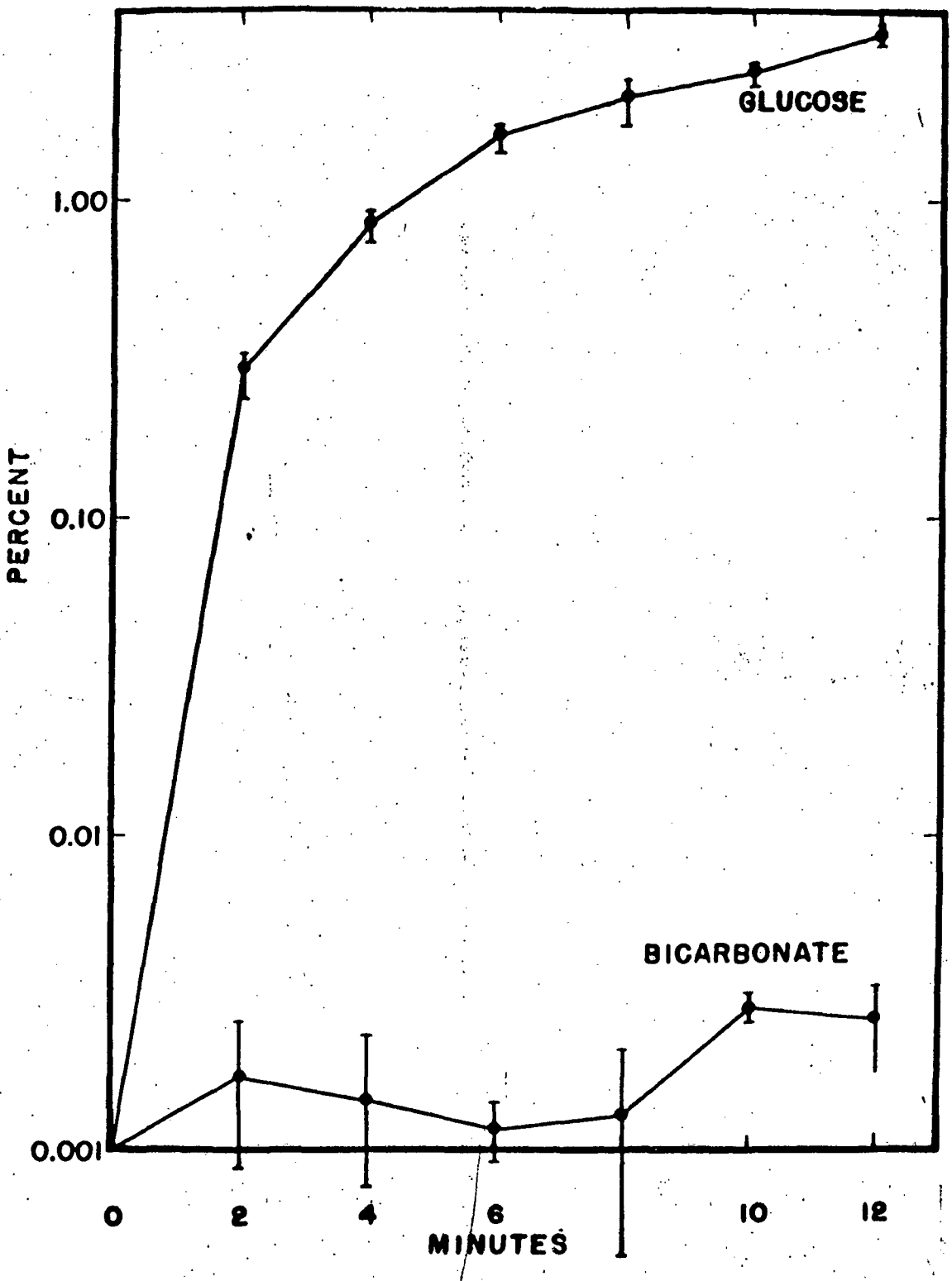

10^5
10^4
10^3
10^2
COUNTS PER MINUTE
ORGANIC ACIDS
AMINO ACIDS
CELLULAR RESIDUE
LIPIDS
SUGARS
0
2
4
6
8
10
12
MINUTES

Figure 4 Percentage of total ^{14}C recovered as sugars from uniformly labeled glucose or bicarbonate-^{14}C uptake in the heterotrophic culture of Chlorella fusca. (The standard deviation about each means is distributed on the graph). (Culture was at 29° and 3% CO_2-in-air.


GLUCOSE
BICARBONATE
1.00
0.10
0.01
0.001
PERCENT
0
2
4
6
10
12
MINUTES

to slow the reverse reactions of glycolysis and the pentose shunt (17). In glucose uptake, the sugar phosphate will be labeled directly; in the CO_2 experiments, labeling would be difficult because of this resistance to the reverse of glycolysis, accounting in part for the differences noted above. Furthermore, only half of the triose phosphate precursors involved in the reverse glycolytic reactions will be labeled when produced from $^{14}CO_2$. Recalling that the main route of dark fixation of $^{14}CO_2$ is via carboxylation of phosphoenol pyruvic acid into oxaloacetic acid, and assuming the reactions of the Krebs cycle continue to succinic acid, equilibration of carbons number 1 and 4 will result in equal distribution of radioisotope between the two carbcxyl groups of both malic and oxaloacetic acids. On subsequent decarboxylation, a pool of pyruvic acid of PEP will be produced only half of which is labeled, the rest of the label being lost as $^{14}CO_2$.

The slight accumulation of radioactivity in the sugars after long periods of $^{14}CO_2$ dark fixation in this study can be explained in the following way: The enzymes of the Calvin Cycle are not lost during heterotrophic growth of Chlorella, but function slowly compared to the autotrophic condition (12) due probably to reduced availability of either NADPH or ATP (1). The NADPH required for the operation of the cycle can be produced in the dark in the chloroplast by the oxidative pentose shunt (9). No oxidative electron transport system has been found in the chloroplast (2) and the pyridine phosphates do not appear to be transported by the chloroplast membrane (10). Therefore, the reduced NADP is most likely retained in the organell and utilized in biosynthetic reactions (2). Since ATP will readily diffuse into the chloroplast, (3) there is the possibility for Calvin Cycle operation in the dark.

The anticipated large amounts of ^{14}C sugars have been found following glucose ^{14}C uptake. They are probably mainly the sugar phosphates of the

glycolytic pathway and the pentose shunt. Labeled sucrose also may account for appreciable amounts, since this sugar has been found in large quantities in Chlorella (14, 22).

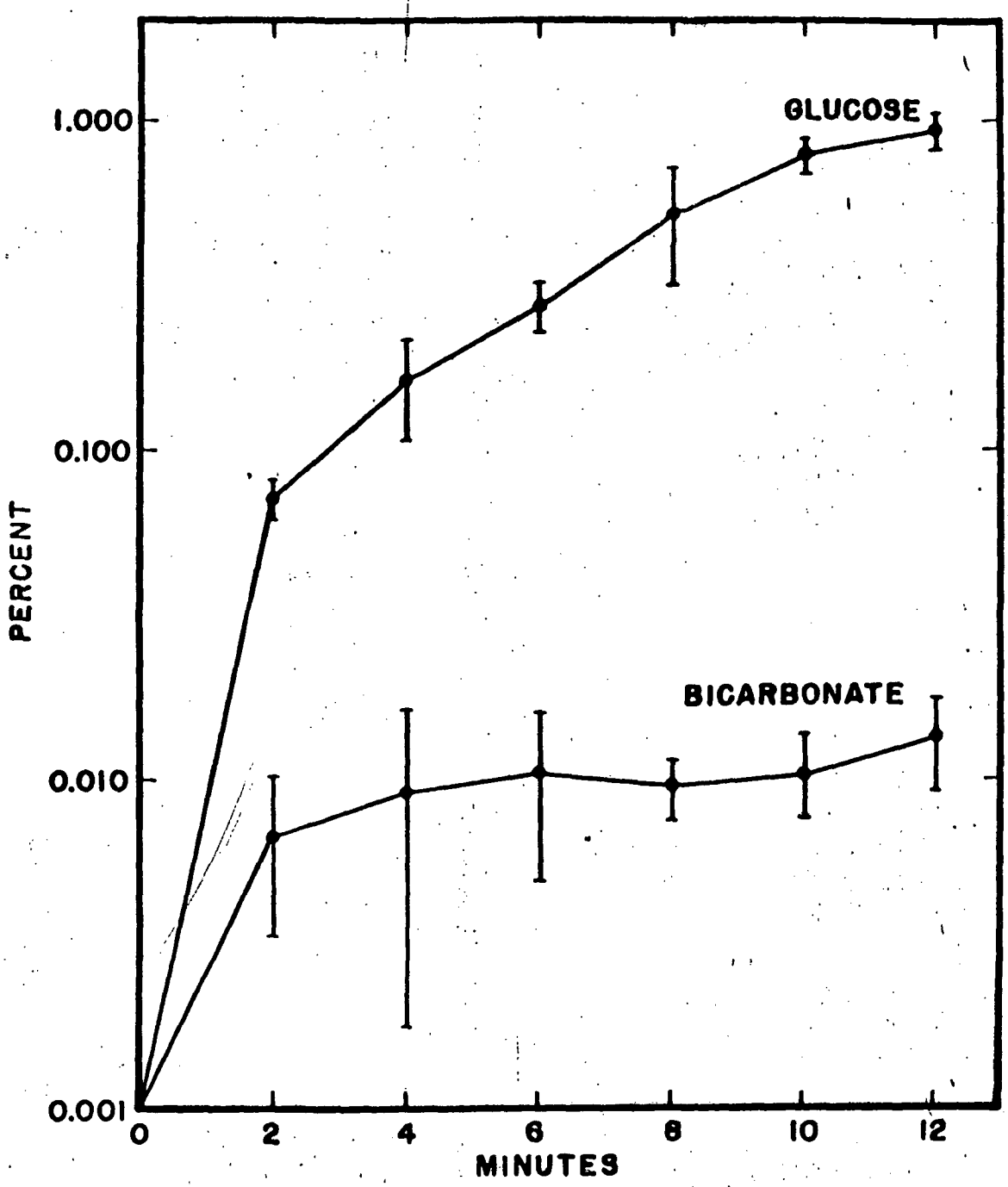
Comparison of the lipid fraction of cells exposed to $^{14}CO_2$ or glucose ^{14}C

The ^{14}C lipid portion of glucose ^{14}C assimilation increased linearly (Fig. 5). Labeled acetyl CoA and labeled glycerol are probably continuously supplied by oxidation of glucose-^{14}C.

Incorporation of radioactivity in lipids during dark fixation of $^{14}CO_2$ is slow by comparison, but significant (Fig. 5). The pathway is not straight forward: Although CO_2 is directly utilized in fatty acid production, (incorporated into malanyl CoA), its addition is transitory, and $^{14}CO_2$ will be again lost in the subsequent reaction. Entry into lipid biosynthesis through a route such as oxaloacetic acid ⟶ pyruvic acid ⟶ acetyl CoA also is not a possibility. Labeled carbon of malic or oxalacetic acid, the first products of dark carbon fixation, becomes the labeled carbon of the carboxyl group of either phosphoenol pyruvic acid or pyruvic acid, and is lost through decarboxylation to acetyl CoA. Thus entry of label into fatty acids is not possible by these mechanisms.

The label in the lipid fraction of CO_2 incorporation would seem to arise in two ways: (1) through labeled PEP or pyruvic acid (from decarboxylation of labeled OAA or malic acid) into glycerol (2) as mentioned above, some operation of the Calvin cycle is anticipated in heterotropic cultures, and can be expected to yield uniformly labeled PGA after some time. This could of course lead to the incorporation of label in acetyl CoA (and subsequently the fatty acids) or into glycerol. The second increase in the radioactivity of lipids at 10 minutes could be explained in this way. The concomitant increase in labeled sugars at 8 minutes seems to substantiate this hypothesis.

Figure 5 Percentage of total ^{14}C recovered as lipids from uniformly labeled glucose or bicarbonate-^{14}C uptake in the heterotrophic culture of Chlorella fusca. (The standard deviation about each means is distributed on the graph). (Culture was at 29° and 3% CO_2-in-air)


GLUCOSE
BICARBONATE
1.000
0.100
0.010
0.001
PERCENT
0
2
4
6
8
10
12
MINUTES

Comparison of the ethanol insoluble fraction of cells exposed to $^{14}CO_2$ or glucose-^{14}C

Figure (6) compares the incorporation of radioactivity into the ethanol insoluble residue fractions of cells after uptake of either $^{14}CO_2$ or glucose. Figure (2) shows that the incorporation of radioactivity into the cell residues after glucose uptake increased concomitantly with that of the sugar fraction. In order to better understand the changes observed, an attempt was made to separate the components of the ethanol insoluble cell residues, but this proved a very difficult problem. Although quantitative estimations are impossible on the basis of our experiments, it was established that the ethanol insoluble fraction consists primarily of polysaccharids of the cell wall, starch* and protein. A trace of organic acids was also shown. There was no evidence for the presence of lipids, although small amounts are probably present. The radioactivity of these crude fractions from the glucose experiments decreased in order of sugars, organic acids and amino acids.

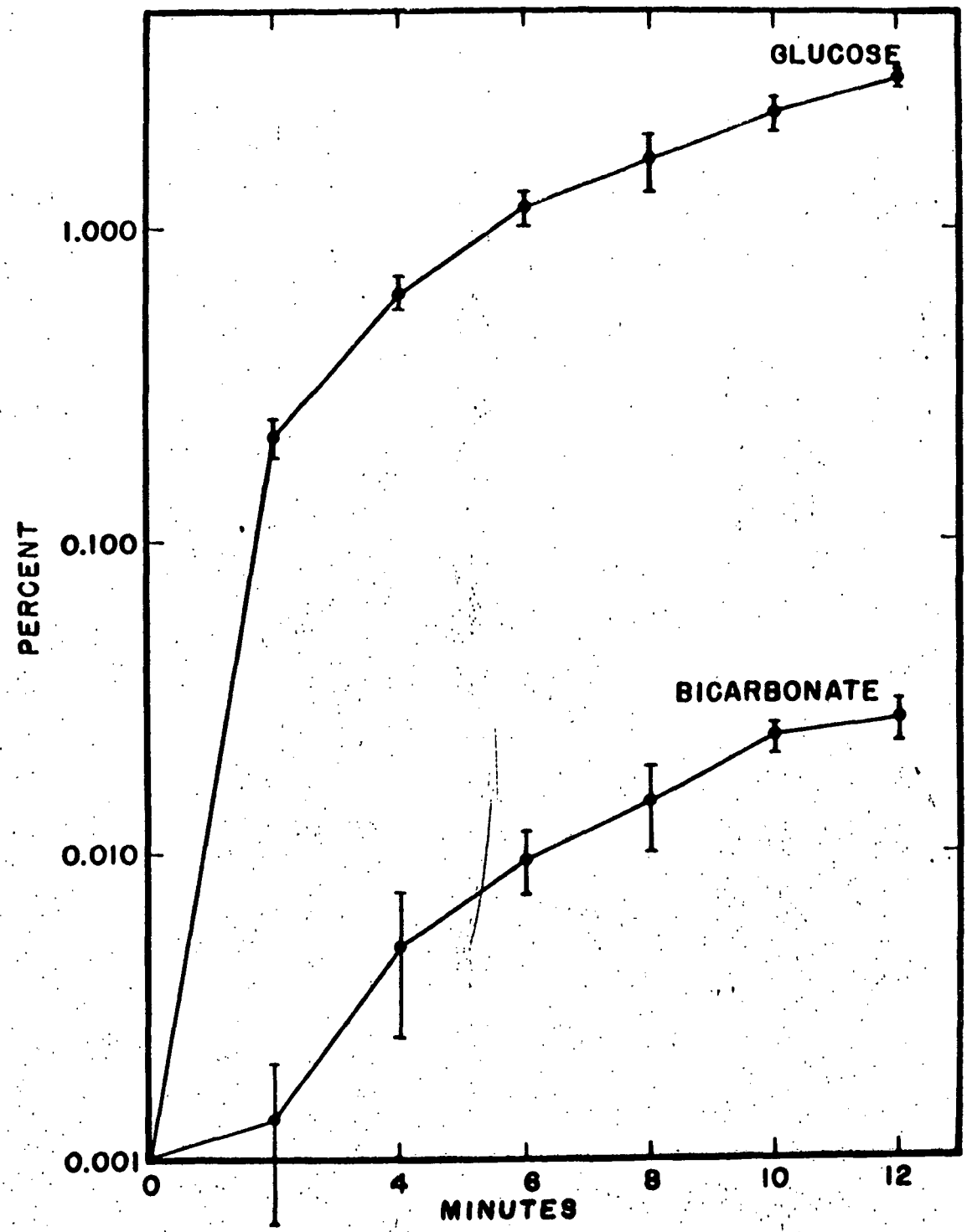
The radioactivity of the cell residue fraction after CO_2 uptake is believed due mainly to protein since a considerable amount of labeled amino acids were detected with less radioactivity in the organic and sugar fractions. The radioactivity of cell residues increased somewhat similarly to the soluble ^{14}C amino acids (Fig. 3) substantiating this observation.

Comparison of the organic acid fraction and the amino acid fraction of cells exposed to the $^{14}CO_2$ or glucose-^{14}C

The labeled organic acids of glucose-14 C uptake, mainly the intermediates of the Krebs cycle, derived, no doubt, from the breakdown of glucose-^{14}C. Because they are the major precursors of several amino acids such as aspartic acid and glutamic acid, the incorporation of ^{14}C into the organic acids was

* All residues turned dark brown when KI was added.

Figure 6 Percentage of total ^{14}C recovered as ethanol insoluble fractions from uniformly labeled glucose or bicarbonate-^{14}C uptake in the heterotrophic culture of Chlorella fusca. (The standard deviation about each means is distributed on the graph). (Culture was at 29° and 3% CO_2-in-air).


GLUCOSE
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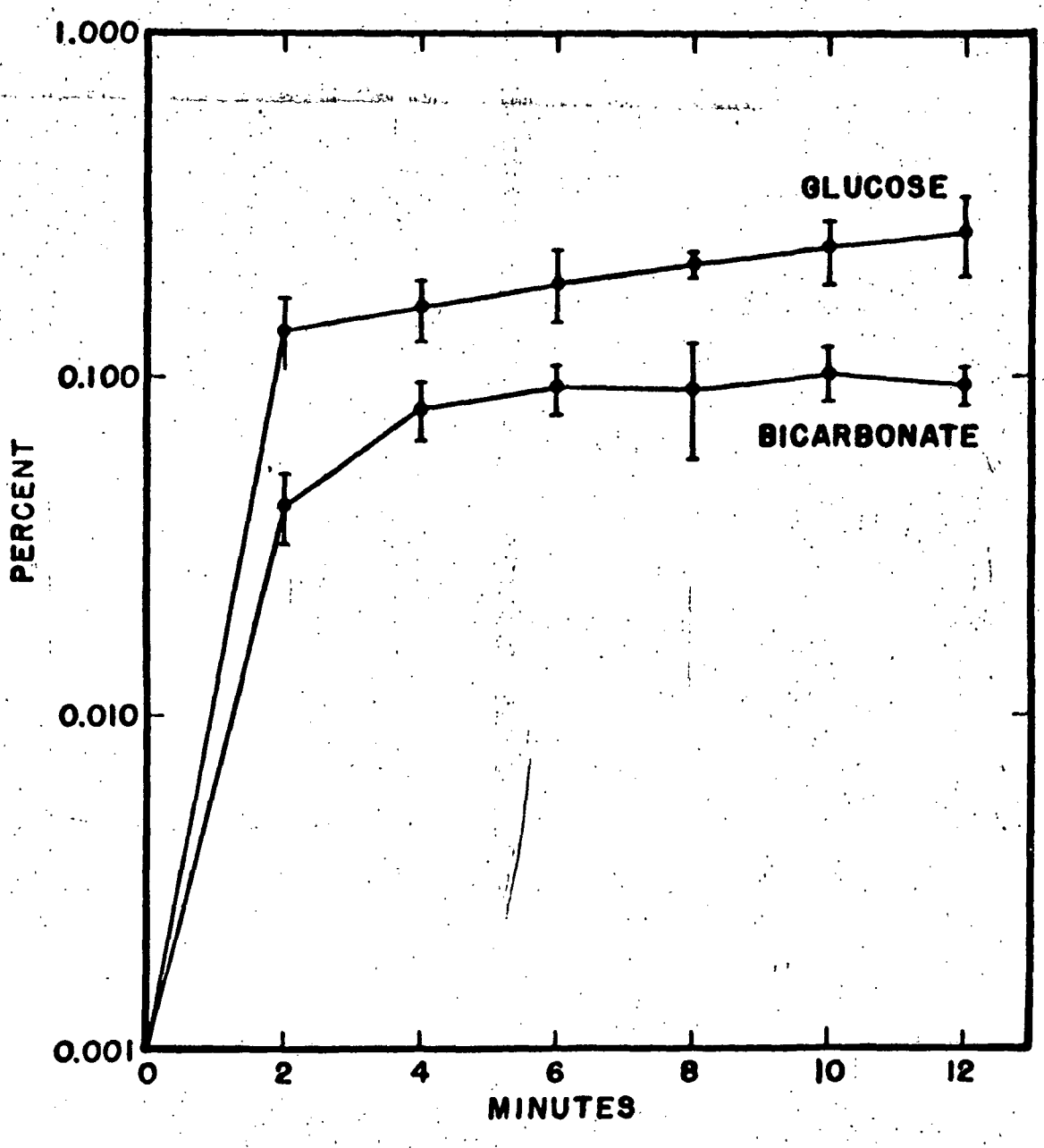
MINUTES

followed by that of the amino acids (Figs. 2 and 7). Reductive amination of ketocarboxylic acids caused the amount of ^{14}C amino acids to increase rapidly (Fig. 8). After the initial formation of glutamate and aspartate, the amino group may become distributed among other amino acids by transamination. Labeled carbon skeletons can be supplied from the intermediates of pathways of glucose ^{14}C assimilation which probably accounts for the fact that after four minutes the labeled amino acids were in excess of the amount of ^{14}C-organic acid, as seen in Figure 2.

As expected, the organic acid fraction of $^{14}CO_2$ uptake was the most heavily labeled component recovered (Fig. 3), since the carboxylic acids of the Krebs cycle probably initially received the label. At six minutes, an equilibrium of ^{14}C was reached in the organic acids (Fig. 7). A rapid turn-over of related amino acids occurs from the ketocarboxylic acids in the Krebs cycle as evidenced by the rise in label in the amino acid pool (Fig. 8). In most organisms, $^{14}CO_2$ uptake results in heavy labeling of aspartic acid, glutamic acid and alanine (18). However, CO_2 fixed in the dark by Chlorella fusca does not contribute to the synthesis of alanine. (Grunberg and Galloway, unpublished data). The literature also reveals that other amino acids are not labeled, probably because of the fact of labeled carbon skeletons.

In conclusion, glucose is the main source of both carbon and energy for heterotrophically grown Chlorella. It is rapidly assimilated and mainly forms ethanol soluble carbohydrates and ethanol insoluble polysaccharids (Fig. 9). Compared to glucose assimilation, CO_2 fixation in the dark is a slow process (Fig. 1), and the carbon flows primarily into organic acids and related amino acids (Fig. 9). Qualitatively, the requirement for CO_2 in heterotrophic cultures seems absolute. There are metabolic fates alternate to those of the Krebs cycle for all of its intermediates, particularly oxaloacetic acid, α-keto glutaric acid, and succinyl CoA. Synthesis of aspartic acid from oxaloacetic acid

Figure 7 Percentage of total ^{14}C recovered as organic acid from uniformly labeled glucose or bicarbonate-^{14}C uptake in the heterotrophic culture of Chlorella fusca. (The standard deviation about each means is distributed on the graph). (Culture was at 29° and 3% CO_2-in-air).


1.000
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GLUCOSE
BICARBONATE
0
2
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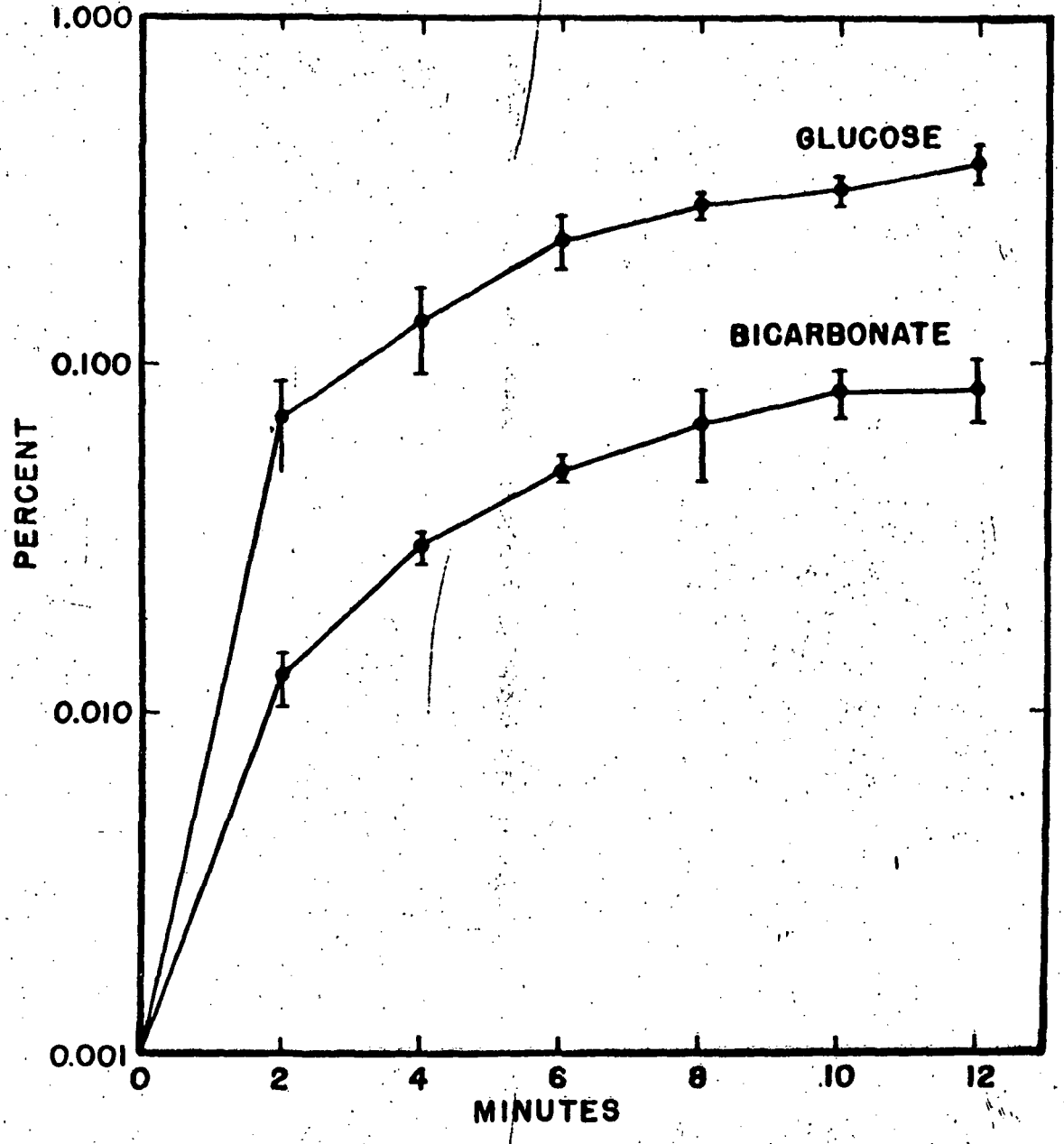
12
MINUTES

Figure 8 Percentage of total ^{14}C recovered as amino acid from uniformly labeled glucose or bicarbonate-^{14}C uptake in the heterotrophic culture of Chlorella fusca. (The standard deviation about each means is distributed on the graph). (Culture was at 29° and 3% CO_2-in-air).


1.000
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GLUCOSE
BICARBONATE
0
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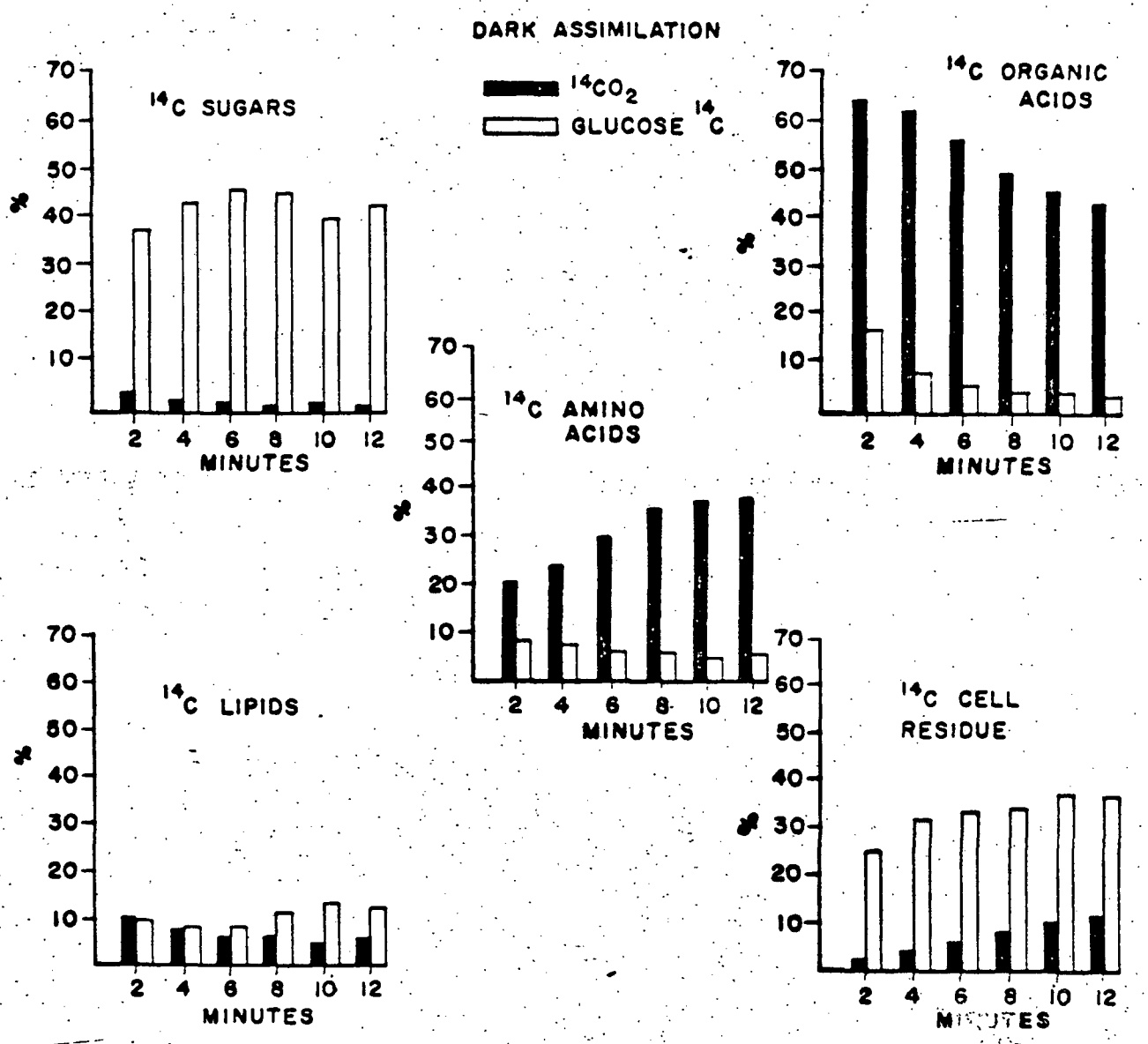
12
MINUTES

Figure 9 Percentage of recovered ^{14}C found in various fractions of extracted cells of Chlorella fusca after heterotrophic assimilation of uniformly labeled glucose and ^{14}C-bicarbonate (Culture was at 29° and 3% CO_2-in-air).


DARK ASSIMILATION
$^{14}CO_2$
GLUCOSE ^{14}C
^{14}C SUGARS
^{14}C ORGANIC ACIDS
^{14}C AMINO ACIDS
^{14}C LIPIDS
^{14}C CELL RESIDUE
%
70
60
50
40
30
20
10
2 4 6 8 10 12
MINUTES

and of glutamic acid from α-ketoglutaric acid would, inevitably, decrease the rate of the cycle operation unless these losses are offset by insertion of carbon into the cycle by a means other than through acetyl CoA (29). In heterotrophically growing Chlorella fusca, the intermediates of the cycle are replenished by CO_2 fixation. The results are consistent with the suggestion that dark fixation of CO_2 is required ultimately to renew the carbon of the Krebs cycle lost in synthetic events (25).

LITERATURE CITED

1. Bassham, J. A., and M. Calvin. 1957. The path of carbon in photosynthesis. Prentice-Hall, Englewood Cliffs, New Jersey.
2. Bassham, J. A. and M. Kirk. 1963. Synthesis of compounds from $^{14}CO_2$ by Chlorella in the dark following preillumination. In: studies on microalgae and photosynthetic bacteria (special issue) Plant Cell Physiol. pp. 493-503.
3. Bassham, J. A., and M. Kirk. 1968. Dynamic metabolic regulation of the photosynthetic carbon reduction cycle. In: comparative biochemistry and biophysics of photosynthesis p. 365-378.
4. Bassham, J. A., M. Kirk, and R. G. Jensen. 1968. Photosynthesis by isolated chloroplasts. I. Diffusion of labeled photosynthetic intermediates between isolated chloroplasts and suspending medium. Biochem. Biophys Acta: 53 p. 211-226.
5. Convin, D. T., and H. Beevers. 1960. Sucrose synthesis from acetate in germinating Caston bean: Kinetic and pathway J. Biol. Chem. Vol. 236 No. 4 p. 988-237.
6. Danforth, W. F. 1962. Substrate assimilation and heterotrophy. p. 99-123. In: Physiology and Biochemistry of Algae. Lewin, R. A. ed. Academic Press, N.Y.
7. Fletcher, J. S. and H. Beevers. 1970. Acetate metabolism in cell suspension cultures. Plant Physiol. 45: 765-772.
8. Gibbs, M. 1962. Respiration p. 61-90. In: Physiology and Biochemistry of Algae. Lewin, R. A. Academic ed. Academic Press. N.Y.
9. Gould, E. S. and Bassham, J. A. 1965. Inhibitor studies on the photosynthetic carbon reduction cycle in Chlorella pyrenoidosa. Biochem. Biophys. Acta 102, 9-19.

10. Heber, U., and J. A. Santarius. 1965. Compartmentation and reduction of pyridine nucleotodes in relation to photosynthesis. Biochem. Biophys. Acta 109. 391-408.

11. Jacobi, G. 1962. Enzyme systems p. 125-140. In: Physiology and biochemistry of algae. Lewin, R. A. ed. Academic press. N.Y.

12. Latzko, E. and M. Gibbs. 1969. Enzyme activity of carbon reduction cycle in some photosynthetic organisms. Plant Physiol. 44. 295-300.

13. Levedahl, B. H. 1968. The biology of Euglena. Vol: II pp. 85-96. Academic Press. N.Y. London.

14. Linberg, B. 1955. Low-molecular carbohydrate in algae VIII. Investigation of two green algae. A. Chem. Scond. 9 169.

15. Lynch, V. H. and M. Calvin. 1952. Carbon dioxide fixation by microorganisms. J. Bacteriol, 63. 525-531.

16. Lynch, V. H. and M. Calvin. 1953. Carbon dioxide fixation by Euglena. Ann. N. Y. Acad. Sci. 56. 890-900.

17. Moses, V., O. Holm-Hansen, and M. Calvin. 1959. Non-photosynthetic fixation of carbon dioxide by the three organisms. J. Bacterial, 77. 70-78.

18. Osmund, Holm-Hansen. 1962. Assimilation of carbon dioxide. p. 28-45. In: Physiology and Biochemistry of algae. Lewin, R. A. ed. Academic Press. N.Y.

19. Ohmann, E. 1969. Reinigung und Erganschaftes von phosphoenol pyrute carboxylase. aus Euglena gracilis. Opeon, J. Brockem 10. 43-45.

20. Pederson, T. A., M. Kirk and J. A. Bassham. 1966. Light-dark transients in levels of intermediate compounds during photosynthesis in air adapted Chlorella. Physiol. Plant. 19: 219-231.

21. Richter, G. 1959. Comparison of enzymes of sugar metabolism in photosynthetic algae. Anacyctic nidulans and Chlorella pyrenoidosa. Naturivissenschaften 46, 604.

22. Rodriguez - Lopez, M. 1966. Utilization of sugar by Chlorella under various conditions. J. Gen. Microbiol. 43. 139-143.

23. Shihira, I., and R. W. Krauss. 1965. Chlorella: Physiology and Taxcnomy of forty-one isolates. Port City Press, Baltimore, Md. p. 97.

24. Siegel, M. R. and H. D. Sisler. 1968. Fate of the Phthalimide and Trichloromethylthio ($SCCl_3$) Moieties of Folpet in Toxic Action on Cells of Saccharomyces pastorianus. Phytopathology, Vol. 58, No. 8, 1123-1128.

25. Splittstoesser, W. E. 1966. Dark CO_2 fixation and its role in the growth of plant tissue. Plant Physiol. 41. 755-759.

26. Syrett, P. J. 1951. The effect of cyanide on the respiration and oxidative assimilation of glucose by Chlorella vulgaris. Ann. Botany (London) 15. 473-492.

27. Thomas, W. H. and Krauss, R. W. 1965. Nitrogen metabolism in Scenedesmus as affected by environmental changes. Plant Physiol. 30. 113-122.

28. Togasaki, R. K. and M. Gibbs. 1967. Enhanced dark CO_2 fixation by preilluminated Chlorella pyrenoidosa and Anacystis nidulans. Plant Physiology 42: 991-996.

29. Wiame, J. M. 1957. Le role biosynthetique du cycle des acid tricarboxyliques. Avan. Enzymol. 18. 241-280.

SUMMARY

The assimilation of glucose and CO_2 in the heterotrophic culture of Chlorella fusca (Shihira and Krauss) was investigated. After addition of radioactivity, extracts of the cells made at two minute intervals to twelve minutes were separated into organic acids, amino acids, sugars, lipids, and an ethanol insoluble fraction, and the radioactivities were determined using a scintillation spectrometer.

The ^{14}C from glucose at 12 minutes was distributed as follows: sugars, 43%: insoluble residues (primarily polysaccharides), 37%; lipids, 12%; amino acids, 5%; and organic acids, 3%. The ^{14}C from CO_2 was primarily recovered as organic acids (43%) and amino acids, (38%), followed by insoluble residue fraction, (12%). Lipids essentially did not change after a rapid incorporation in the first two minutes until a raise at ten minutes and accounted for 6% of the total radioactivity at 12 minutes. The least amount of ^{14}C (1%) was incorporated into sugars. The kinetics of the incorporation of ^{14}C into the lipids and the sugars was similar.

Differences in the distribution of ^{14}C assimilated from glucose-^{14}C and $^{14}CO_2$ made it clear that there are different roles for the two carbon sources in heterotrophy. Glucose is considered the major source of carbon and energy as evidenced by its far greater incorporation.

The large incorporation of CO_2 into the organic acid fraction is consistent with its envisioned role in the replenishment of intermediates drained from the Krebs cycle for synthetic purposes. Furthermore, entry of labeled carbon into the sugar and lipid fractions lends substance to the concept of the dark operation of the Calvin cycle.