A STUDY OF PHYCOPHYSIOLOGY IN CONTROLLED ENVIRONMENTS

TWENTY-SECOND SEMIANNUAL STATUS REPORT

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

RESEARCH GRANT NO. NGR-21-002-003

TO:

THE UNIVERSITY OF MARYLAND

DEPARTMENT OF BOTANY

TECHNICAL REPORT

No. 1022

PREPARED BY DR. ROBERT W. KRAUSS

GRANT DIRECTOR AND PROFESSOR OF PLANT PHYSIOLOGY

COLLEGE PARK, MARYLAND

JUNE 15, 1971

Twenty-second Semiannual Status 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

June 15, 1971

Introduction

This report is concerned with the six-month period from 16 December 1970 to 15 June 1971. The research was under supplements to Research Grant NsG 70-60 begun on 1 April 1960 and now listed as Research Grant NGR 21-002-003.

This report is intended to give a brief summary of the studies under way during the period as well as the final details of experiments which have resulted in two papers submitted for publication for which support under NSR-21-002-003 has been acknowledged.

Personnel

Name	Title	Appointment
Dr. R. W. Krauss	Director and Professor	4/1/60 - present
Dr. C. Sorokin	Research Professor	4/1/60 - present
Dr. R. A. Galloway	Professor	4/1/60 - present
Dr. G. W. Patterson	Associate Professor	7/1/65 - present
Technical Assistants:		
A. Osretkar	Research Assistant	4/5/64 - present
O. Owens	Research Assistant	6/15/70 - present
Part-time and Student Labor:		
S. Nishino	Undergraduate Assistant	2/24/69 - present
Daniel Cacchione	Undergraduate Assistant	9/21/70 - present

Research Summaries

The primary overall objective of the research in this laboratory is to obtain fundamental data concerning the growth and metabolism of the unicellular green algae. These organisms are the most likely to provide a source of biological oxygen for man venturing for long periods in space. They absorb CO_2 and release O_2 at the optimal rate when they are growing at their maximum rate. The food value of the algal product is also potentially ideal for human consumption. They currently form a major part of the life support system on Earth so that research directed at the understanding of their metabolism is of value for terrestrial life as well.

The research currently under way can be summarized under three categories biochemical conversions, chemical composition, and cell growth and division.

Biochemical Conversions

During studies employing the Recyclostat, a device built to provide continuous culture of the green alga Chlorella, it became clear that nutrient balances were not maintainable with the levels of nitrogen we had expected to be sufficient for growth. The algae appeared to consume larger amounts of nitrogen than could be accounted for in analyses of the cells or the medium in which they were grown. The observations of this phenomenon has been given in previous NASA reports, but during the last report period the problem has been solved. The major mechanism for nitrogen loss is now known.

The procedure for this study can be summarized as follows. Cultures of Chlorella sorokiniana Shihira and Krauss grown in a continuous culture device, called a Recyclostat, consumed more nitrogen than required for

growth. Analyses of cells and media grown on nitrate and urea demonstrated significant losses of nitrogen. Average losses, as determined by a micro-Kjeldahl method and confirmed by the modified micro-Dumas method, were 26.9% for cultures supplied nitrate and 28.7% for those supplied urea. Cultures grown in test tubes on fixed amounts of nitrogen showed average nitrogen losses of 21.7% from nitrate cultures and 25.0% from urea cultures. At high levels of urea, nitrogen losses were less than at low levels. These cultures had a continuous supply of CO_2-in-air. To remove the avenue for loss of volatile nitrogenous substances in the gas stream, cultures were grown in sealed flasks, autotrophically and heterotrophically, with both nitrate and urea as nitrogen sources. Nitrogen losses were similar to those in which there was a gas supply.

Analyses of metabolic intermediates of cultures grown on nitrate revealed the presence of nitrite. Nitrite accumulation was positively correlated with growth rate of the culture. Traces of hydroxylamine were also found in cultures grown in the Recyclostat. However, quantities of both nitrite and hydroxylamine were too small to account for the missing nitrogen.

Cultures, grown in sealed flasks, were analyzed for gaseous compounds. Analyses involved gas-liquid chromatography, mass spectrometry, and Raman spectroscopy. Nitrous oxide between 1.2 and 2.4 mg per 100 ml of medium were observed when analyzed by gas-liquid chromatography. Mass spectroscopy indicated an average of 7.1 mg nitrous oxide per 100 ml for urea cultures and 7.6 mg for nitrate cultures. Quantitative calculations from Raman spectroscopy indicated 6.0 mg nitrous oxide per 100 ml of medium in both cases. Some nitrogen was unaccounted for. This fraction may represent undetected compounds or the discrepancy may be the sum of sampling and analytical errors. Nevertheless, the presence of nitrous oxide as shown by several procedures,

indicates the ability of Chlorella to convert fixed nitrogen to nitrous oxide.

This discovery is a major break-through in our understanding of algal metabolism and its significance cannot be underestimated. For the first time it identifies a nitrogen lesion from cells which was not formerly known in nature. It identifies a biological source of nitrous oxide found in the earth's atmosphere and appears a logical explanation for the ability of natural waters to rapidly purify themselves of nitrogenous pollutants. Furthermore, it identifies a problem in dealing with photosynthetic gas exchangers--that of accounting for the extra nitrogen consumed by the alga. This is not expected to pose a major design problem nor is the disposal of N_2O in the effluent gas stream likely to present difficulty. However, engineering design for biological life support systems must take this into account. Full details of several years of experiments on this topic are included in the manuscript at the end of this report.

Chemical Composition

Studies in this laboratory have concentrated on the organism components of algal cells other than fats, proteins and carbohydrates. Of special concern have been the sterols and hydrocarbons. These compounds, many similar to powerful human hormones, have been under investigation because of their possible impact on man should Chlorella cells become components of his diet. Implicit in all of these studies has been the need to establish the biosynthetic pathway for sterols. Inasmuch as any temporary unexpected blocks to normal growth and metabolism might produce large amounts of an otherwise undetected sterol. It is essential to know the intermediates. For this purpose, intermediates are identified by the insertions of biochemical blocks.

One of these is the hypocholesterolemic drug AY-9944 (trans-1,4-bis-(2 chlorobenzylamino-methyl) cyclohexane dihydrochloride). From experiments with this drug, compounds have been isolated which have been identified as key intermediates in the synthesis of normal algal sterols. Of the sixteen identified, several are new to nature and provide clues to the mechanism of sterol synthesis. Details are given in the manuscript included in this report.

Cell Growth and Division

High-temperature algae.

The experience of the past twenty years indicates that the hereditary nature of the organism, together with the optimal environmental conditions, are among the most important factors contributing to a highly productive algal culture. Our high-temperature organism Chlorella sorokiniana, strain 7-11-05, is a well-known and highly productive green alga. A newly developed strain, Chlorella 1-9-30, may even surpass Chlorella 7-11-05 in its growth rate and other metabolic characteristics. High temperature algae may be particularly useful in the following areas:

1. As organisms used in basic research (extended temperature and light intensity ranges, high-performance, relatively short life cycle, etc.).
2. In mass culture for the purpose of production of food, fodder and oxygen (as gas exchangers) or as components of biological systems used in sewage treatment plants.
3. As members of phytoplanktonic populations in areas of thermal pollution (Atomic power plants).

The effects of cell secretions on algal growth.

Preliminary observations indicate that substances produced by algal cells may strongly affect the performance of the same organism and of other strains brought into contact with the biotically active substances. These substances may be released within the cells and thus affect the cell growth internally, or they may be released into the medium and then act on the same and other cells externally. The effects of substances produced by algal cells may be inhibitory in nature or they may be stimulatory. Studies of the inhibitions produced by algal cells and the attempts to control and to eliminate the inhibiting action would improve the conditions for algal growth and increase the growth rate, yield and duration of algal culture. Growth factors capable of improving the efficiency of algae open even more exciting perspectives in regard to the possible performance and metabolic activity of algae in mass culture used as photosynthetic gas exchangers or as the sources of food or fodder.

The physiology and biochemistry of the division cycle.

Cell developmental studies conducted by using the synchronization technique during the past 17 years indicate that in the course of their development cells undergo remarkable changes in many of their physiological and biochemical characteristics. This knowledge opens wide opportunities for the studies of the metabolic mechanisms and the effects of conditions controlling the growth of algal cells. One of the most exciting theoretical problems is the mechanism and factors which turn an old metabolically sluggish cell after it divides into an active vigorously growing cell. Practical applications of these studies may be in application of synchronized culture for mass culture. If used only during most active period during their growth period,

these cultures far out-perform nonsynchronized cultures.

Dark Fixation of CO_2.

Studies during this period have continued on the ability of algae to fix CO_2 in the dark. Many intermediate steps have been identified and the mechanism in Chlorella is being elucidated to provide a comparison to the process in higher plants. The intermediates are being catalogued and implications for the system are being assembled in a manuscript to be included in the next report.

NITROUS OXIDE AND OTHER NITROGEN LOSSES FROM CULTURES OF *CHLORELLA*[1,2]

Anthony Osretkar[3] and

Robert W. Krauss

Department of Botany, University of Maryland

College Park, Maryland 20742

FOOTNOTES

[1]This investigation was supported in part by grants from the National Science Foundation. GI-29906 and from the National Aeronautics and Space Administration NSR-21-002-003.

[2]Scientific Article No._____Contribution No._____of the Maryland Agricultural Experiment Station, Department of Botany

[3]Present address: Department of Biology, Montgomery College, Rockville, Maryland 20850.

ABSTRACT

Cultures of Chlorella sorokiniana Shihira and Krauss grown in a continuous culture device, called a Recyclostat, consumed more nitrogen than required for growth. Analyses of cells and media grown on nitrate and urea demonstrated significant losses of nitrogen. Average losses, as determined by a micro-Kjeldahl method and confirmed by the modified micro-Dumas method, were 26.9% for cultures supplied nitrate and 28.7% for those supplied urea. Cultures grown in test tubes on fixed amounts of nitrogen showed average nitrogen losses of 21.7% from nitrate cultures and 25.0% from urea cultures. At high levels of urea, nitrogen losses were less than at low levels. These cultures had a continuous supply of CO_2-in-air. To remove the avenue for loss of volatile nitrogenous substances in the gas stream, cultures were grown in sealed falsks, autotrophically and heterotrophically, with both nitrate and urea as nitrogen sources. Nitrogen losses were similar to those in which there was a gas supply.

Analyses of metabolic intermediates of cultures grown on nitrate revealed the presence of nitrite. Nitrite accumulation was positively correlated with growth rate of the culture. Traces of hydroxylamine were also found in cultures grown in the Recyclostat. However, quantities of both nitrite and hydroxylamine were too small to account for the missing nitrogen.

Cultures, grown in sealed flasks, were analyzed for gaseous compounds. Analyses involved gas-liquid chromatography, mass spectrometry, and Raman spectroscopy. Nitrous oxide between 1.2 and 2.4 mg per 100 ml of medium were observed when analyzed by gas-liquid chromatography. Mass spectroscopy indicated an average of 7.1 mg nitrous oxide per 100 ml for urea cultures and 7.6 mg for nitrate cultures. Quantitative calculations from Raman spectroscopy indicated

6.0 mg nitrous oxide per 100 ml of medium in both cases. Some nitrogen was unaccounted for. This fraction may represent undetected compounds or the discrepancy may be the sum of sampling and analytical errors. Nevertheless, the presence of nitrous oxide as shown by several procedures, indicates the ability of Chlorella to convert fixed nitrogen to nitrous oxide.

INTRODUCTION

The process of denitrification has long been involved to explain loss of gaseous N from soils or culture solutions. In this process, nitrate serves as a hydrogen acceptor--substituting for oxygen. Gas losses occur primarily as N and/or N_2O. This contrasts with assimilatory reduction of nitrate and nitrite to ammonia. However, dissimilatory reduction of nitrate does not always result in production of gas. There may be a reduction to ammonia in quantities greater than that required to meet organic nitrogen requirements. Denitrification has been observed almost exclusively in bacteria and is thought to be exclusively anaerobic.

There have been some reports of N losses from autotrophic organisms. As early as 1886, Atwater and Rockwood (4), reported appreciable N losses from cowpeas during germination and early growth of seedlings. Some years later in 1906, Wilfarth et al. (35), reported losses of N from barley, spring wheat, peas and mustard, but the nature of the loss was not determined. Irving and Hankinson (12) immersed Elodea in solutions of nitrate and asparagine, and reported the production of gaseous N. Davidson (6), apparently repeating some of the earlier work on cowpeas, grew both wheat and cowpea seedlings in Kjeldahl flasks under sterile and non-sterile conditions. His results contradicted the evidence for gaseous N loss. In 1937, Pearsall and Bellimore [illegible], in experiments with Chlorella and Narcissus leaves, reported N losses amounting to as much as 65%. With glucose-supplemented Chlorella, N loss was high in the dark on $NaNO_3$, and less in light. There was little or no loss with NH_4NO_3 where NH_4 was utilized preferentially. In Narcissus leaves, losses occurred only when the inorganic N source, NH_4NO_3, was supplied. There was no loss from media containing urea, asparagine, or alanine. They hypothesized the following reactions:

$$HNO_3 \rightleftharpoons HNO_2 \rightleftharpoons NH_3$$

$$HNO_2 + R\text{-}CH\text{-}HN_2COOH \longrightarrow N_2 + H_2O + R\text{-}CH\text{-}OH\text{-}COOH$$

Combined with the data of Irving and Hankinson, this suggests:

$$HNO_3 \rightleftharpoons HNO_2 \rightleftharpoons NH_3 \rightleftharpoons \text{Amide N}$$
$$\downarrow \qquad\qquad \uparrow\downarrow$$
$$N_2 \longleftarrow \text{Amino-N} \rightleftharpoons \text{Protein N}$$

Eggleton (7) suspected a N loss on grass plots supplied $(NH_4)_2SO_4$, $NaNO_3$, or $NaNO_2$. Using Chlorella, Allison, et al. (2) reported average losses of only 5% with one exceptionally high figure of 14%. Losses occurred with NH_4NO_3 as the N source. There was no loss with KNO_3, urea, alanine, NH_4SO_4 or asparagine. The test organisms were Chlorella vulgaris and Chlorella pyrenoidosa, grown in light up to 18 days, and in darkness for as long as 102 days. Bongers (5), using N-deficient algae, reported no N losses. Thus there is conflicting evidence for the existence of N losses attributable to both higher plants and algae.

Previous work in this laboratory, using controlled nutrient supplies to continuous cultures, revealed that more N was required to sustain growth than could be subsequently accounted for in either the algae or the media. There was also a constant tendency for pH to rise, even though balance sheets showed that it should have remained constant because of the addition of a nutrient solution with HNO_3 as the N source (19). It seemed that cells were absorbing, but not incorporating all of the N into cellular constituents.

The investigations reported in this paper were designed to examine the details of this phenomenon, and to quantify, as far as possible, the fate of N compounds supplied as nutrients to algal cultures.

MATERIALS AND METHODS

CULTURE METHODS

Cultures of Chlorella sorokiniana Shihira and Krauss (29), were grown in the liquid media shown in Table 1. The N source was modified in different experiments by changing the concentration of KNO_3 or by substituting urea at different levels. Media and vessels were autoclaved at 20 lbs. for 15 minutes prior to inoculation.

Test tube cultures employed tubes measuring 25 x 200 mm which were closed with cotton-plugged bubbler tubes to provide cultures with a 1% CO_2-in-air mixture. The test tubes were maintained in Lucite water baths at 39 C $\pm$ 0.5C. Illumination was provided by two banks of fluorescent lights consisting of four, cool-white, "Power-Groove", General Electric, fluorescent tubes per bank. Changes in illuminance were effected by wire screen filters. A Weston Illumination Meter, placed inside the bath, measured illuminances given in the tables. Cultures were kept at maximal growth rates by daily transfers. A Bausch and Lomb Spectronic 20 Colorimeter at 550 nm measured optical density.

Continuous cultures were grown in an apparatus, conceived and built in this laboratory, which has been named the Recyclostat (18). The system operates as follows: the algal culture is maintained in a glass chamber illuminated by a high intensity "Quartzline" iodine vapor lamp mounted within the culture chamber. The lamp is cooled by a continuous flow of cold water. A temperature probe, connected to a temperature regulator, regulates the flow of water to a coolant jacket inside the culture jacket. A 1% CO_2-in-air mixture is bubbled through the culture medium. Optical density is recorded by photodiodes mounted on either side of a small bulge at the bottom of the chamber. The culture is agitated by a magnetic stirrer. When the population density reaches a pre-set level, the photodiode signals for introduction of an aliquot of culture medium

TABLE I

Inorganic Medium for the Autotrophic Culture of

Chlorella sorokiniana Shihira & Krauss

Salts	Grams of salt per liter
KNO_3	2.00
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.8
K_2HPO_4	0.2
EDTA·NaFe	0.0385
" Na_2Mn	0.0071
" Na_2Ca	0.0077
" Na_2Co	0.0093
" Na_2Cu	0.0077
" Na_2Zn	0.0067
MSO_3	0.0015

EDTA = Ethylenediaminetetraacetate

which is pumped through a peristaltic pump from a heated reservoir and pasteurized as a precaution to maintain the sterility of the medium. When the proper amount of fresh medium has been introduced to dilute the culture to a pre-set level, an equivalent amount of culture is forced out of the exhaust port by the small, but constant, CO_2-in-air pressure maintained in the culture chamber. Effluent cells and medium may be collected at once in a sterile flask for analysis, or they may be passed through an air trap and filtered through a bacteriological Millipore filter which provides for continuous harvest. The medium can then be passed through another microbiological filter, and back into the reservoir from which it can be recycled to the culture. Increments of fresh or recycled medium are recorded on a strip chart recorder which establishes the time of addition. Cells and medium within the culture chamber can be sampled independently by introducing a sterile hypodermic needle through a rubber, self-sealing serum stopper, mounted in a sampling port. Illuminance regulated by a Variac ranges to 10,000 ft-c.

For studies involving growth of cultures in sealed vessels, two kinds of culture vessels and techniques were employed. The first was a 200 ml long, single-necked flask with a 29/42 standard-taper outer joint. This was fitted with a close standard-taper inner joint which was, in turn, fitted with two lengths of glass tubing. One length of tubing was centered in the joint and extended below the level of medium in the flask--approximately 10 mm above the joint. The second piece of tubing was attached to one side of the joint and extended 10 mm from an opening at the surface. One hundred ml of medium was added to each of the flasks; a teflon-coated stirring bar was placed in the bottom and cotton was fitted into the ends of the glass tubing. Flasks were steam-sterilized. After sterilization and inoculation, argon was bubbled through the long glass tube and medium, and exhausted from the second tube. Bubbling continued for at least 30 minutes to remove atmospheric gas from the flask. Afterward, the exhaust stopcock was closed, and a mixture of 92% A, 6% O_2, and 2% CO_2 was bubbled into the flask. A gauge pressure of 1(psi was reached. A rubber self-sealing, serum stopper was fitted to the end of th

exhaust tube to allow sampling by means of a sterile syringe. The flask was placed into a thermostated, Lucite, water-bath mounted on magnetic stirrers. The flasks were positioned directly over the stirrers. Light intensity was 1000 ft-c as measured by a Weston Illumination Meter.

The second type of sealed culture vessel was a 500-ml Erlenmeyer flask with a 29/42 standard-taper joint similar to the first type, except that the stop-cocks were replaced with screw clamps. Cultures were grown on a shaker, agitated at 69 R.P.M. with an amplitude of 66 mm, and housed in a New Brunswick Psychrotherm Controlled Environment incubator. Temperature was maintained at 39 C ± 0.5 C at an illuminance of 780 ft-c. Cultures grown heterotrophically were handled in a similar fashion but double-wrapped with aluminum foil to exclude light.

ANALYSES

Nitrite. Samples were prepared for analysis by centrifuging at 3500x G for 15 minutes to remove cell and cell wall fragments from the supernatant. The procedures of Snell and Snell (30), A.O.A.C. Methods of Analysis (3), and a modification of those of Novack and Wilson (23) were used to determine nitrite by colorimetrically measuring the red azo dye formed in the presence of sulfanilic acid.

Hydroxylamine. Samples were prepared as described above and analyzed utilizing the methods of Novack and Wilson (23), which involve oxidation of hydroxylamine to nitrous acid that is determined colorimetrically. The method of Frear and Burrell (9) was also employed. In this method, hydroxylamine reacts quantitatively with an excess of 8-quinolinol to form the stable 5, 8-quinolinequinone-5-(8-hydroxy-5 quinolylimide) which can be measured colorimetrically.

Total Nitrogen. Samples were prepared by centrifuging aliquots of the culture, at 3500x G for 15 minutes, to separate cells from the medium. These were then frozen and dried in a Thermovac Model FD-Port freeze drier. Dry weights were obtained, and then dried cells and supernatant were transferred to micro-Kjeldahl flasks. A semi-micro adaptation of the Ranker (26) method for total-N was employed using the Kemmerer-Hallett (14) distillation unit. This method also measures nitrate N by retaining nitrate with salicylic acid until it is reduced by the $Na_2S_2O_3 \cdot 5H_2O$. A catalyst mixture of Na_2SO_4, $CuSO_4$, and Se insures inclusion of amino, amide, and ammonia N. However, NO_2 and NH_2OH are excluded. For verification of the Kjeldahl method, each set of samples was checked against those determined by a Perkin-Elmer 240 Elemental Analyzer. For some samples, analyses were conducted on an Aminco C-H Analyzer to establish C/H/N ratios.

Ammonia. The method is applicable to dissolved ammonia. Samples were prepared as described above. Aliquots of 15 to 20 ml of supernatant were transferred to a steam-distillation unit to which 20 ml of 40% NaOH was added. The mixture was steam-distilled into 10 ml of 2% H_3BO_3 with 5 drops of bromcresol-green methyl-red indicator. The H_3BO_3 solution was titrated with H_2SO_4 and the ammonia calculated as N (3).

GAS CHROMATOGRAPHY

Nitrogen analyses. Gas samples were obtained by growing cultures, at 39 C and 1000 ft-c, in test tubes fitted with neoprene stoppers. The neoprene stoppers were fitted with inlet and outlet glass tubing, so that six tubes were connected in a series with gas flow in one direction. A 2% CO_2-6% O_2-92% A gas mixture was bubbled through the cultures at a pressure of 10 psi. The outlet port of the last tube in the series was led to a test tube suspended in liquid N in a Dewar flask. All gasses were liquified with the exception of N. Any N_2 was collected from above the liquified gases and transferred to vacuum flasks fitted with rubber, self-sealing, serum-stoppers. Samples were drawn through

serum-stoppers by means of a gas-tight syringe and injected into a Fisher Model 29 Gas Partitioner at room temperature. The columns consisted of Porapak S in the first position, and Molecular Sieve 13-X in the second position. The detector was a thermal conductivity cell; the carrier gas was He.

Cultures grown in sealed flasks were analyzed for gaseous N, Samples were injected into a Glowall Chromalab Gas Liquid Chromatograph. Columns consisted of a 6' x 1/8" glass column packed with Molecular Sieve 13X, 60 to 80 mesh, connected to a second 6' x 1/8" glass column packed alternately with Porapak Q, 50 to 80 mesh, or Porapak R, 50 to 80 mesh. Columns were maintained at 30 C with the flash-heater off. Carrier gas was A ionized at 125 C at a detector voltage of 900 V.

Nitrous Oxide. Free gas samples were withdrawn from sealed flasks and injected into the GLC. Columns were Porapak Q, 50 to 80 mesh, and Porapak R, 50 to 80 mesh. The method was essentially that described by Whilwhite and Hollis (34). The method described for gaseous N also gave a measure of N in sealed flask samples.

Those cultures suspected of containing dissolved N_2O were centrifuged to separate cells from supernatant. An aliquot of supernatant was then injected into a GLC as described above. The flashheater at 150 C vaporized the sample. The column was Porapak R, 50 to 80 mesh, hald at 100 C. The detector was held at 135 C at 900 V. Immediately prior to appearance of the water peak, the column was disconnected from the detector for 20 to 30 minutes to prevent collection of water vapor on the detector. An alternative for drying, which was equally satisfactory, was to pass the vaporized supernatant through a U tube suspended in a Dewar flask containing dry ice and acetone at 20 C and thence into the column.

A further modification was to fit a 50 ml Erlenmeyer flask with a neoprene stopper and equipped with a U tube. The supernatant was placed in the flask, and the other open end of the inverted U tube was immersed in absolute ethyl alcohol in a second flask. The supernatant was heated to nearly boiling to drive off dissolved gasses and concentrate them in alcohol. The alcohol solution was injected into the flash-heater and vaporized.

Mass Spectrometry. All samples were prepared by centrifugation as described previously. Spectra were obtained on an LKB, type 9000, Gas Chromatograph-Mass Spectrometer. The separator temperature was 280 C; filament trap current was 60 μ amps.; and, electron energy was 70 volts. Where GLC was employed, the procedure was that given earlier. For direct insert, the sample was injected directly into the 300 C vaporization chamber. Masses of 2 to 100 were recorded on light-sensitive strip chart paper, except where they were examined to mass 300 to scan for any other compounds which may have been present.

Raman Spectroscopy. The instrument was a Spex 1401 Double Spectrometer using a laser beam at 4880 Å. Samples were prepared as described above and placed in 10 x 10 mm quartz cuvettes. They were placed in the light beam, and scattering was measured at an absorption of 1287 cm^{-1} and at a very weak band occurring at 2214 cm^{-1}.

RESULTS AND DISCUSSION

In view of the apparently high rate of N uptake observed in preliminary experiments, it was obvious that balance sheets should be prepared to describe the fate of all nutrient N. Therefore, N analyses were made of cells and supernatants from the Recyclostat, culture tubes, and sealed flasks. Determinations were also made of N supplied to the medium. These data indicated that significant amounts of N were lost from cultures during growth of the algae. Table II gives the balance sheet from two cultures grown in the Recyclostat, and harvested directly. These are representative of numerous experiments with cultures grown o KNO_3 and urea as N sources. The average loss of N, which was unaccounted for by the micro-Kjeldahl method, was 26.9% for the cultures supplied KNO_3 and 28.7% for those supplied urea. Variation was encountered within subsequent experiments However, the average losses, considering 22 analyses from 6 experiments with cultures grown in the Recyclostat, showed a lower figure for those supplied urea rather than KNO_3. Averages for all experiments were 32.7% loss for KNO_3 and 20.2% for urea. Total N analyses by the semimicro-Kjeldahl method were reproducible within an accuracy of 2%. Total N concentration of cells remained well within the 10% of dry weight maximum observed by many researchers. This was also true for other experiments involving N balance sheets--including cultures grown in 4.0 and 8.0 g KNO_3/l as the N source. N increments were supplied throu addition of fresh nutrients calculated to match N removed by cell growth and harvest.

With KNO_3-supplied cultures, pH was controlled by direct injection of nitric acid solution. This was comprised of 64.3 ml of 70% HNO_3, 0.18 gm KH_2PO_4, 0.9 gm $MgSO_4 \cdot 7H_2O$, and 0.006 gm MgO per liter. It was included in the calculations as an additional N source.

TABLE II

Micro-Kjeldahl nitrogen analyses of cultures of *Chlorella sorokiniana* grown in the Recyclostat.

N Source	Illuminance, ft-c	Days after Inoculation	N Increments, mg.	Total N supplied, mg.	Cellular Dry Weight, mg.	N Recovered, mg.			Apparent % N Loss
						Supernatant	Cells	Overflow	
KNO_3	800	2	182.0*	182.0	650	71.4	49.0	9.4	28.8
"	1200	3	31.4	213.4	1150	59.0	74.0	49.4	14.2
"	2300	6	165.5	378.9	2010	79.0	157.0	4.6	38.0
"	2800	8	93.5	472.4	2320	86.9	241.0	19.9	26.5
Urea	800	2	305.5*	305.5	1200	113.5	87.5	17.0	28.8
"	1600	3	22.5	328.0	1860	129.4	143.6	13.7	12.5
"	2300	4	22.5	350.5	2440	87.1	167.3	9.7	24.6
"	2800	5	22.5	373.0	2600	69.6	161.3	9.4	35.2
"	3000	6	15.0	388.0	2790	51.7	161.6	8.8	42.6

*Initial Nutrient Level

To measure the effect of any N loss from fixed amounts of nutrient N (in contrast to the steady state resupply system of the Recyclostat), cultures were grown in test tubes supplied with either KNO_3 or urea. Results of these experiments are shown in Table IV. Average N losses were 21.66% for KNO_3 cultures and 25.03% for urea cultures. These data confirm a level of apparent N loss quantitatively similar to those of the Recyclostat cultures. To determine if there was a correlation between N losses and N concentrations, cultures were grown in test tubes at three concentrations cf urea--70, 90, and 117 mg. Results, indicated less N loss with increasing amounts of N in the medium. Though unexpected, this observation had also been made on preliminary Recyclostat cultures when excessive amounts of N were provided. The experiments described above were performed with a continuous supply of 2% CO_2-in-air bubbled through cultures. If the N source were converted to a volatile substance, it could have been carried off in the gas stream. To obviate this possibility, numerous cultures were grown with KNO_3 and urea as N sources both autotrophically and heterotrophically in sealed flasks at varying N levels. Cultures were sacrificed for analyses after from 3 to 17 days. Growth rates were expectedly less than those for comparable cultures gassed with CO_2-in-air. However, similar quantities of N were unaccounted for. Losses ranged from 3.0% in the young cultures to as high as 47% of the initial nutrient N in the older cultures Apparently a similar system, operating under a variety of conditions, is responsible for conversion of N to a form which is not detectable by standard methods.

Hypotheses to account for the balance sheet discrepancies arise from a consideration of pathways of N reduction or oxidation by cells. Urease has not been detected in Chlorella (10,33). Apparently urea is assimilated without

TABLE III

Micro-Kjeldahl Nirtogen analyses of cultures of Chlorella sorokiniana grown in test tubes bubbled with 1% CO_2-in-air at 1000 ft-c. with fixed initial N concentrations.

N Source	Hr after Inoculation	Cellular Dry Weight, mg.	N Recovered, mg.		Total N Recovered, mg.	% N Loss
			Supernatant	Cells		
7.0 mg. as KNO_3	48	35.7	2.8	3.0	5.8	17.0
"	72	48.9	1.0	4.3	5.3	25.0
"	96	62.1	0.7	4.7	5.4	23.0
23.5 mg. as Urea	48	44.3	15.1	3.8	18.9	19.5
"	72	67.4	11.7	5.2	16.8	28.5
"	96	57.7	12.5	4.6	17.1	27.1

preliminary breakdown to ammonia. Therefore, consideration of N loss through metabolic intermediates will be confined to those cells grown on KNO_3.

It is generally assumed that, before assimilation, nitrate-N is reduced to ammonia-N in four states--each of which require two electrons (15).

$$HNO_3 \longrightarrow HNO_2 \longrightarrow H_2N_2O_2 \longrightarrow NH_2OH \longrightarrow NH_3$$

The presence of NO_2^- in culture media is well established (10,31,32). It has been reported that NO_2^- can be utilized at or below 0.001 M, but that higher concentrations are inhibitory (8,24,36). NO_2^- is not usually detected by the micro-Kjeldahl methods. In view of this, analyses were performed on cultures, grown in the Recyclostat, to determine whether NO_2^- excretion could be responsible for the apparent N loss in KNO_3-grown cultures. Typical analyses are given in Table IV. The amounts are in agreement with those reported earlier (5). NO_2^- accumulation was closely correlated with the condition and vigor of the culture--a healthy viable culture producing a maximum amount of NO_2^-. This was so reliable an index as to serve as a measure of vigor of a given culture. NO_2^- analyses of cultures grown in test tubes showed a maximum NO_2 level of 1.8 mg 1L at an illuminance of 3,000 ft-c. Bongers (5) reported that increasing illuminance decreases NO_2^- production. In the cultures studied here, NO_2^- production is proportional to cell growth. Greater amounts of NO_2^- accumulate as the cell population increases during normal growth.

Although NH_2OH is an intermediate of NO_3^- reduction (21), it is too toxic to be expected as a by-product in culture media. Concentrations of 3×10^{-5} M were reported to be toxic to species of Chlorella and Scenedesmus (5,11,16). Furthermore, Syrett (32) reports that neither $H_2N_2O_2$ nor NH_2OH have been reported to accumulate in algal cultures. Nason, et al. (22) showed the presence of NH_2OH in Neurospora cultures. Escherichia coli utilizes $H_2N_2O_2$ or NH_2OH as N sources (21). In this study only, trace amounts of NH_2OH were

TABLE IV

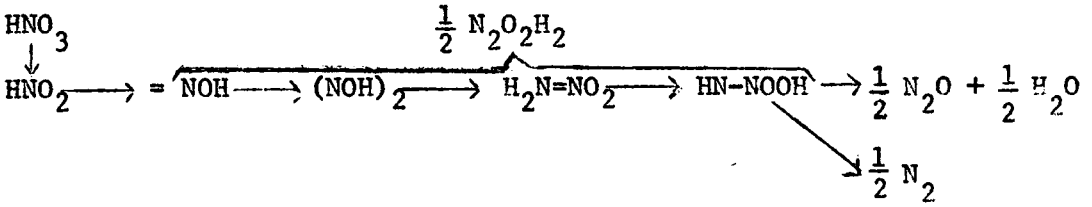
NO_2 in the supernatant of cultures of Chlorella sorokiniana grown in the Recyclostat supplied 2.0 g./L. N as KNO_3 at increasing levels of illuminance and optical density

Illuminance, ft-c.	Optical Density	Days after Inoculation	NO_2, g./L	Total N as NO_2, mg.
800	4.1	2	8.25	2.5
1200	5.1	3	8.20	2.5
1600	5.3	4	8.00	2.9
2000	8.0	5	7.00	2.1
2300	7.9	6	7.50	2.3
"	8.4	7	3.20	1.0
2800	9.0	8	4.50	1.9
"	8.2	9	4.00	1.2
"	7.9	10	2.15	0.6
"	8.0	11	3.20	1.0
"	7.8	13	0.00	0.0

occasionally found in media from both test tube and Recyclostat cultures.

Although significant quantities of NO_2^- were identified, and traces of NH_2OH were occasionally found, the small amounts detected cannot account for the unexplained losses from nitrate-grown cultures. Whether N loss involves any of the intermediates of NO_3^- reduction is not certain, but intermediates have been detected in some organisms where N loss has been observed (13,27).

The next approach was to analyze for gaseous N losses. Denitrification occurs in certain bacteria. One scheme for the denitrification pathway is that proposed by Kluyver and Verhoeven (17):



Other pathways have been suggested. They differ as to whether N_2O is (17,27) or is not (1,28) a normal intermediate. Nevertheless, these hypotheses suggested logical gaseous compounds as possible avenues for N loss.

Cultures in culture tubes were bubbled with a 2% CO_2, 6% O_2, 92% A gas-mixture. After 18 to 22 hours from inoculation, samples were collected from the gas stream which had passed through the culture. Control samples were collected just before entering the cultures. Samples were then examined in a gas partitioner. In cultures grown with urea as the N source, there appeared a slight peak with the retention time of N_2. This peak appeared in several samples. It occurred in cultures supplied urea rather than in those grown on KNO_3. However, concern about possible contamination from atmospheric N_2 led to other methods of analysis.

To eliminate either loss, or contamination, cultures were grown in sealed flasks. Gas samples were removed from the flasks and injected directly into

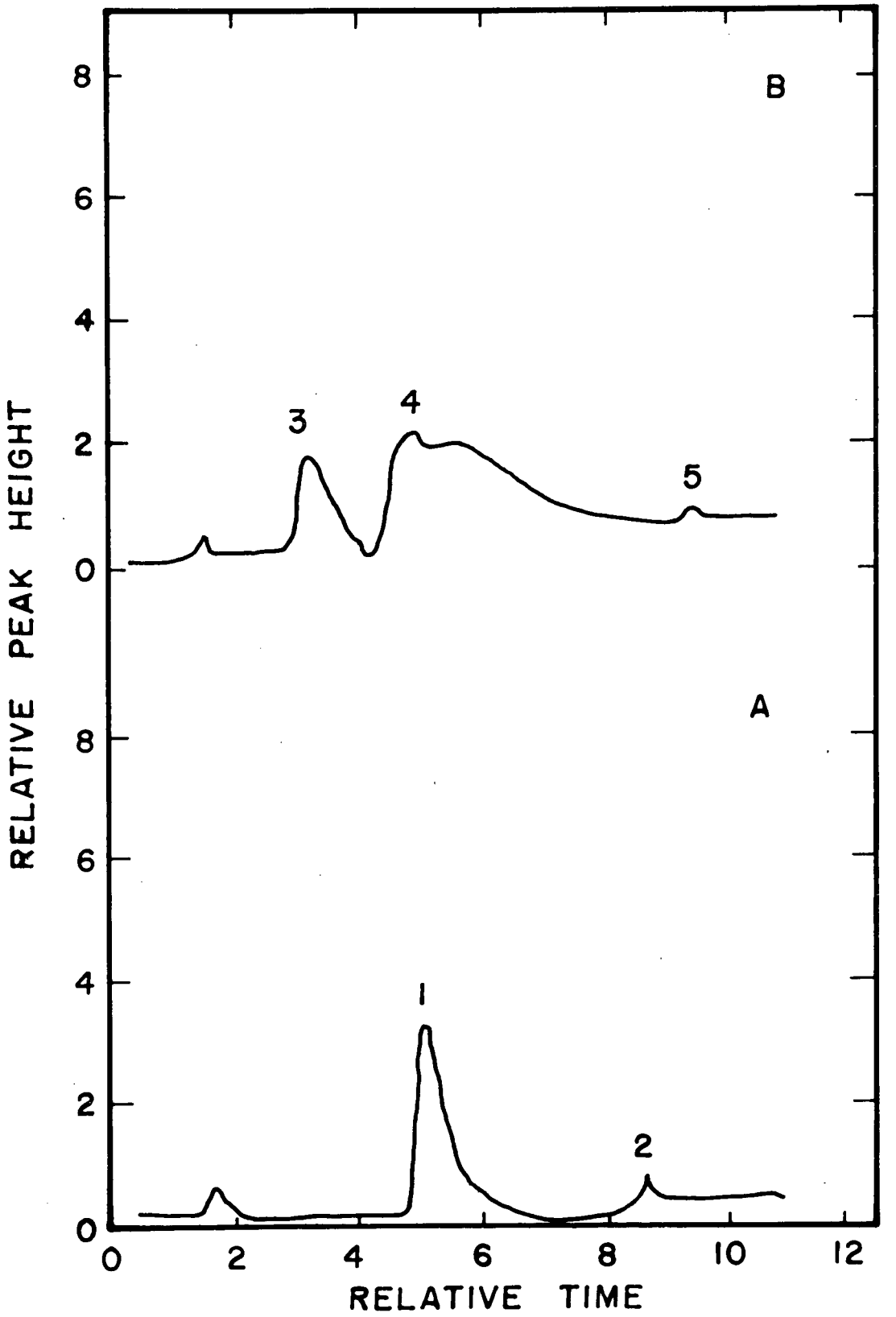
a GLC. This was done daily using cultures gorwn with KNO_3 or urea as N sources, and maintained in light or in darkness on glucose for various periods of time. Good separation was obtained, but no N_2 and only traces of N_2O were detected. One exception occurred in a culture grown autotrophically on urea. A significant peak for N_2O occurred five days after inoculation, and remained for two days before gradually disappearing.

In view of its solubility in water (56.7 cm^3/100 ml at 25 C), if all missing N were in the form of N_2O most could have been dissolved in the medium. N_2O can be treated essentially as an ideal gas with $\frac{PV}{RT} = 0.9931$.

The problem was either to measure gas in the medium or to remove it from the medium first. Several methods were employed. One of these involved injection of a sample directly into the chromatograph. The medium was then vaporized, passed through a cold trap, and passed through the column for separation. For standardization, seven equal-sized samples of a saturated N_2O-in-water solution were injected. Areas of the peaks were measured and an average obtained. Peplicate samples of medium, in which cultures had grown, were then injected and average peak heights for these were determined. The data indicate amounts of 1.2 to 2.4 mg N_2O per 100 ml of medium. Later data indicated that vaporization was not total, and that these figures were probably too low. For cultures grown in sealed flasks with KNO_3 as the N source, this amount would account for a significant portion of the missing N.

Increased analytical efficiency was achieved by heating the medium and driving the gas into a smaller volume of absolute alcohol which was then injected into the chromatograph. Alcohol had a lower boiling point and consequently was easier to vaporize. It also had a longer retention time on the column before the solvent front passed through.

Figure 1. Gas-liquid chromatogram peaks of samples injected into a column which was attached to a mass spectrometer. The samples were vaporized and then passed through the column for separation. The numbers over the peaks identify each peak with reference to mass spectra. Sample A was that of a nitrous oxide-in-water solution, while Sample B was that of the culture medium in which Chlorella had been grown.



18-A

In the course of early experiments, when N analyses were confounded by atmospheric N, cultures were supplied with N^{15}. Gas over the cultures was then injected directly into a mass spectrometer and the ratios of N^{14} to N^{15} measured. While the resulting data did not reveal the presence of increased N, an additional method of analysis was devised for correlation of the data obtained from GLC.

Columns were removed from the GLC and attached to the gas-liquid chromatograph-mass spectrometer. The sample was then injected using the previous procedure. Results are obtained graphically as GLC peaks. An example is shown in Figure 1 in which the sample was injected as a liquid. The numbers over the peaks identify each peak with reference to the mass spectra. The mass number for each peak can be determined as the sample passes through the column. Retention time can then be correlated with the mass of the group or groups contained within that peak.

Using these techniques, analyses were made for N_2O. N_2O (mass 44) fractionates in the MS to NO (mass 30) and N (mass 14), but only a small amount does so. To estimate the amount for this instrument, a number of samples of N_2O of known concentration were injected, and the percent of mass 30 to mass 44 was determined. This mass characteristic then served as a guide for any mass 44/mass 30 combinations which might arise. Only those with the correct characteristics were considered.

Figure 2 shows three spectra representing three of the GLC peaks shown in Figure 3. The peak heights of the spectra represent relative amounts of each mass present. These compare the N_2O standard (peak 1) with two peaks (peaks 4 and 5) from the sample cultured autotrophically with urea as the N source. Peak number 4 has an Rf identical to the standard, and also has masses 30 and 44 present. While the relative heights are less, they do occur in the correct ratio. Peak number 5 is a later peak and represents an air peak--completely lacking mass 30.

Figure 2. Mass spectra representing the materials from cultures of Chlorella located under each of the three peaks presented in Figure 1. The nitrous oxide standard spectrum - peak 1 - shows relatively large peaks for masses 44, 30, and 14. The light-grown urea culture - peak 4 - shows smaller, but definite peaks for those masses. Peak 5, which represents an air peak from the urea culture has a smaller mass 44 peak (CO_2), and no mass 30 peak.

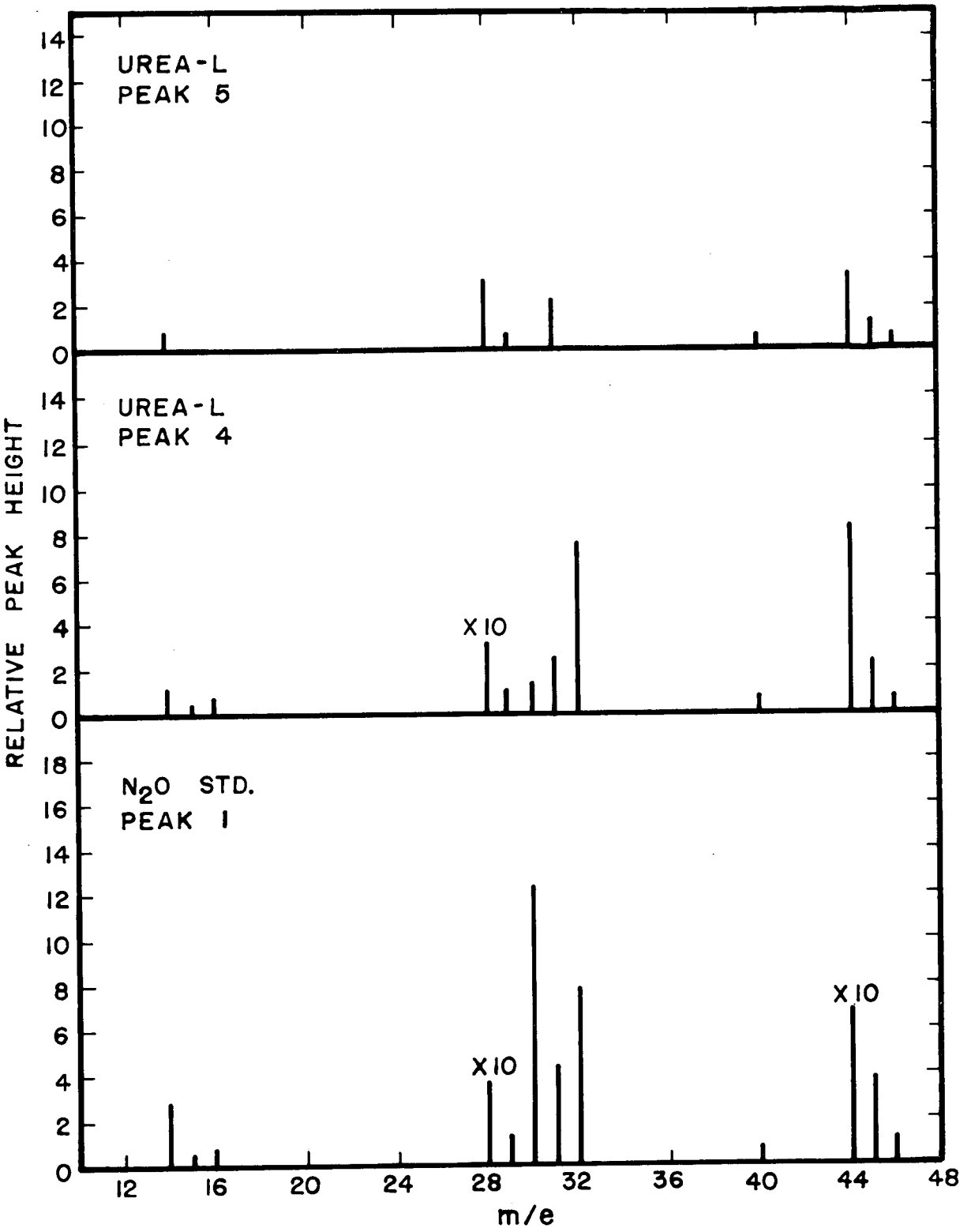

UREA-L
PEAK 5
UREA-L
PEAK 4
X10
N_2O STD.
PEAK 1
X10
X10
RELATIVE PEAK HEIGHT
m/e
0
2
4
6
8
10
12
14
16
18
12
16
20
24
28
32
36
40
44
48

Figure 3 compares a dark-grown urea culture with a light-grown KNO_3 culture using an N_2O spectrum for reference. These were injected as liquid samples and again demonstrate a similar pattern. Figure 4 compares gas from over a culture with a sample of gas from a flask of medium without cells. The control gas has the spectrum of the flushing gases--CO_2; O_2 and A.

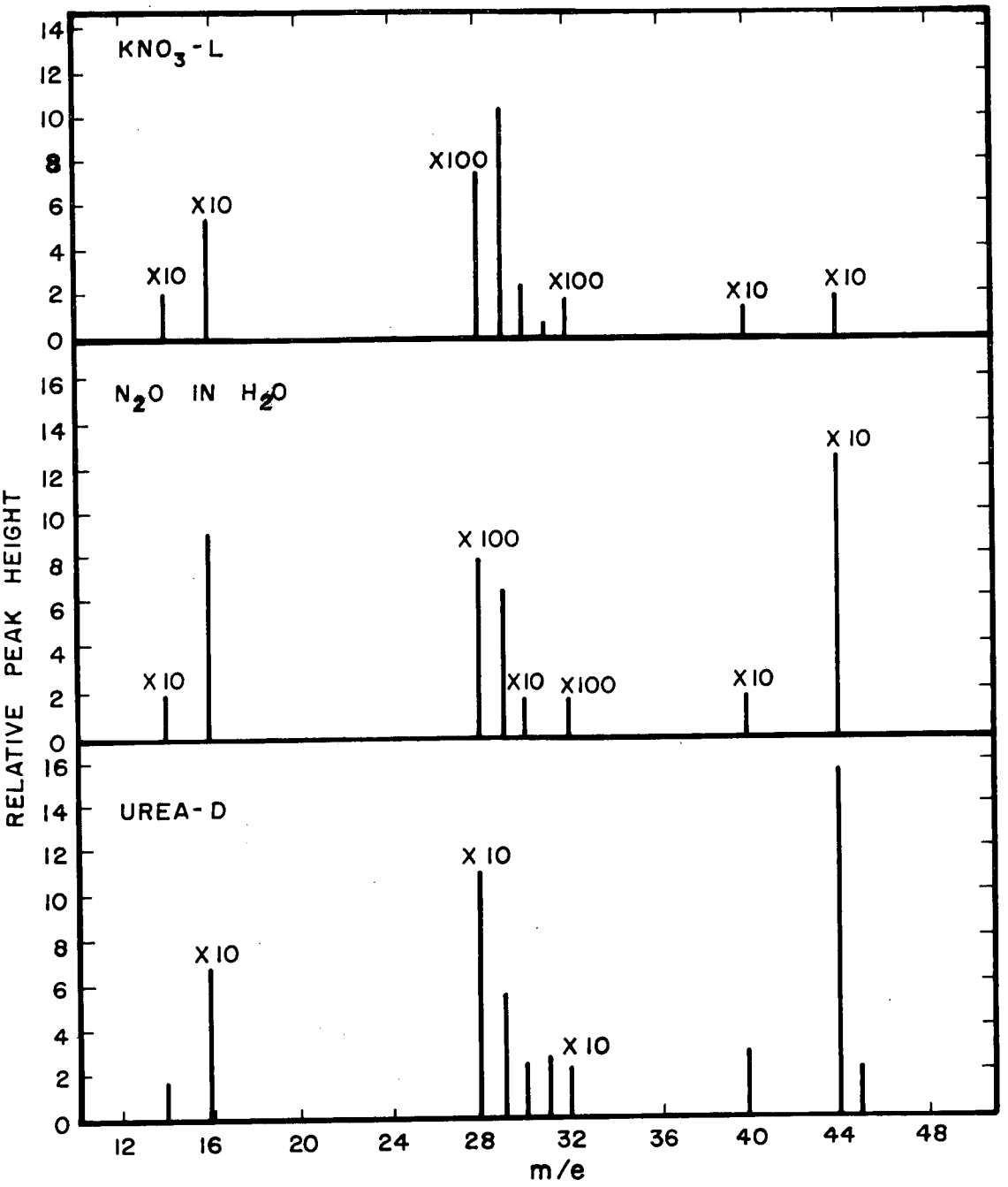
The instrument was also used for direct inserts--bypassing the GLC column. The sample was injected into a chamber held at 300 C where it was vaporized, passed directly to the separator, and one spectrum was obtained for the entire sample. Figure 5 compares a KNO_3 medium without cells with a medium which had grown cells.

It was suggested earlier that incomplete vaporization occurred in vaporizing liquid samples for GLC determinations. This was observed during several analyses of liquid samples when mass spectra were run continuously through to the water front as it came off the column. When a saturated N_2O solution was measured, the largest amount of the gas came off as expected, but decreasing amounts continued to come into the water front--indicating that some was still dissolved in water vapor. When culture samples were analyzed, the same was found to be true. No precise determination can be made, but it appears most is vaporized and found in the major peak.

To determine qualitatively the amount of N_2O present in the samples, four analyses were selected randomly. The relative peak heights of the standards with known concentrations of N_2O were determined and compared with the samples. Calculations were made which indicated an average of 7.1 mg N_2O / 100 ml for urea cultures and 7.6 mg for KNO_3 cultures. These values are roughly two thirds more than was shown with GLC data.

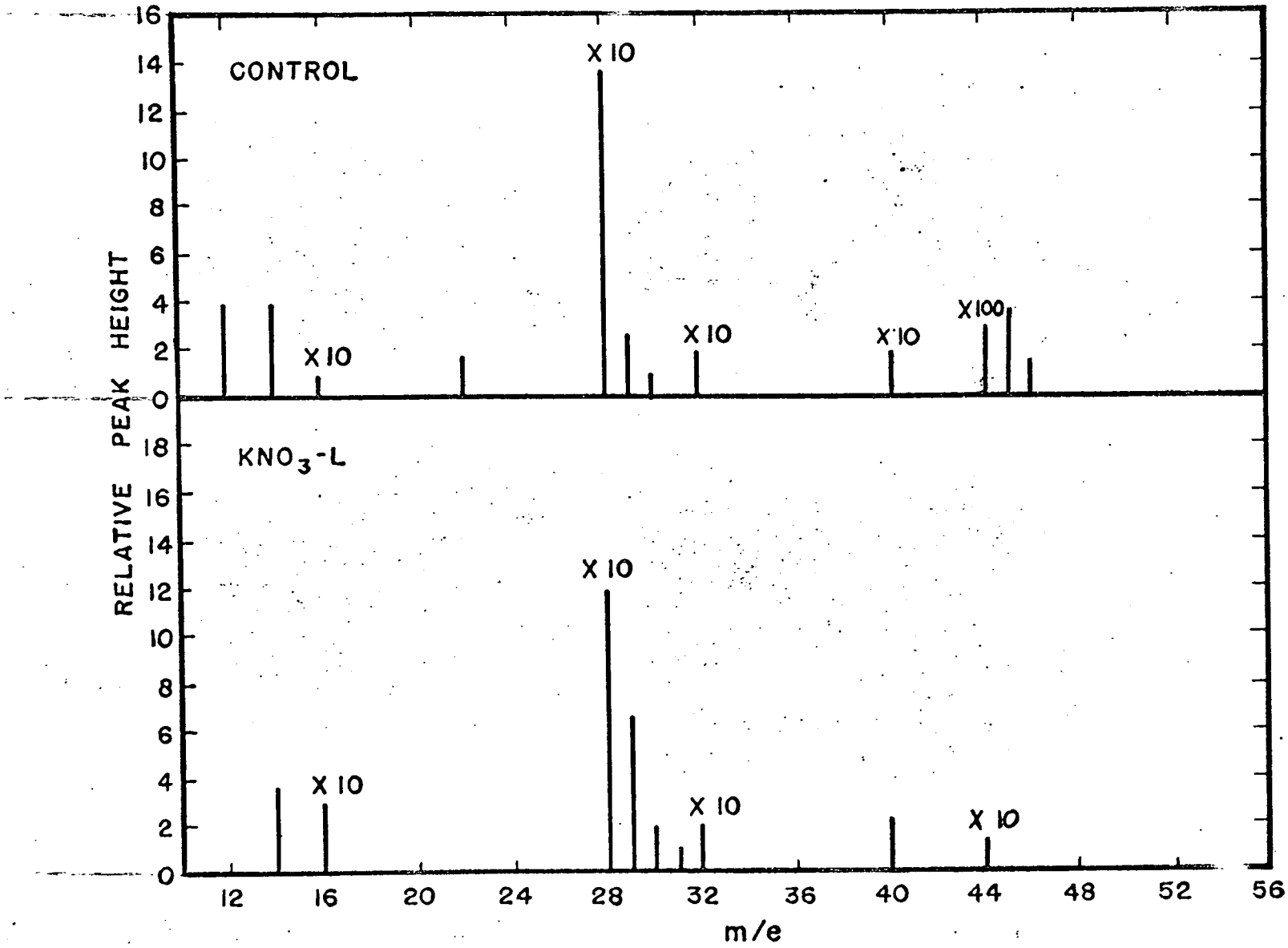
As an additional verification, several samples were analyzed by Raman Spectroscopy. As before, culture samples were compared with medium saturated with N_2O. The first sample, a urea sample grown autotrophically, gave a positive

Figure 3. Mass spectra comparing a dark-grown urea culture of *Chlorella* with a light-grown nitrate culture. A nitrous oxide spectrum is given as a reference. All are similar with respect to the presence of masses 44, 30, and 14, although relative peak height differ.


KNO3 - L
X10
X10
X100
X100
X10
X10
N2O IN H2O
X 10
X 100
X10
X100
X10
X 10
UREA - D
X 10
X10
X 10
RELATIVE PEAK HEIGHT
m/e
12
16
20
24
28
32
36
40
44
48

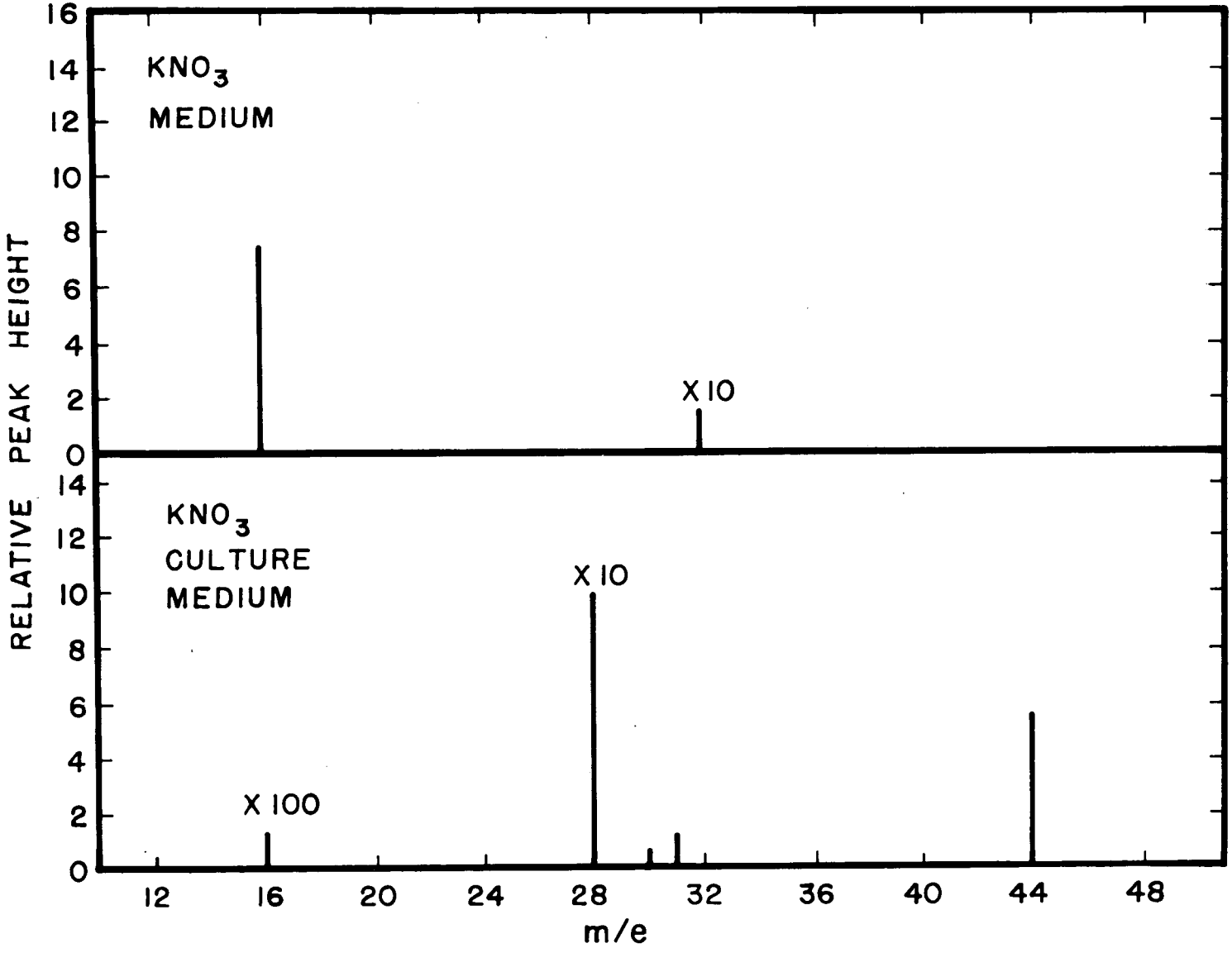
32-A

Figure 4. Mass spectra comparing gas taken from over a sealed culture of Chlorella with gas taken from a flask of medium without cells. The control spectrum indicates the presence of large quantities of flushing gases--argon, CO_2, and oxygen. These ratios are progressively altered in the culture medium as cells gorw due to photosynthesis.


CONTROL
X 10
X 10
X 10
X100
X 10
KNO3-L
X 10
X 10
X 10
X 10
RELATIVE PEAK HEIGHT
m/e
0
2
4
6
8
10
12
14
16
18
12
16
20
24
28
32
36
40
44
48
52
56

93-A

Figure 5. Mass spectra comparing a nitrate-supplied medium from cultures of Chlorella with the medium without cells. Data were obtained by direct insert into a mass spectrometer. Peaks 44 and 30 are absent on the control, but are obvious in the medium which had supported cell growth.


RELATIVE PEAK HEIGHT
KNO3
MEDIUM
X 10
KNO3
CULTURE
MEDIUM
X 10
X 100
m/e

24-A

indication of N_2O. The second sample was a light-grown KNO_3 culture from which the gases had been driven into alcohol to concentrate the gas to provide a clear background for the instrument. This second sample also gave a positive response at the correct wavelength, but the peak was smaller than that of the previous sample. Inasmuch as alcohol has absorption bands near this region, the data are not clear enough to assure the presence of N_2O. The final sample was a dark-grown urea sample which also indicated a peak for absorption. This culture was part of the experiment which produced the first sample, so that the gas would be expected to be present. Semiquantitave calculations on these samples indicate 6.0 mg N_2O / 100 ml--a figure close to those results from MS data.

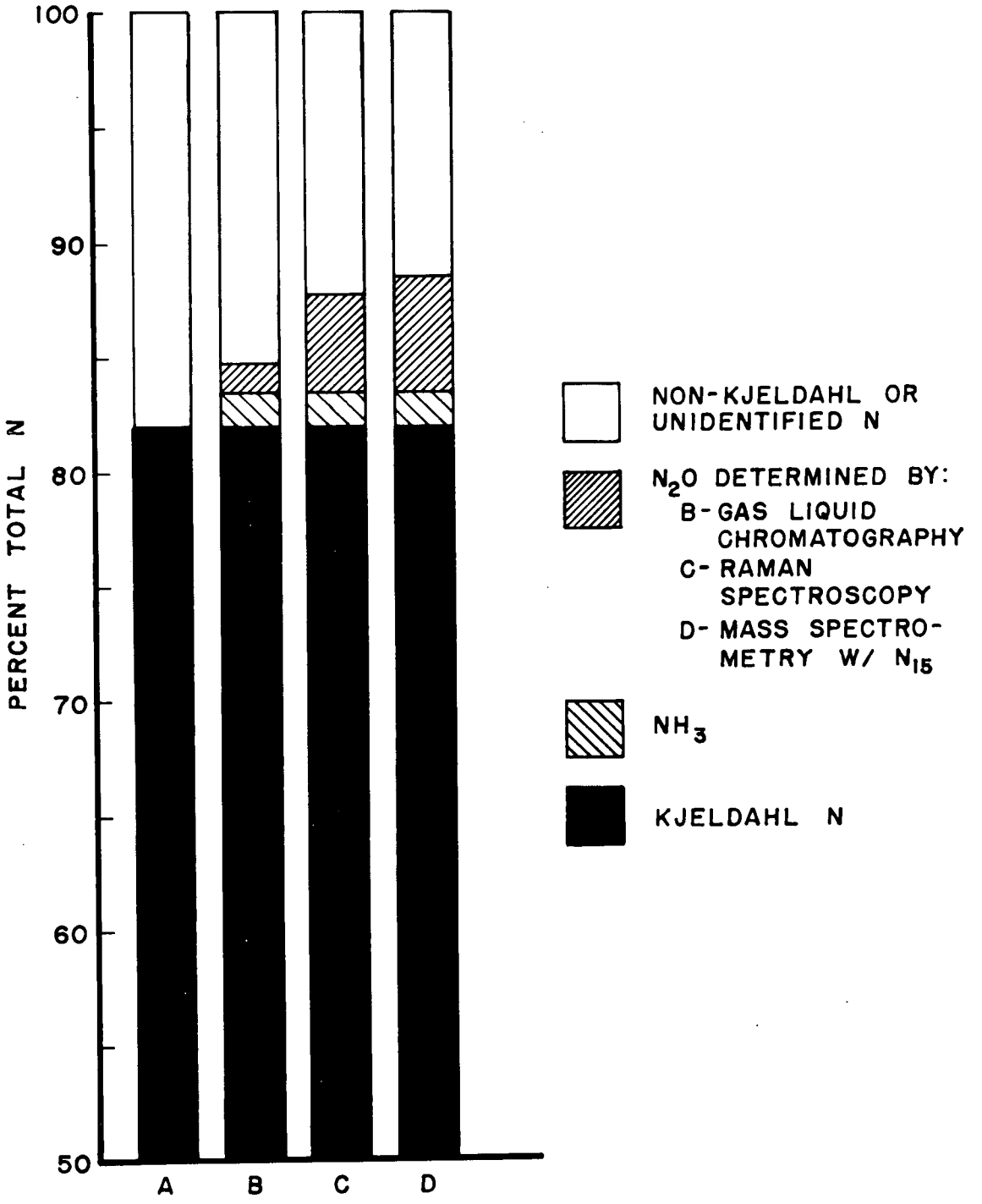
Hattori (10) reports no trace of ammonia in cells or medium of N-starved cultures of Chlorella ellipsoidea with either KNO_3 or urea as the N source. Stiller (31) demonstrated that Chlorella sorokiniana produced ammonia when grown on KNO_3, when CO_2 was absent or limiting, and Little and Mah (20) report ammonia production in cultures of Chlorella ellipsoidea in medium containing excess urea and limiting amounts of glucose. Studies were therefore included to determine if ammonia were present. In related experiments, gas effluents were trapped in H_3BO_3 solutions, but no significant amounts of NH_3 were detected. For urea cultures in sealed flasks, gas was bubbled through sterilized urea medium--pH 7.5. The effluent gas was passed through a 2% H_3BO_3 solution. After 30 minutes, there was no detectable NH_3. Twenty ml of a 40% NaOH solution was added and bubbled for an additional 30 minutes. The result was only 0.7 mgm N / 100 ml. It would seem then that little if any NH_3 is produced in sterilization and/or lost in flushing the chamber in the sealed flask cultures.

Some NH_3 is undoubtedly produced in the urea cultures, either by the cells or by the sterilization process, but the amounts appear to be small. Little and Mah (20) reported a maximum of 34 µg NH_3-N, and Stiller reported 4.52 to 5.42 µmoles produced by the algae.

A summary of all data is shown in Figures 6 and 7. Figure 6 represents cultures grown on urea. Figure 7 gives the data for KNO_3 grown algae. Some N still remains unaccounted for. The remaining N is either in a form undetected by the analytical procedures available or may represent the sum of sampling and analytical errors in the procedures which were difficult and complicated.

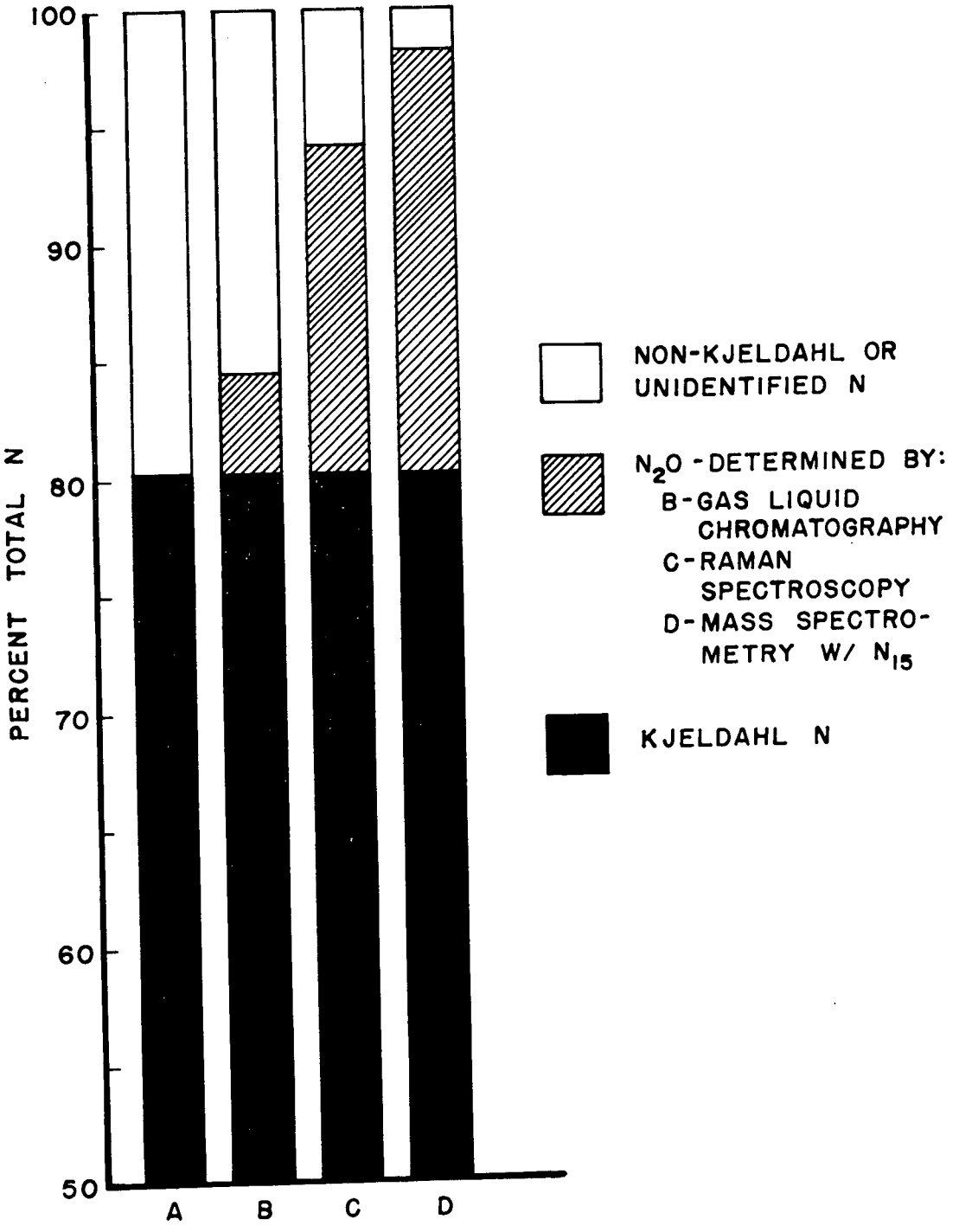
However, major gaseous loss has been confirmed by three distinct analytical procedures and there is no doubt that Chlorella is capable of significantly reducing the N levels of the media on which it grows by conversion of a significant amount of N to N_2O.

Figure 6. A summary of the data, comparing nitrogen supplied to the medium with that accounted for, in cultures of Chlorella sorokiniana, grown on urea.


100
90
80
70
60
50
PERCENT TOTAL N
A
B
C
D
NON-KJELDAHL OR UNIDENTIFIED N
N_2O DETERMINED BY:
B- GAS LIQUID CHROMATOGRAPHY
C- RAMAN SPECTROSCOPY
D- MASS SPECTROMETRY W/ N_{15}
NH_3
KJELDAHL N

21. A.

Figure 7. A summary of the data, comparing nitrogen supplied to the medium with that accounted for, in cultures of Chlorella sorokiniana, grown on KNO_3.


100
90
80
70
60
50
PERCENT TOTAL N
A
B
C
D
NON-KJELDAHL OR UNIDENTIFIED N
N_2O - DETERMINED BY:
B - GAS LIQUID CHROMATOGRAPHY
C - RAMAN SPECTROSCOPY
D - MASS SPECTROMETRY W/ N_{15}
KJELDAHL N

58-A

LITERATURE CITED

1. Allan, M. B. and C. B. Van Niel. 1952. Experiments on bacterial denitrification. J. Bacteriol. 64: 397-412.

2. Allison, F. E., K. S. Love, L. A. Pinck, and V. L. Gaddy. 1948. Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna. Plant Physiol. 23: 496-504.

3. American Organization of Agricultural Chemists Methods of Analysis, Eighth Edition. 1955. A. O. A. C., Washington, D. C. pp. 217-218.

4. Atwater, W. O. and E. W. Rockwood. 1886. II. On the loss of nitrogen by plants during germination and growth. Amer. Chem. Jour. 8: 327-343.

5. Bongers, L. H. J. 1956. Aspects of nitrogen assimilation by cultures of green algae. Mededel. Landbouwhogeschool Wageningen. 56: 1-52.

6. Davidson, J. 1923. Is gaseous nitrogen a product of seedling metabolism? Bot. Gaz 76: 95-101.

7. Eggleton, W. G. E. 1935. The assimilation of inorganic nitrogenous salts, including sodium nitrate by the grass plant. Biochem. J. 29: 1389-1397.

8. Fogg, G. E. and M. Wolfe. 1954. The nitrogen metabolism of the Blue-Green Algae (Myxophyceae). Symposium Soc. Gen. Microbiol. No. 4: 99-125.

9. Frear, D. S. and R. C. Burrell. 1955. Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27: 1664-1665.

10. Hattori, A. 1957. Studies on the metabolism of urea and other nitrogenous compounds in Chlorella ellipsoidea. I. Assimilation of urea and other nitrogenous compounds by nitrogen-starved cells. J. Biochem. (Tokyo) 44: 253-273.

11. Inada, Y. 1958. Stimulatory and inhibitory effect of light on the nitrate assimilation by Chlorella ellipsoidea. J. Gen. Appl. Microbiol. (Japan) 4: 153-162.

12. Irving, A. A. and R. Hankinson. 1907. The presence of a nitrate reducing enzyme in green plants. Biochem. J. 3: 87-96.

13. Iwasaki, H. and T. Mori. 1958. Studies on denitrification. III. Enzymatic gas production by the reaction of nitrite with hydroxylamine. J. Biochem. (Tokyo) 45: 133-140.

14. Kemmerer, G. and L. T. Hallett. 1927. Improved micro-Kjeldahl ammonia apparatus. Ind. and Eng. Chem. 19: 1295-1296.

15. Kessler, E. 1957. Stoffwechselphysiologische untersuchungen and hydrogenase enthaltenden Grunalgen. II. Dunkel-Reduktion bon nitrat und nitrit mitmolekularen wasserstoff. Arch. Mikrobiol. 27: 166-181.

16. Kessler, E. 1964. Nitrate assimilation by plants. Ann. Rev. Plant Physiol. 15: 57-72.

17. Kluyver, A. S. and W. Verhoeven. 1954. Studies on true dissimilatory nitrate reduction. IV. An adaption in Micrococcus denitrificans. Antonia Van Leeuwenhock J. Microbiol. Serol. 20: 241-262.

18. Krauss, R. W. 1966. The physiology and biochemistry of algae with special reference to continuous-culture techniques for Chlorella. From: Bioregenerative Systems, N.A.S.A. Special Report (NASA SP-165), pp. 97-109.

19. Krauss, R. W. 1968. A study of phycophysiology in controlled environments. Department of Botany, University of Maryland, Technical Report No. 1016, Sixteenth Semiannual Status Report to the National Aeronautics and Space Administration.

20. Little, L. W. and R. A. Mah. 1970. Ammonia production in urea-grown cultures of Chlorella ellipsoidea. J. Phycol. 6: 277-280.

21. McNall, E. G. and D. E. Atkinson. 1957. Nitrate reduction. II. Utilization of possible intermediates as nitrogen sources and as electron acceptors. J. Bacteriol. 74: 60-66.

22. Nason, A., R. G. Abraham, and B. C. Averbach. 1954. The enzymic reduction of nitrous oxide to ammonia by reduced pyridine nucleotides. Biochim. Biophys. Acta. 15: 159.

23. Novack, R. and P. W. Wilson. 1948. Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. J. Bacteriol. 55: 517.

24. O'Kelley, J. C. 1968. Mineral nutrition of algae. Ann. Rev. Plant Physiol. 19: 89-112.

25. Pearsall, W. H. and M. C. Billimoria. 1937. Losses of nitrogen from green plants. Biochem. Jour. 31: 1743-1750.

26. Ranker, E. R. 1927. A modification of the salicylic-thiosulfate method suitable for the determination of total nitrogen in plants, plant solutions, and soil extracts. J. Assoc. Offic. Agr. Chemists 10: 230-251.

27. Renner, E. D. and G. E. Becker. 1970. Production of nitric oxide and nitrous oxide during denitrification by Corynebacterium nephridii. J. Bacteriol. 101: 821-826.

28. Sachs, L. E. and H. A. Barker. 1952. Substrate oxidation and nitrous oxide utilization in denitrification. J. Bacteriol. 64: 247-252.

29. Shihira, I. and R. W. Krauss. 1965. Chlorella - Physiology and Taxonomy of Forty-One Isolates. Port City Press, Baltimore, Md. 97 pp.

30. Snell, F. D. and C. T. Snell. 1953. Colorimetric Methods of Analysis, Vol. II, Third Edition. D. Van Nostrand and Co., Inc., New York, N.Y. pp. 803-804.

31. Stiller, M. 1966. Hydrogenase mediated nitrite reduction in Chlorella. Plant Physiol. 41: 348-352.

32. Syrett, P. J. 1962. In R. A. Lewin (Ed.). Physiology and Biochemistry of Algae. Academic Press, New York, N.Y. pp. 171-183.

33. Walker, J. B. 1952. Arginosuccinic acid from Chlorella. Proc. Natl. Acad. Sci. U.S. 38: 561-566.

34. Whilwhite, W. F. and O. L. Hollis. 1968. The use of porous polymer beads for analysis of the Martian atmosphere. J. Gas Chrom. 6: 84-88.

35. Wilfarth, H., H. Romer, and G. Wimmer. 1906. Uber die Nahrstoffaufnahme der pflanzen inverschiedanen zeitan ihres wachstums. landw. Versuch. Sta. 63: 1-70.

36. Zobell, C. E. 1935. The assimilation of ammonium-nitrogen by Nitzchia closterium and other marine phytoplankton. Proc. Natl. Acad. Sci. U.S. 21: 517-522.

INHIBITION OF STEROL BIOSYNTHESIS IN CHLORELLA ELIPPSOIDEA BY AY-9944*

Leal G. Dickson+ and Glenn W. Patterson++

Department of Botany, University of Maryland
College Park, Maryland 20742, U.S.A.

---

*Scientific Article No. A1775, Contribution No. 4565 of the Maryland Agricultural Experiment Station.

+Present address: Department of Biology, Walla Walla College, College Place, WA. 99324

++To whom all correspondence should be addressed

ABSTRACT

When Chlorella ellipsoidea was grown in the presence of 4 ppm AY-9944, complete inhibition of Δ^5-sterol biosynthesis was achieved. However, total sterol production remained unaltered. As a result, a number of sterols accumulated which appear to be intermediates in sterol biosynthesis. These sterols were described and identified as (24S)-5α-ergost-8(9)-en-3β-ol, (24S)-5α-stigmast-8(9)-en-3β-ol, 4α-methyl-(24S)-5α-ergosta-8, 14-dien-3β-ol, 4α-methyl-(24S)-5α-stigmasta-8,14-dien-3β-ol, 4α-methyl-(24S)-5α-ergost-8(9)-en-3β-ol and (24S)-4α-methyl-5α-stigmast-8(9)-en-3β-ol. The occurrence of these sterols in Chlorella ellipsoidea is the first time they have been noted in biological material. The accumulation of these sterols in treated cultures indicates that AY-9944 is an extremely effective inhibitor of the Δ^8 ____ Δ^7 isomerase and the Δ^{14} reductase of these plants. The occurrence of small amounts of other sterols in treated cultures has led to a proposed pathway for the biosynthesis of sterols in Chlorella ellipsoidea.

KEY WORDS AND KEY PHRASES

plant sterol biosynthesis

Chlorella ellipsoidea

cholesterol

ergostenols

methyl-sterols

stigmastenols

AY-9944

Δ^7 reductase

ergostadienols

stigmastadienols

sterol biosynthesis inhibitor

INTRODUCTION

It has been pointed out that the hypocholesterolemic drug, AY-9944, (trans-1,4-bis-(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride), inhibits the biosynthesis of cholesterol in animals by preventing the reduction of 7-dehydrocholesterol to cholesterol (1-5). Since AY-9944 has been regarded as a quite specific Δ^7-reductase inhibitor in animals, and the effect of AY-9944 on plant sterol biosynthesis was unknown, it was of interest to determine the effect of AY-9944 on the biosynthesis of Δ^5-sterols in a unicellular green alga, Chlorella ellipsoidea. Assuming that AY-9944 acts similarly in algae and animals, and assuming algal Δ^5-sterols to be synthesized via the pathway expected for higher plants, we expected that AY-9944 treatment would alter sterol composition in favor of the $\Delta^{5,7}$ and Δ^7-sterol intermediates. However, from our earlier data (6,7,8), and from the work described here, it seems certain that rather than being a Δ^7-reductase inhibitor, AY-9944 inhibits the reduction of the Δ^{14}-bond of $\Delta^{8,14}$-sterol intermediates in the biosynthesis of sterols in C. ellipsoidea. In earlier reports (6,7) we identified two novel sterols, (24S)-5α-ergosta-8,14-dien-3β-ol and (24S)-5α-stigmasta-8, 14-dien-3β-ol from AY-9944-treated cells of Chlorella ellipsoidea. In this paper we identify a total of sixteen sterols from AY-9944-treated cultures--several of which have not been previously found in nature. A quantitative analysis of all sterols found in control and treated cells of C. ellipsoidea is reported and a proposed scheme of sterol biosynthesis is this organism if presented.

EXPERIMENTAL PROCEDURES

Chlorella ellipsoidea Gerneck, Indiana Culture Collection No. 247 was grown heterotrophically in 15-l carboys on basal inorganic medium plus

0.5 percent glucose. A constant air flow from an oil-free compressor provided oxygen and kept the cells in suspension. Unpon inoculation, 4 ppm (8.6μM) AY-9944 was added to treated cultures. Sterols were extracted from freeze-dried cells with cholcroform:methanol, (2:1), and partially purified by digitonin precipitation as described by Doyle, et al. (9). Further purification and separation was accomplished using alumina, $AgNO_3$= impregnated silica gel G, and Anasil B column Chromatography (7). This routine resulted in separation of the sterols as seen in Figure 1. Qualitative and quantitative gas-liquid chromatographic (GLC) analyses, GLC-MS analyses, and physical data were also obtained as previously described (7).

RESULTS AND DISCUSSION

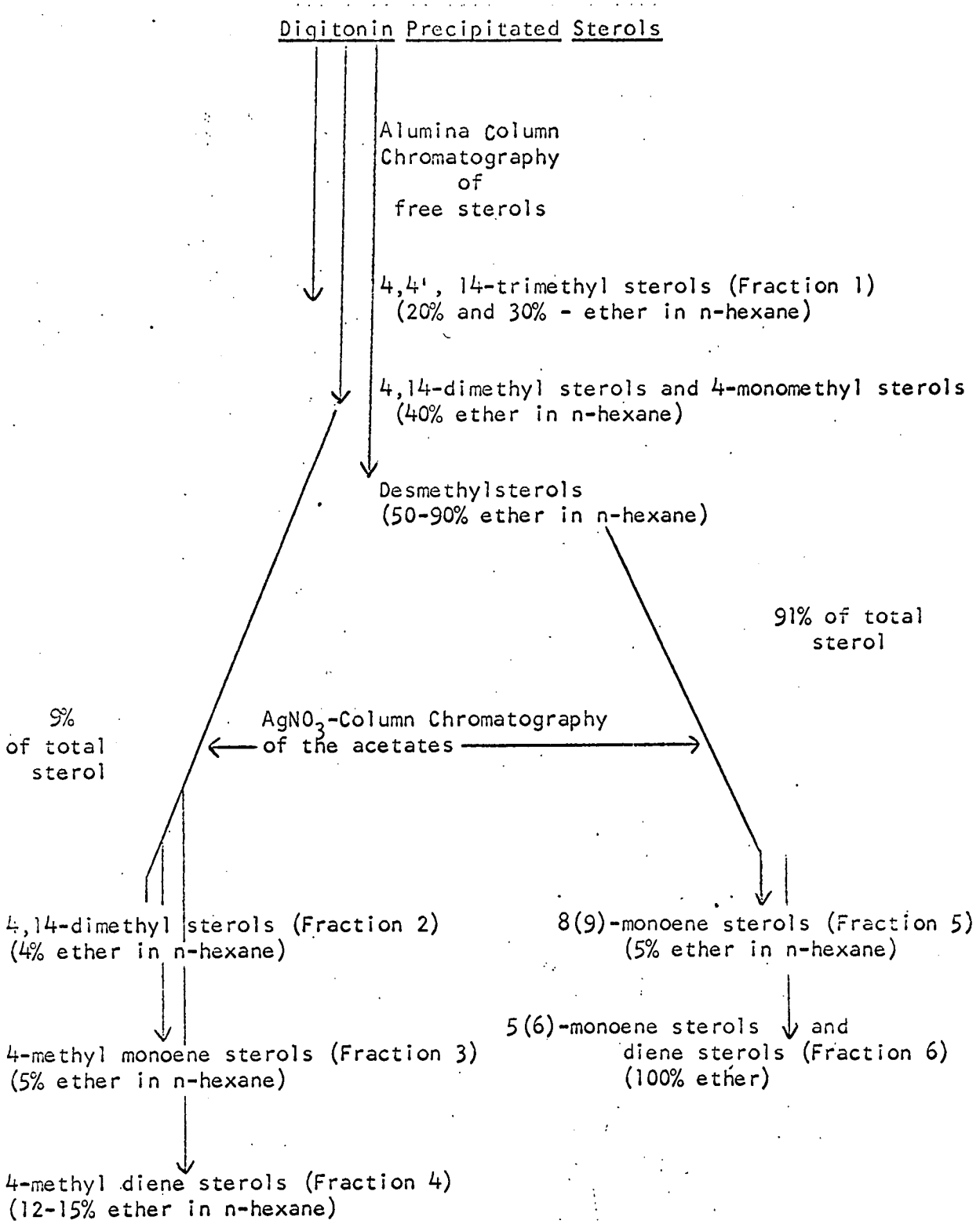
Sterols were extracted from C. ellipsoidea control cultures and from those grown in the presence of 4 ppm AY-9944. Gas-liquid chromatography (GLC) of the digitonin-precipated sterols from the inhibited cultures revealed two major peaks and two minor peaks with relative retention times (RRT) on four GC systems completely unlike those of control and unlike any available authentic standards. Alumina column chromatography, followed by $AgNO_3$- silica gel chromatography of the sterol acetates resulted in a separation of the sterols into six fractions (Fig. 1). In several cases pairs of sterols were found which were C-28 and C-29 homologs. Although the sterols of each pair could not be further separated by thin-layer or column chromatography, the compounds were well resolved by GLC and thus amenable to identification by GLC (four systems) and GC-MS.

Identification of 4α,14α-Dimenthyl Sterols and 24-Methylene Cycloartanol

Fraction one from alumina column chromatography contained very small amounts of 24-methylenecycloartanol although no cycloartenol was detected. Fraction two contained the two sterols, 4α,14α-dimenthyl-(24S)-5A-ergost-

Figure 1 - Separation of Chlorella ellipsoidea sterols using alumina and $AgNO_3$-silica gel chromatography.

5-A


Digitonin Precipitated Sterols
Alumina Column Chromatography of free sterols
4,4', 14-trimethyl sterols (Fraction 1)
(20% and 30% - ether in n-hexane)
4,14-dimethyl sterols and 4-monomethyl sterols
(40% ether in n-hexane)
Desmethylsterols
(50-90% ether in n-hexane)
91% of total sterol
9% of total sterol
AgNO3-Column Chromatography of the acetates
4,14-dimethyl sterols (Fraction 2)
(4% ether in n-hexane)
8(9)-monoene sterols (Fraction 5)
(5% ether in n-hexane)
4-methyl monoene sterols (Fraction 3)
(5% ether in n-hexane)
5(6)-monoene sterols and diene sterols (Fraction 6)
(100% ether)
4-methyl diene sterols (Fraction 4)
(12-15% ether in n-hexane)

8(9)-en-3β-ol (dihydroobtusifoliol) and 4α,14α-dimenthyl-(24S)-5α-stigmast-8(9)-en-3β-ol. Identification of these sterols was achieved principally on the basis of relative retention data from four GC systems which showed observed values to be indistinguishable from those reported by Doyle et al. (10). Evidence for a C-4 methyl substituent is provided by the behavior of the sterols on alumina chromatography and differences in behavior of the free sterols and sterol acetates on SE-30(11). GC-MS analyses of these sterols also confirmed the above identifications.

Identification of 4α-Methyl $\Delta^{8(9)}$- Sterols

Identification of the sterols in fraction three as 4α-methyl-(24S)-5α-ergost-8(9)-en-3β-ol and 4α-methyl-(24S)-5α-stigmast-8(9)-en-3β-ol is based on their movement on the alumina and $AgnO_3$- silica gel columns, as well as the very close correlation of the observed and calculated RRTs in the four GC systems (Table 1). The presence of a methyl at C-4 is demonstrated by the free sterol having a higher RRT on SE-30 than the corresponding sterol acetate (11).

To our knowledge, this is the first reported occurrence of the identification of these two sterols from natural sources, although Anding, et al. (12) have recently isolated an "undefined methyl sterol" which they feel may have the structure, 4α-methyl-ergost-8(9)-en-3β-ol.

Identification of 4α-Methyl Dienols

The GC-MS of the two sterols of fraction four provided evidence for a methyl at C-4. The spectra are identical below m/e 285, and differ only by 14 mass units above m/e 285 indicating the presence of homologs containing a methyl and an ethyl, respectively at C-24 (Figures 2,3). The parent ion peaks were at m/e 412 and 426: both have strong fragmentations at m/e 285, indicating loss of a saturated side chain from a diunsaturated nucleus con-

2

TABLE I

Relative retention times of sterols isolated from control or AY-9944-inhibited cultures of Chlorella ellipsoidea

Sterol Acetates	Relative retention times on 4 GC systems[a]				
	SE-30[b]	SE-30[b,f]	QF-1[c]	Hi-Eff-8BP[d]	PMPE[e]
brassicasterol	1.12[h]	1.12	1.09	----[g]	----
Δ^5-ergostenol	1.29	1.29	1.29	1.32	1.29
poriferasterol	1.42	1.42	1.32	1.34	1.31
clionasterol	1.63	1.63	1.56	----	----
cholesterol	1.00	1.00	1.00	----	----
$\Delta^{8,14}$-ergostadienol	1.32	1.32	1.24	1.44	1.38
24S-$\Delta^{8,14}$-stigmastadienol	1.66	1.66	1.48	1.74	1.65
$\Delta^{8(9)}$-ergostenol	1.35	1.35	1.29	1.33	1.38
24S-$\Delta^{8(9)}$-stigmastenol	1.69	1.69	1.56	1.59	1.65
4α-methyl $\Delta^{8,14}$-ergostadienol	1.50	1.54	1.38	1.54	1.42
4α-methyl 24S- $\Delta^{8,14}$-stigmastadienol	1.87	1.93	1.67	1.87	1.69
4α-methyl $\Delta^{8(9)}$-ergostenol	1.55	1.59	1.46	1.44	1.40
4α-methyl $\Delta^{8(9)}$-stigmastenol	1.92	1.97	1.74	1.69	1.66
24-dihydroobtusifoliol	1.51	1.54	1.55	1.28	1.21
4α,14α-dimethyl 24S-Δ^{8}-stigmastenol	1.87	1.93	1.85	1.53	1.44
24-methylene cycloartanol	2.02	2.12	2.22	----	----

[a]Relative to cholesterol acetate.

[b]Column 1.8m x 3.4mm I.D., 3% SE-30 on 100-120 mesh Gas Chrom Q, 20 p.s.i., 244 C.

[c]Column 1.8m x 3.4mm I.D., 1% QF-1 on 100-120 mesh Gas Chrom Q, 25 p.s.i., 231 C.

[d]Column 1.8m x 3.4mm I.D., 3% Hi-Eff-8BP on 100-120 mesh Gas Chrom Q, 25 p.s.i., 238 C.

[e]Column 1.8m x 3.4mm I.D., 2% PMPE on 100-120 mesh Gas Chrom Q, 20 p.s.i., 250 C.

[f]RRT is expressed as free sterol relative to free cholesterol.

[g]Data not obtained due to unresolved mixture on these columns.

[h]In all cases, actual relative retention times, as given, are essentially identical to calculated relative retention times.

Figure 2 - MS of 4α-methyl-5α ergosta-8(9), 14-dien-3β-ol.

8A

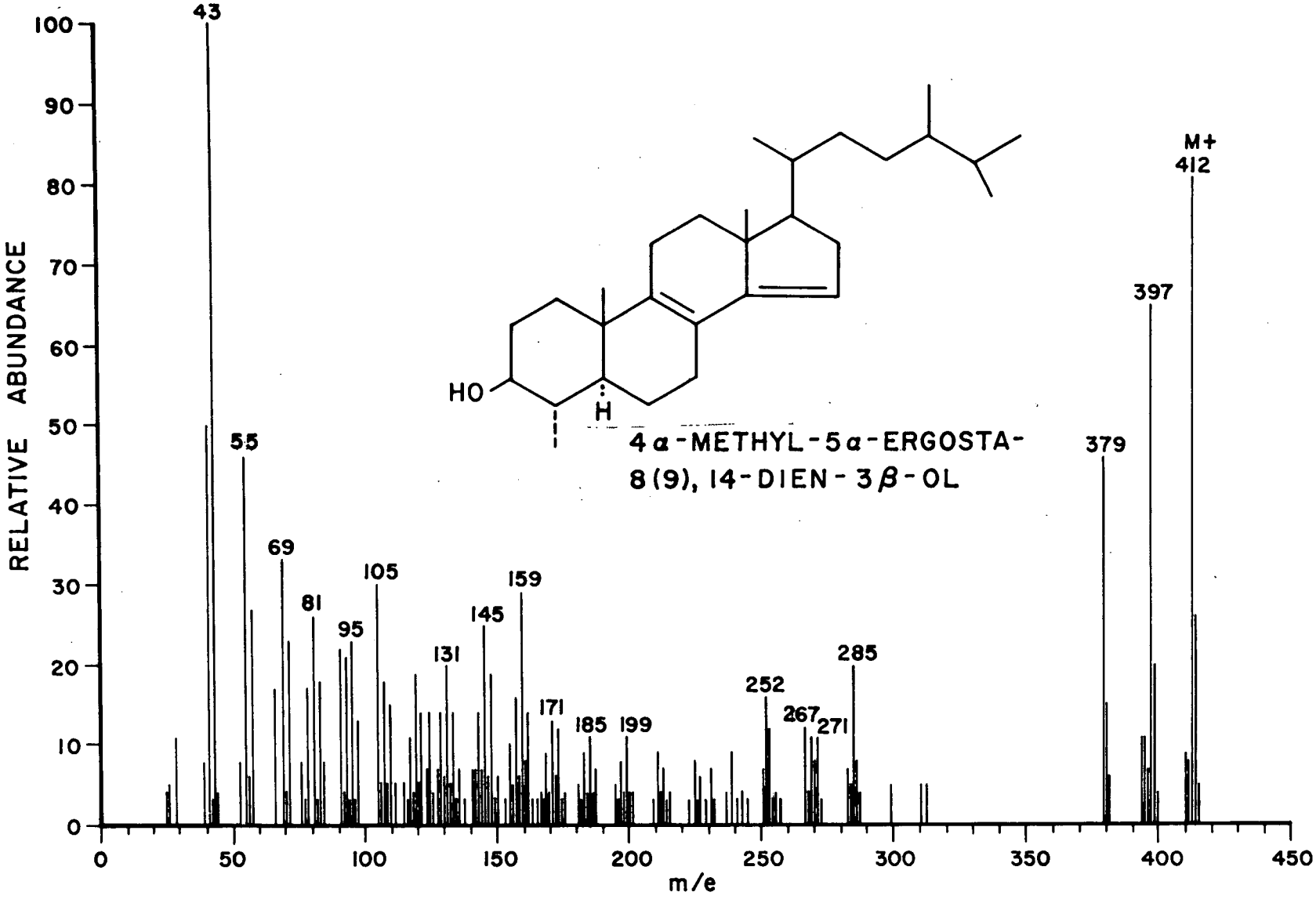
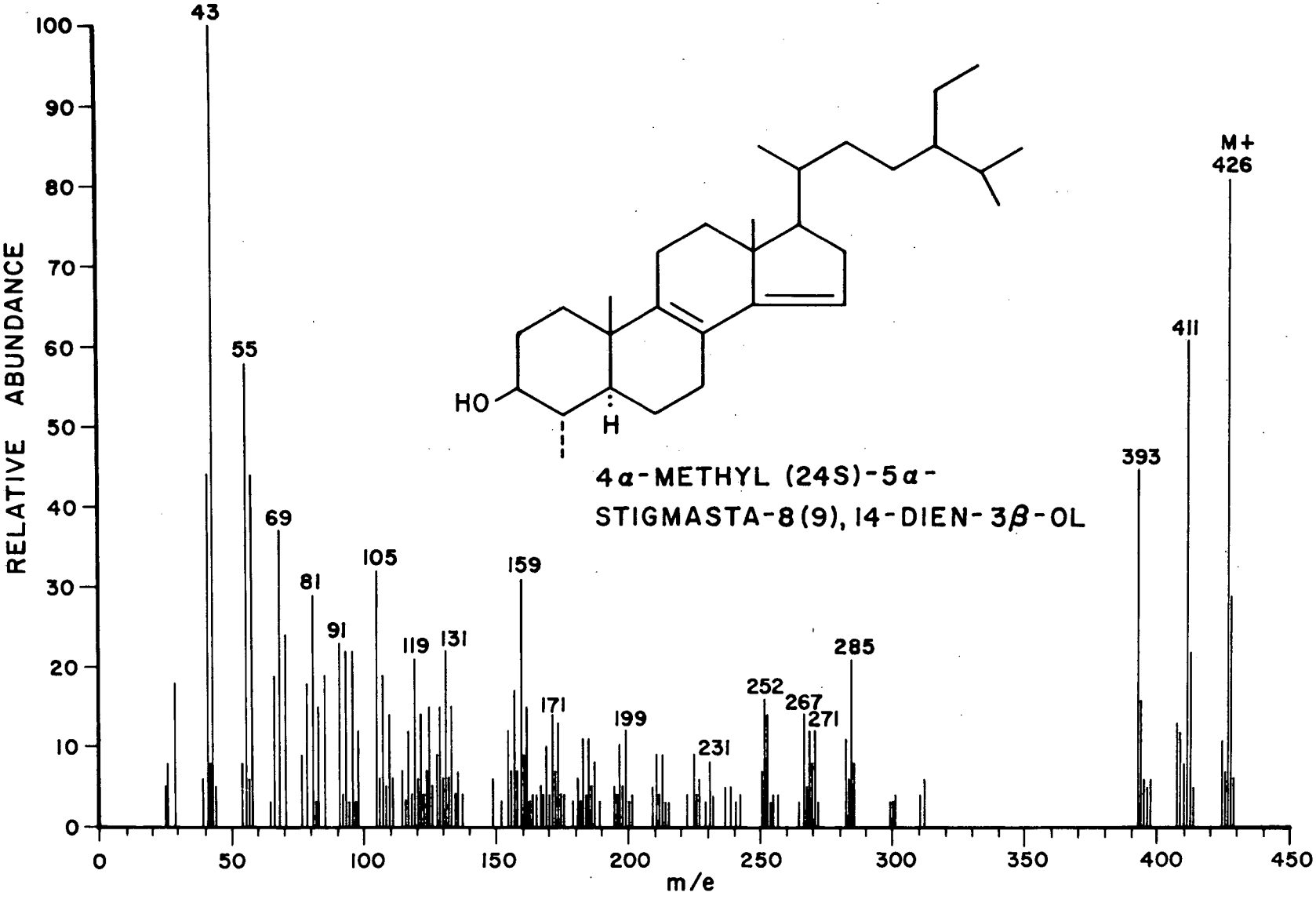

RELATIVE ABUNDANCE
m/e
43
55
69
81
95
105
131
145
159
171
185
199
252
267
271
285
379
397
M+
412
HO
H
4α-METHYL-5α-ERGOSTA-
8(9), 14-DIEN-3β-OL

Figure 3 - MS of 4α-methyl (24S)-5α-stigmasta-8(9),14-dien-3β-ol.

9-A


RELATIVE ABUNDANCE
m/e
43
55
69
81
91
105
119
131
159
171
199
231
252
267
271
285
393
411
M+
426
HO
H
4α-METHYL (24S)-5α-
STIGMASTA-8(9), 14-DIEN-3β-OL

taining an additional methyl. A strong peak is seen at m/e 252, which apparently corresponds to loss of the side chain and part of ring D, since this fragmentation is also seen at m/e 238 in $\Delta^{8,14}$-cholestadienol, $\Delta^{8,14}$-ergostadienol and (24S)$\Delta^{8,14}$-stigmastadienol and appears to be a characteristic of sterols with this double bond system. The saturated nature of the sterol side chain, the methyl at C-4, and the placement of the double bonds at the 8 and 14 positions were verified by GLC (Table 1). A decrease in RRT of the acetate compared to the free sterol is evidence of a methyl group at C-4. Actual RRT values were identical to those calculated by the method of Clayton (14), thus confirming the MS analyses and identifying the sterols of fraction four as 4α-methyl-(24S)-5α-ergosta-8,14-dien-3β-ol and 4α-methyl-(24S)-5α-stigmasta-8,14-dien-3β-ol. An ultraviolet absorption spectrum of a mixture of these sterols gave the expected absorption maximum at 251 nm.

Identification of Desmethyl Sterols

The two sterols of fraction five, comprising 19 percent of the total sterols (Table II), are identified as (24S)-5α-ergost-8(9)-en-3β-ol and (24S)-5α-stigmast-8(9)-en-3β-ol. The mass spectra show molecular weights of 400 ($C_{28}H_{48}O$) and 414 ($C_{29}H_{50}O$), respectively. As seen in Figure 4, the C-28 homolog has a fragmentation peak at m/e 273--suggesting a saturated side chain. The placement of the double bond at the $\Delta^{8(9)}$-position is done on the basis of the IR spectrum, mass spectrum and GLC retention data obtained from a mixture of these two sterols. The IR spectrum lacked bands characteristic of disubstituted and trisubstituted double bonds. The only points on a sterol structure where a tetrasubstituted double bond could occur are at the 8(9), 8(14) and 24(25) positions (providing an alkyl substituent is present at C-24). These are easily distinguishable on the four GLC columns used in this research. The optical rotation (free sterol $[\alpha]_D^{23}$ + 39; acetate,

TABLE II

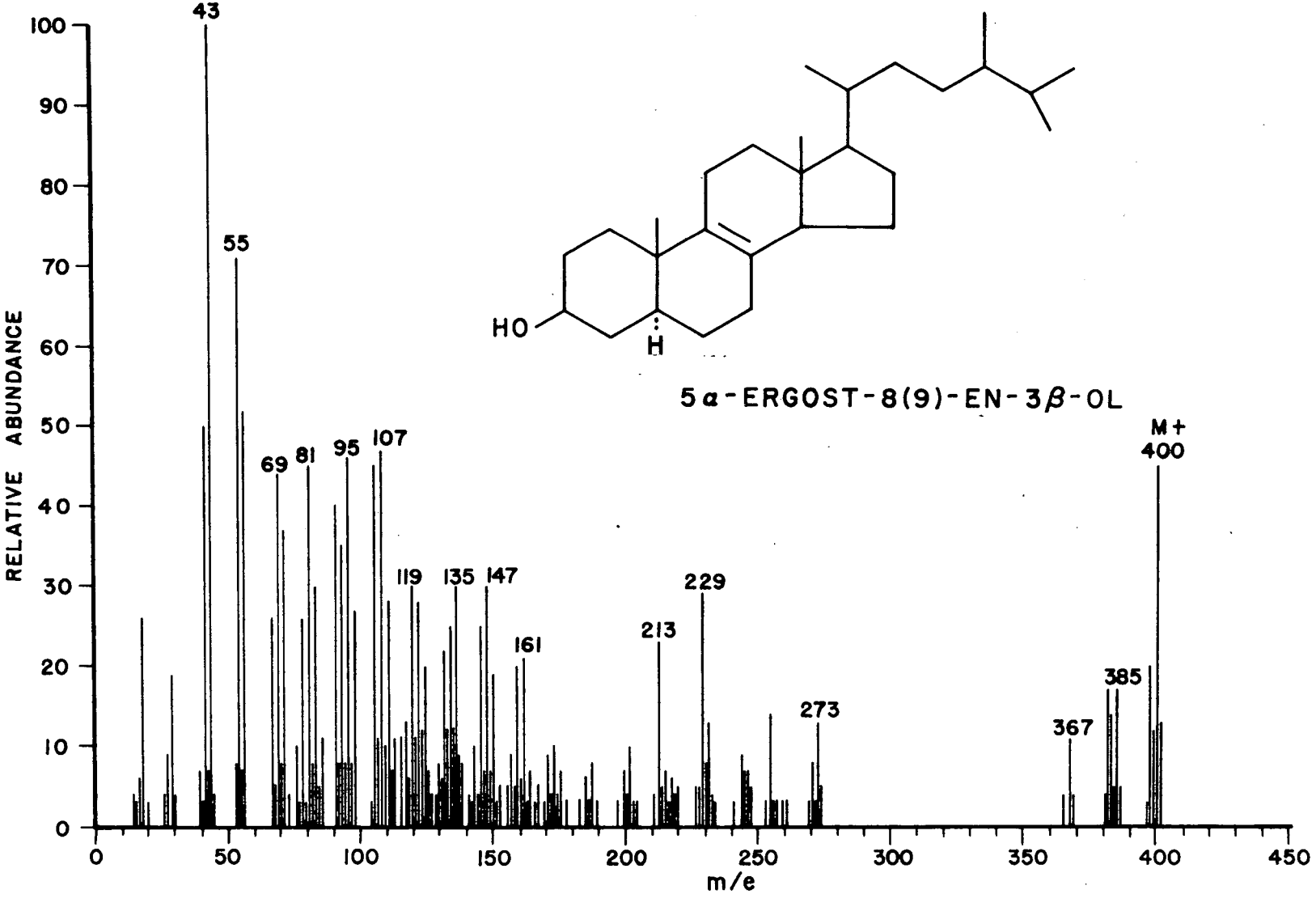
A quantitative comparison of sterols from control and AY-9944-treated cultures of Chlorella ellipsoidea

Sterols	Control		AY-9944-treated	
	% of sample	µg/g DW*	% of sample	µg/g DW*
brassicasterol	5.7	181	0.0	0
Δ^5-ergostenol	21.9	695	0.4	13
poriferasterol	65.6	2065	1.0	30
clionasterol	6.8	216	0.1	3
cholesterol	0.0	0	0.1	2
$\Delta^{8,14}$-ergostadienol	0.0	0	26.4	831
24S-$\Delta^{8,14}$-stigmastadienol	0.0	0	43.2	1371
$\Delta^{8(9)}$-ergostenol	0.0	0	6.1	193
24S-$\Delta^{8(9)}$-stigmastenol	0.0	0	13.2	415
4α-methyl $\Delta^{8,14}$-ergostadienol	0.0	0	4.0	126
4α-methyl 24S $\Delta^{8,14}$-stigmastadienol	0.0	0	3.1	97
4α-methyl $\Delta^{8(9)}$-ergostenol	0.0	0	0.4	13
4α-methyl 24S- $\Delta^{8(9)}$-stigmastenol	0.0	0	0.3	10
24-dihydroobtusifoliol	0.0	0	0.5	17
4α,14α-dimethyl 24S-$\Delta^{8(9)}$-stigmastenol	0.0	0	1.0	30
24-methylene cycloartanol	t**	t	0.1	2
Total	100.0	3157	99.9	3153

*DW = Dry Weight

**t = trace; indicates less than 1 µg/g DW.

Figure 4 - MS of 5α-ergost-8(9)-en-3β-ol.


43
55
69
81
95
107
119
135
147
161
213
229
273
367
385
M+
400
HO
H
5α-ERGOST-8(9)-EN-3β-OL
RELATIVE ABUNDANCE
m/e
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450

$[\alpha]_D^{23}$+ 28) data of this mixture are also within the expected range for $\Delta^{8(9)}$-sterols (13). Except for an increase in 14 mass units, the spectrum (Figure 5) of the stigmastenol compound of fraction five is identical to that of its C-28 homolog. In addition to MS, direct evidence for the proposed structures comes from a comparison of calculated to actual RRT values on the four GC systems. On all systems the calculated values are within 1% of the actual values. These data allow identification of the sterols of fraction five as $\Delta^{8(9)}$-sterols and collectively exclude all other possibilities.

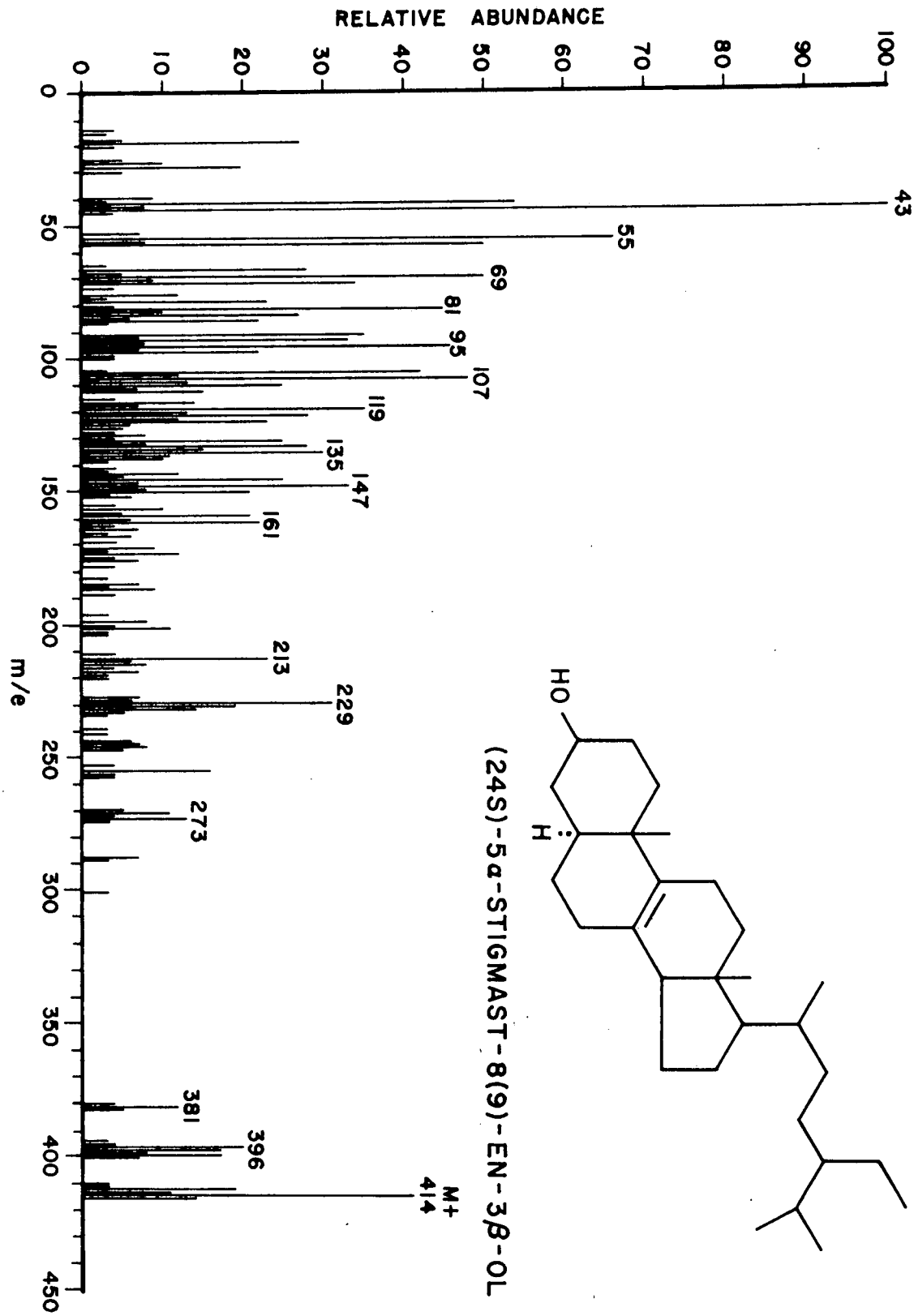
Two of the sterols of fraction six have been previously identified as (24S)-5α-ergosta-8,14-dien-3β-ol and (24S)-5α-stigmasta-8,14-dien-3β-ol (7). These two compounds comprised 69 percent of the total sterols isolated from the treated cultures (Table II).

Extremely small amounts (Table II) of Δ^5-ergostenol, poriferasterol and clionasterol--the predominant sterols of control cultures of C. ellipsoidea (15)--were detected in AY-9944-grown cultures. All had relative retention times on GC which matched authentic standards. GC/MS analysis of poriferasterol and Δ^5-ergostenol confirmed the identification of these sterols. All sterols identified in this work are assumed to have one 24S configuration since the "normal" sterols of this organism have been established to have the 24S configuration (15,16).

Mass spectrographic analysis and GC retention data confirmed the presence of small amounts of cholesterol and brassicasterol in the AY-9944 inhibited cultures. This is the first reported isolation of cholesterol from Chlorella, although its occurrence in Oocystis (17), Ulva, and Chaetomorpha (18) has been reported.

As seen in the quantitative comparison of sterols from control and AY-9944-treated cultures (Table 1), AY-9944 has a drastic effect on sterol

Figure 5 - MS of (24S)-5α-stigmast-8(9)-en-3β-ol.


RELATIVE ABUNDANCE
0
10
20
30
40
50
60
70
80
90
100
m/e
0
50
100
150
200
250
300
350
400
450
43
55
69
81
95
107
119
135
147
161
213
229
273
381
396
M+
414
HO
H
(24S)-5α-STIGMAST-8(9)-EN-3β-OL

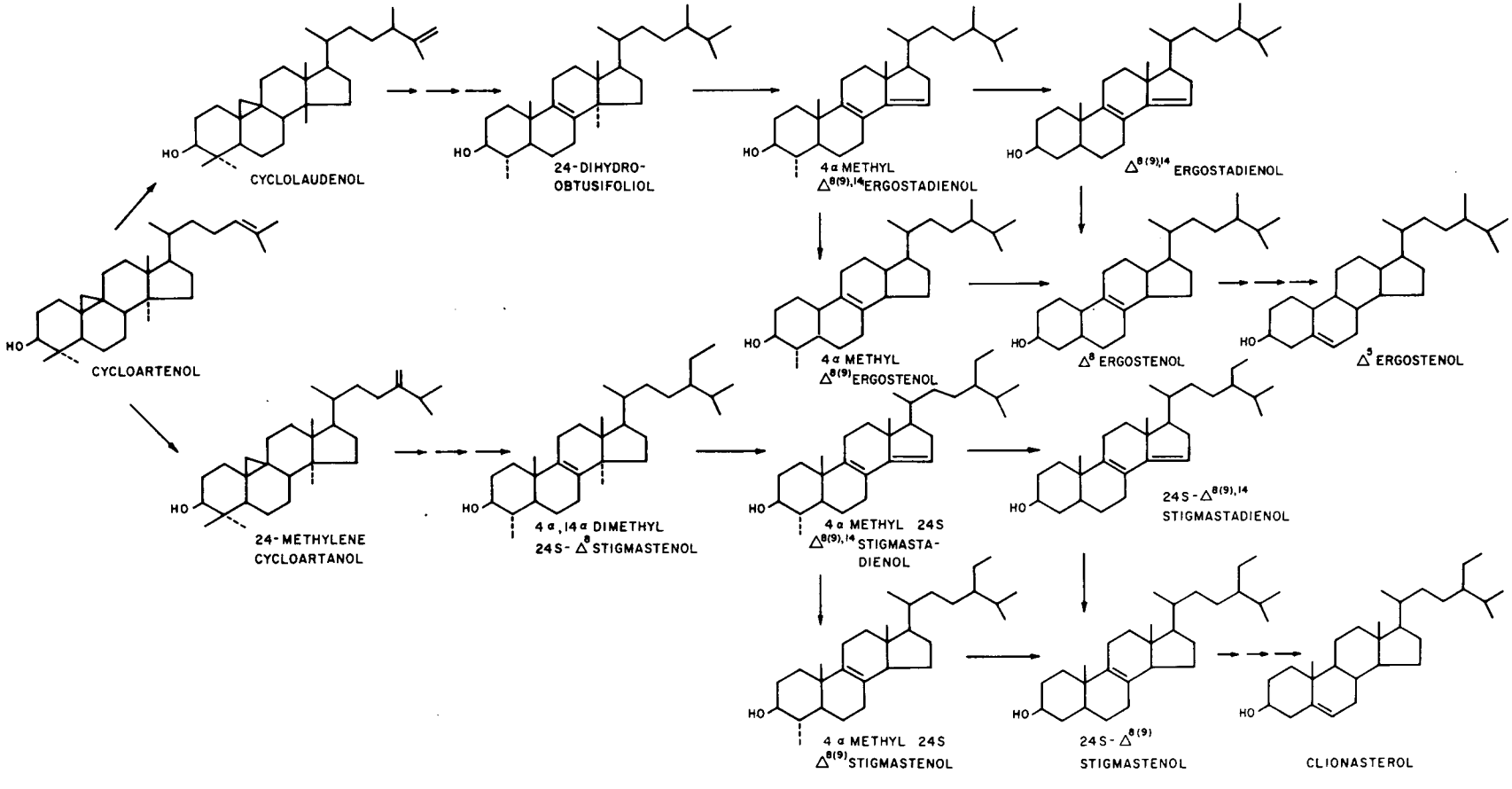
composition which apparently having no influence on sterol production (3.2 mg/g DW in control and inhibited cultures). AY-9944 is virtually a 100 percent-effective inhibitor of Δ^5-sterol biosynthesis in C. ellipsoidea. The total of 5 mg of Δ^5-sterols in treated cultures is calculated to approximate the amount of these sterols which were present in the untreated inoculum used to start the treated cultures.

Instead of the predicted accumulation of the immediate precursors to Δ^5 sterols (i.e., $\Delta^{5,7}$ and Δ^7 sterols), AY-9944 brought about an almost exclusive accumulation of $\Delta^{8(9)}$ and $\Delta^{8,14}$ sterols. An inhibition of the reduction of the Δ^{14} double bond, and an inhibition in the $\Delta^8 \rightarrow \Delta^7$ isomerase is clearly suggested. It is certainly possible to state with confidence that AY-9944 must be more than a Δ^7-reductase inhibitor in plants. It is also clear that any speculation concerning sites of AY-9944 inhibition must be accompanied by a postulated pathway involving the sterols which have accumulated. Accordingly, the sterols described above are included in a proposed scheme for Δ^5-ergostenol and clionasterol biosynthesis, although other reactions are possible (Figure 6). Although no cycloartenol and cycloläudenol were detected in C. ellipsoidea, inclusion of these sterols in the scheme reflects the current thought on sterol biosynthesis in plants. All other sterols in this scheme were found in C. ellipsoidea.

Strong support for such a sequence is taken from the substantial recent literature concerning the occurrence of $\Delta^{8,14}$-sterol dienes in biological material, and their suspected roles as intermediates in sterol biosynthesis. Frost and Ward (19) isolated 5α-stigmasta-8,14 24(28)-trien-3β-ol from Vernonia seed oil. Canonica, et al. (20) Akhtar, et al. (21) and Watkinson, et al. (22) have shown conclusively that cholesta-8,14-dien-3β-ol is converted to cholesterol in rat liver homogenate enzyme system. Akhtar, et al.

Figure 6 - A proposed pathway of sterol biosynthesis in *Chlorella ellipsoidea*.

16 - A


HO
CYCLOLAUDENOL
24-DIHYDRO-
OBTUSIFOLIOL
4α METHYL
Δ8(9),14ERGOSTADIENOL
Δ8(9),14 ERGOSTADIENOL
CYCLOARTENOL
4α METHYL
Δ8(9)ERGOSTENOL
Δ8 ERGOSTENOL
Δ5 ERGOSTENOL
24-METHYLENE
CYCLOARTANOL
4α,14α DIMETHYL
24S-Δ8STIGMASTENOL
4α METHYL 24S
Δ8(9),14STIGMASTA-
DIENOL
24S-Δ8(9),14
STIGMASTADIENOL
4α METHYL 24S
Δ8(9)STIGMASTENOL
24S-Δ8(9)
STIGMASTENOL
CLIONASTEROL

(23) and Canonica, et al. (24) have shown that the loss of a methyl at C-14 is accompanied by a simultaneous oxidation at C-15.

Assuming such a pathway exists in plants (Chlorella in particular), then the two suggested sites of inhibition just mentioned, the inhibition of the Δ^{14}-reductase step, and interruption of the $\Delta^{8}\rightarrow\Delta^{7}$-isomerase step, should again be considered. A choice of the former as the primary site of inhibition is preferable when one considers that 77 percent of the accumulated sterols are $\Delta^{8,14}$-sterols and that a reduction of the 14(15) double bond is likely to require NADPH and be similar in other respects to the Δ^{7}-reductase system reported in animals. An accumulation of a $\Delta^{8,14}$-sterol has also recently been observed as a result of AY-9944 treatment in an animal system (25).

However, the accumulation of the $\Delta^{8(9)}$-sterol monoenes (21 percent of accumulated sterols), which appear after the sterol dienes in the postulated sequence, indicates an additional inhibition at the Δ^{8}-Δ^{7}-isomerase step by AY-9944. Alternatively, if AY-9944 inhibits only the isomerase reaction, then the equilibrium of the previous reaction (reduction of the C-14 double bond) must be strongly in favor of the sterol diene.

From Table II it is observed that the inhibited cultures produced practically no Δ^{22}-sterols, while 77 percent of the total sterol of control cultures had a 22(23) double bond. Either AY-9944 has a secondary effect in preventing insertion of this double bond, or the introduction of the 22,23 double bond may not normally occur until later in the pathway.

Dealkylation of the methyl groups on the sterol nucleus in C. ellipsoidea appears to proceed as in higher plants, i.e., the 4α-methyl is the last group to be removed. However, in C. emersonii (sterols are Δ^{7}), Doyle, et al. (9) found that the 14Δ-methyl was removed last. Thus, sterol composition and the

pathway of biosynthesis of sterols in Chlorella ellipsoidea is much more similar to those of higher plants than is the case with Chlorella emersonii.

ACKNOWLEDGMENT

The authors thank S. R. Dutky and M. J. Thompson of ARS, USDA for their interest in this work as well as aid with mass spectra and numerous helpful suggestions. We also thank Ayerst Research Laboratories for a generous supply of AY-9944. This work was supported in part by Grant #NGR-21-002-003 from the National Aeronautics and Space Administration.

LITERATURE CITED

1. Chappel, C., J. Dubuc, D. Dvornik, M. Givner, L. Humber, M. Kraml, K. Voith, and R. Gaudry. Nature 201, 497 (1964).

2. Dempsey, M. Ann. New York Acad. Sci. 148, 631 (1968).

3. Dvornik, D., M. Kraml, J. Dubuc, M. Givner, and R. Gaudry. J. Amer. Chem. Soc. 85, 3309 (1963).

4. Dvornik, D., M. Draml, and J. Bagli. J. Amer. Chem. Soc. 86, 2739 (1964).

5. Kraml, M., J. Bagli, and D. Dvornik. Biochem. Biophys. Res. Comm. 15, 455 (1964).

6. Dickson, L. G., G. W. Patterson. Plant Physiol. (Suppl.) 47 p. 22 (1971).

7. Dickson, L. G., G. W. Patterson, C. F. Cohen, and S. R. Dutky. Phytochemistry, in press.

8. Dickson, L. G. Ph.D. Thesis, University of Maryland, 1971.

9. Doyle, P. J., G. W. Patterson, S. R. Dutky, and C. F. Cohen. Phytochem. 10, 2093-2098 (1971).

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

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

12. Anding, C., R. D. Brandt, and G. Ourisson. Eur. J. Biochem. 24, 259 (1971).

13. Bergmann, W. Ann. Rev. Plant Physiol. 4, 383 (1953).

14. Clayton, R. B. Biochem. 1, 357 (1962).

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

16. Thompson, M. J., S. R. Dutky, G. W. Patterson, and E. L. Gooden. Phytochemistry 11, 1781 (1972)

17. Orcutt, D. and B. Richardson. Steroids 16, 429 (1970).

18. Ikekawa, N., N. Morisaki, K. Tsuda, and T. Yoshida. Steroids 12, 41 (1968).

19. Frost, D. J. and J. P. Ward. Rec. Trav. Chim. 89, 1054 (1970).

20. Canonica, L., A. Fiecchi, M. Kienle, A. Scala, G. Galli, E. Paoletti, and R. Paoletti, J. Amer. Chem. Soc. 90, 6532 (1968a)

21. Akhtar, M., I. Watkinson, A. Rahimutula, D. Wilton, K. Munday, Biochem. J. III, 757 (1969).

22. Watkinson, I., D. C. Wilton, K. Munday, and M. Akhtar. Biochem. J. 121, 131 (1971).

23. Akhtar, M., I. Watkinson, A. Rahimtula, D. Wilton, and K. Munday, Chem. Commun. 1406 (1968).

24. Canonica, L., A. Fiecchi, M. Kienle, A. Scala, G. Galli, E. Paoletti, and R. Paoletti. J. Am. Chem. Soc. 90, 3597 (19686).

25. Schroepfer, G. J., Jr., B. N. Lutsky, J. A. Martin, S. Huntoon, B. Rourcans, W. H. Lee and J. Vermillion. Proc. Royal Soc. Lond. B. 180, 125-146 (1972).