EORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

[X] ORIGINAL [ ] REVISION NO. _____

Project No. G-32-682 DATE 7/26/82

Project Director: Dr. Thomas G. Tornabene School/~~Lab~~ Applied Biology

Sponsor: Midwest Research Institute of the Solar Energy Research Institute Division (SERI)

Type Agreement: Subcontract No. XK-2-02149-01 under DOE Prime No. EG-77-C-01-4042

Award Period: From 6/21/82 To ~~8/19/83~~ 9/30/83 (Performance) 8/19/83 (Reports)

Sponsor Amount: $236,077 Contracted through:

Cost Sharing: _____ GTRI/~~EXT~~

Title: Chemical Profiles of Microalgae, with Emphasis on Lipids

ADMINISTRATIVE DATA OCA Contact Faith G. Costello x-4820

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Defense Priority Rating: N/A Security Classification: (See below) *

RESTRICTIONS

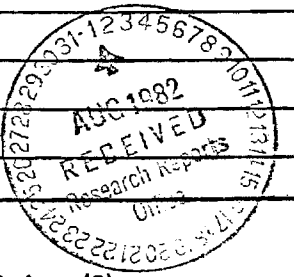
See Attached DOE Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval — Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with Government. Advance notification is required for th e purchase of any items over $1,000 except those specified on page 2, Article 4 of Subcontract Schedule.

COMMENTS:

* Publicity releases of any nature in connection with this Subcontract require prior review and approval of the SERI Public Information Office.



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ORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 11/17/83

oject No. G-32-682 School/~~XXX~~ Applied Biology

ıcludes Subproject No.(s) N/A

roject Director(s) Dr. T.G. Tornabene GTRI / ~~GIT~~ ~~XXX~~

Sponsor SERI

Title "Chemical Profiles of Microalgae, with Emphasis on Lipids"

Effective Completion Date: 9/30/83 (Performance) 9/30/83 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
- [X] Final Invoice or Final Fiscal Report
- [ ] Closing Documents
- [X] Final Report of Inventions
- [X] ~~Govt.~~ Property Inventory & Related Certificate
- [ ] Classified Material Certificate
- [ ] Other

Continues Project No. Continued by Project No. G-32-605

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Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Oct. 15, 1982 - Nov. 15, 1982

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

Nov. 14, 1982

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards identifying algae that have the highest lipid producing potential.

Progress Report:

Cultivation of Dunaliella, Isochrysis and Botryococcus are in progress. Isochrysis has been grown in nitrogen rich and nitrogen deficient media. Total lipid protein, carbohydrate and ash compositions were determined. A marginal shift towards a higher total lipid production was observed. A substantial shift towards increased neutral lipid production did occur, however, demonstrating significant alteration in the relative intensities of the individual lipids. The individual lipids have now been purified by standard chromatographic procedures. The procedures for identifying the components are in progress.

Two kilograms each of nitrogen fed and starved Gracilaria was received from John Ryther. They are being lypophilized at the present time. Multiple extracts will be made on each of the cells to characterize the protein, carbohydrate and protein productions. We are currently trying to establish some simplified screening procedure for evaluating the pharmacological properties of each extract (this is still in the design stage). The lipid and carbohydrate analyses will begin as soon as the algae are dried, ashed and quantitated.

Lipopolysaccharide (Endotoxins) were isolated from three different strains of blue-green algae, microcystis, aerugines, NRC-1, LIV-006 and LIV 027. They were purified by CSCL gradient ultracentrifugation. The chemical composition of these endotoxins was determined. These endotoxins consist of about 28 to 34% total carbohydrates. They consist of typical endotoxin sugar 2-Keto-2-deoxyoctonate (KDO), colitose, glucosamine, phosphate, etc. Lipid A moiety and carbohydrate moiety of these LPS preparations were cleaved by 1% acetic acid hydrolysis and their individual compositions determined. The polysaccharide moiety is being examined in detail by GLC. Polysaccharide and lipid A moeities are being further separated into different fractions by Sephadex Column Chromatography for structural investigations. Toxicity in two strains of mice with LPS and lipid A are being tested. Limulus lysate gelation assay for endotoxin potential is also being examined. Studies are also planned for effect of these LPS and lipid A preparations on neutropenia in mice, etc.

Total lipids were extracted by Bligh and Dyer procedure. They were separated by using Biosil A and Florisil column into polar (phospholipids) and neutral lipids. Total neutral and polar lipids are being identified.

This study with the blue-green algae are being conducted because so little is known about these fast growing bacterial-like algae and they produce substantial quantities of highly potent toxins. We are using these algae in an attempt to establish screening systems for measuring algal toxins. We feel that this is an important area since many of the

algal systems being examined for lipid (oil) production are also intended for feed stock.

I had attempted to grow Botryococcus in 1962 with only limited success. Dr. Ben-Amotz and I are again attempting to uncover the mystery why the alga grows so slowly under controlled conditions. There is actually little time spent on this system; however, if we could, by chance, improve the replication rate, the effort is well worth it since this alga can produce up to 80% of its weight in hydrocarbon oils. There is some hope for success, at least, on my part, since Dr. Ben-Amotz, is one of the best men in the world for conducting such studies.

Chemical Profiles of Microalgae with Emphasis on Lipids

Progress Report

for period July 15, 1982 - August 15, 1982

Thomas G. Tornabene

School of Applied Biology
Georgia Institute of Technology
Atlanta, Georgia 30332

August 15, 1982

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose

The research is directed towards identifying photosynthetic algae that have the highest lipid producing potential.

Progress Report

The last four weeks has been spent on getting the lab in full operation. This constituted the preparation of standards, chomatographic columns, and standardization of analytical equipment. Four different algal species have been cultivated and extracted for total lipids, bound-lipids and carbohydrates. The test conducted on 3 of the algal strains were cesium gradient profiles of carbohydrates, Limmulus test for toxicity, hydrolyses and separation of carbohydrate and bound-lipids and separation of neutral pigments from the total lipid extracts. The extraction and analyses of the lipid content of a Dunaliella sp. is in its initial stages.

The desert algae Dunaliella bardawil, D. salina and Isochrysis galbara are being cultivated in artifical hypersaline medium under controlled conditions of light intensity, temperature and shaking. Attempts are being carried out to propagate the algae to high density (10^7 cells/ml) under conditions optimized for maximal lipid production and for other valuable biological products. Preliminary experiments are directed to determine baseline data for each species of a common denominator to include, nutrient requirement, specific growth rate, pigment content, and proximate chemistry. More species of desert algae have been collected or ordered and they are on the way to the School of Applied Biology, Georgia Tech. It is hoped that one or more species of these algae will provide valuable biomass source that is (are) rich in high yields of lipids.

The preparation of course manual is underway; it is expected to be completed by mid-September.

Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Aug. 15, 1982 - Sept. 15, 1982

Thomas G. Tornabene

School of Applied Biology
Georgia Institute of Technology
Atlanta, Georgia 30332

Sept. 14, 1982

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose

The research is directed towards identifying photosynthetic algae that have the highest lipid producing potential.

Progress Report

The course manual for training technicians in lipidology is with the typist. Corrected copies should be sent to Larry Raymond and staff in a week or two.

Lipid characterization of Dunaliella and Isochrysis is underway. As cell accumulations are obtained for the various species, lipids characterizations will be initiated. At present we are cultivating continually two Dunaliella, and Isochrysis species under varying growth conditions.

The Isochrysis sp #2307 was obtained from the University of Texas culture collection (UTEX) as a marine species adapted to warm temperatures.

The alga was cultivated in 0.5 mM NaCl, 5 mM $FeCl_3$ 30 μM EDTA, 20 mM $NaHCO_2$, trace metal mix, vitamin mix and 20 mM Tris-Cl, pH 7.2.

Algae were grown in a temperature controlled growth room under illumination with cool white fluorescent lamps at 25°C with mixing.

Cells were counted with a hemacytometer. Chlorophyll and accessory pigments were extracted with DMSO and acetone (after Burnison B.K. 1980. Modified DMSO extraction for chlorophyll analysis of phyloplankton. Can. J. Fish. Aquat. Sci. 37: 725-733).

The algae multiplied with a doubling time of about 20 hrs. to a maximal cell concentration of 2 x $10^{>7}$ ml^{-1}. Maximal chl *a* and fucoxanthin levels were 20 μg ml^{-1} representing a fucoxanthin-to-chl *a* ratio of about 1.

Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Sept. 15, 1982 - Oct. 15, 1982

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

Oct. 14, 1982

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards identifying algae that have the highest lipid producing potential.

Progress Report:

The course manual for training technicians in lipidology is completed. We are ready to conduct the short course. SERI should pick the dates for the training of the personnel and to inform those individuals who are to attend. A final printing of the manual will be delayed until after the first training course. If any difficulties arise from the procedures presented, suitable corrections will be made and then a final version will be printed.

Lipid characterization of lipids of Dunaliella and Isochrysis is underway. It is still too early to attempt to delineate the composition of Isochrysis (we already have published the lipid identities of Dunaliella). Variations in growth conditions of Isochrysis and Dunaliella are in progress. Nutrient limitations and their effects on lipid synthesis are our principal efforts at this time. We are attempting to pinpoint the trigger for switching all cellular biosynthesis to predominantly lipid synthesis. Some progress has been made in understanding the growth parameters for maximizing β-carotene production.

Drs. Ben-Amotz and Tornabene presented seminars at a special symposium on aquatic species sponsored by the American Society for Microbiology on Oct. 1-3, 1982. This meeting was held at Jekyll Island, Georgia.

Dr. Ben-Amotz participated in a SERI sponsored site visit to New Mexico to evaluate programs at New Mexico State University.

Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Nov 15, 1982 - Dec. 15, 1982

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

Dec. 15, 1982

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards identifying algae that have the highest lipid producing potential.

Progress Report:

Lipid studies on Isochrysis and Dunaliella are continuing. Nitrogen limited cultures of Isochrysis contain a neutral lipid compound that was not readily seen in cells on adequate-N-containing media. The compound which is a major constituent in the lipids was purified. NMR and IR spectra were recorded on this compound. Mass spectral patterns will be obtained next week. The identity of the compound is yet unknown.

A short week short course on lipid chemistry was presented to 7 individuals in aquatic species program, Dec. 13-17, 1982.

Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Dec 15, 1982 - Jan 15, 1983

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

Jan 15, 1983

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards, identifying algae that have the highest lipid producing potentials.

Progress Report:

Algae are being cultivated under stress to force the cellular metabolisms from normal cell growth and reproduction to carbon storage (preferably lipid-carbon storage). We now have several "fresh water algae" (Isochrysis and Botryococcus) growing at sea-water salt concentrations and the halophile, Dunaliella, growing at extreme salt concentrations (4 M NaCl). It has taken several months to accomplish the algae adaptations to higher salt concentrations. These algae are about ready for harvesting and subsequent analytical evaluations.

Nitzshia, Gracillaria, Scenedesmus, Chlamydomonas, Isochrysis, Dunaliella and Botryococcus (at different growth parameters) are under investigation. The thin-layer profiles of acetone and methanol eluates collected from silicic acid columns are shown in xerox copies 1 and 2, respectively. The multiple component samples are now being identified. The components in the polar lipid fractions, however, are not particularly interesting. The polar components are relatively similar and typical of functional lipids in living microorganisms. The non-polar fractions are, in part, more diverse. The yield of hydrocarbons synthesized by the algae are given in Table 1. Substantial and significant differences are recorded between Isochrysis NE (nitrogen enriched) and Isochrysis D (nitrogen deficient). While the productivity of Isochrysis NE is only 0.5% of the organic dry weight, it is obvious that such cultivation procedures directly effect lipid biosynthesis. We are hopeful that other cultivation parameters will be more effective in changing the cellular carbon flow to lipid biosynthesis; the cultivation parameters being tested are age, nitrogen imbalance, temperature and salinity.

The nature of the hydrocarbons identified are relatively typical of algae. Gracillaria contains virtually all hetadecane; Scenesdesmus and Chlamydomonas contain principally heptadecene (monounsat.); Nitzshia and Dunaliella bardawil contain components that range from C_{13} - C_{30} with no predominant component.

HYDROCARBONS OF ALGAE

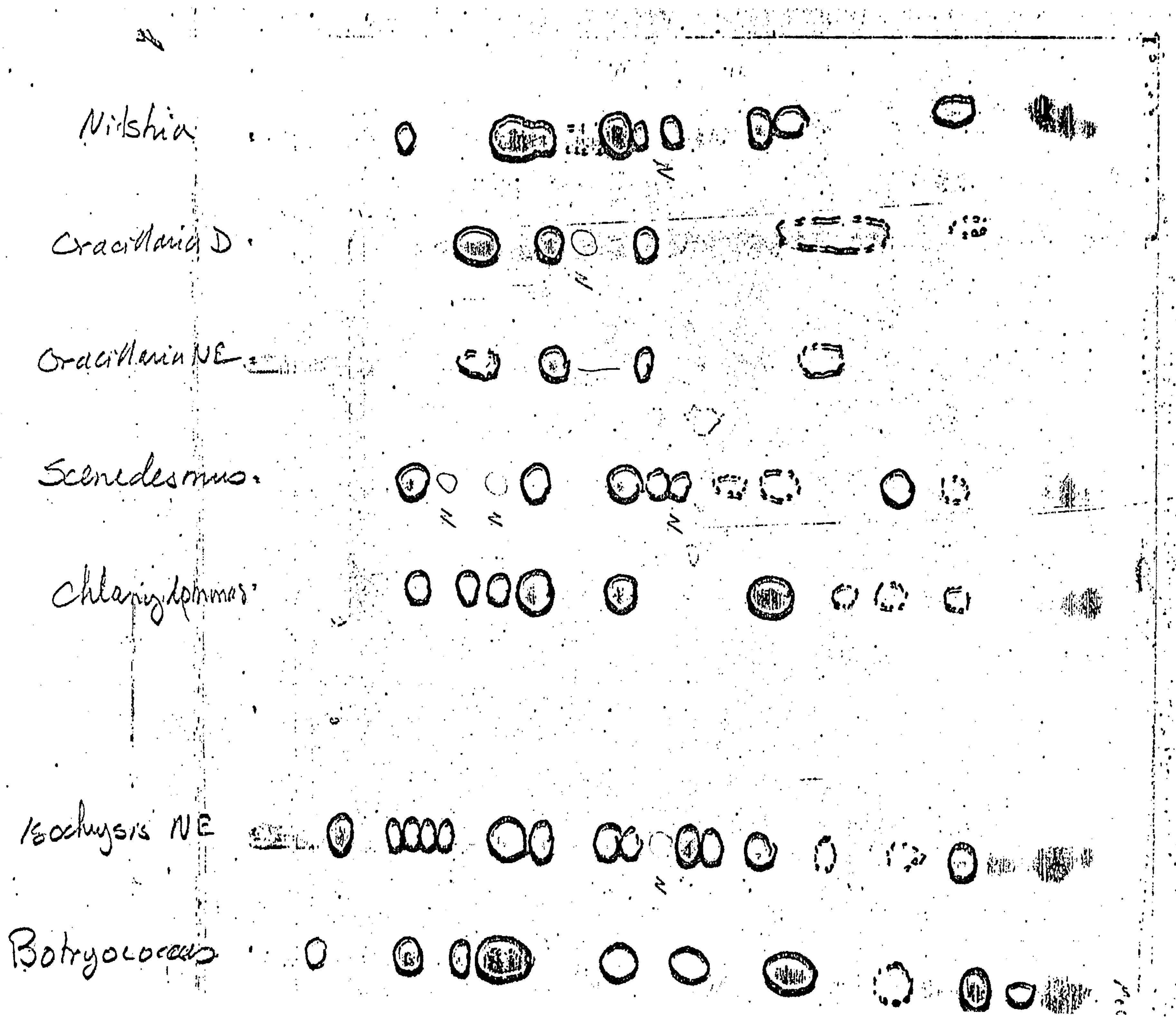
Organism	Hexane		Hydrocarbons	
	Total Dry wt(mg)	Total Org wt(mg)	wt(mg)	% Org wt.
Botryococcus brauni	390	?		
Dunaliella bardawil	330	278	0.464	0.166
Dunaliella salina	-	-		
Gracillaria NE	1472	?	1.129	(0.076)
Gracillarid D	2008	?	0.388	(0.019)
Isochrysis sp. NE	1975	1599	1.266	0.080
Isochrysis sp. D	920	470	2.32	0.494
Nitzschia sp	1168	?	3.25	0.000 ?
P202 Chlamydomonas	254	228	0.107	0.047
Scenedesmus	804	724	0.869	0.120

Iodine
comm plle

Acetone
eluate

MeOH eluate

Polar lipid c [illegible]
Iodine, Ninhydrin
Comm plate



Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period Jan 15, 1983 - Feb 15, 1983

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

Feb 15, 1983

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards, identifying algae that have the highest lipid producing potentials.

Progress Report:

This progress report is essentially a continuation of last months descriptive efforts. Algae are being cultivated under stress to force the cellular metabolisms from normal cell growth and reproduction to carbon storage (preferably lipid-carbon storage) and the lipid contents of Nitzshia, Gracillaria, Scenedesmus, Chlamydomonas, Isochrysis, Dunaliella and Botryococcus (at different growth parameters) are under investigation. We have been cleaning-up loose ends on chemical quantitations of lipid component in preparation of the paper to be presented in San Diego at the ASP Subcontractor's review. The data in relative detail will be formally presented. We are also attempting to completely identify the major oily neutral lipid in isochrysis that occurs in nitrogen deficient cells and which represents a major component of the organic cell dry weight. We have NMR, IR, UV, GLC and M.S. data. Compound reduction and derivatizations are being conducted in an attempt to pin-point the compounds identity. We hope to also unveil this compound at the San Diego meeting. I will also report on the chemical composition of Amacroalgae that does not produce significant lipid contents.

1. Chemical Profile of Algae with Emphasis on Lipids of Microalgae

(Progress Report to SERI, March 1983)

ABSTRACT

Analysis of proximate chemistry composition with emphasis on the neutral lipids was carried out as a comparative study among different algae. Total lipid content varied from a high of about 50% lipids/organic weight in Botryococcus, 26% in Isochrysis, 19% in Chlamydomonas P202, 18% in Dunaliella, 12% in Nitzschia, 9.3% in Scenedesmus to a low of 2.0% in Gracilaria. Nitrogen deficiency caused lipid accumulation in Isochrysis, lipid elimination in Gracilaria, and no effects on the lipid content of Dunaliella. The distribution of the lipid classes 60% neutral lipids and 40% polar lipids in Botryococcus; 30% neutral lipids and 70% polar lipids in Isochrysis; 15% neutral lipids and 85% polar lipids in Chlamydomonas P202; 10% neutral lipids and 90% polar lipids in Scenedesmus and about 2% neutral lipids and 98% polar lipids in Dunaliella salina, Gracilaria and Nitzschia. Dunaliella bardawil grown under the conditions of nitrogen deficiency accumulated about 50% neutral lipids most of it as β-carotene. Long chain aliphatic hydrocarbons (>C-30) have been identified in the hexane eluate of Botryococcus and Isochrysis. Medium chain aliphatic hydrocarbons (C-18 to C-20) have been identified in the hexane eluate of Nitzschia, and short chain aliphatic hydrocarbons ($\leq$ C-17) appeared in the hexane fractions of Chlamydomonas P202, Dunaliella, Gracilaria and Scenedesmus. The major component of the neutral lipid fraction in Isochrysis appeared in the benzene eluate and accounted for about 3% of the algal organic weight. This component has been isolated, purified and analyzed by TLC, MS, IR, and NMR.

INTRODUCTION

Lipids and fatty acids are ubiguitous to algae. There are significant differences, however, in quantity and nature of the lipids, in genera of algal classes as well as in strains of the same class [1]. Algal lipids construct a major part of the cellular membranes and they accumulate in many species as storage products. It is well documented

that ecological and physiological factors influence the chemical composition of algae. The lipid compositions are specifically responsive to a number of conditions. Some of these conditions can be controlled and manipulated experimentally. In this particular study our specific objective was to conduct lipid experiments that would challenge growth parameters of algae in an attempt to identify conditions that would alter the lipid chemistry of the algae toward higher production of neutral lipids.

MATERIALS AND METHODS

All methods were as previously described in the Aquatic Species Program for SERI Workshop held in December 1982 at the School of Applied Biology, Georgia Institute of Technology. Derivitized lipids were analyzed on Varian 3700 gas-liquid chromatograph connected to a CDS 401 Varian computer. Gracilaria was obtained from J.H. Ryther; Chlamydomonas P202 and Scenedesmus were obtained from J.R. Benemann; and Nitzschia sp. was obtained from W.H. Thomas.

RESULTS

Proximate Chemical Composition

The proximate chemical compositions of a few algae grown under nitrogen sufficient conditions are illustrated in Table 1. Botryococcus braunii grown on freshwater medium presented a slow growth rate of about 6 days/division. At the harvesting time the algal concentration was

TABLE 1

Proximate Cellular Composition of a Few Unicellular Algae Grown Under NO_3^- - Sufficient Conditions

Species	% Organic Weight					% Dry Weight
	% Protein	% Glycerol	% Carbohydrate	% Lipid	% Unknown	% Ash
Botryococcus braunii, Fresh water	16.5	>0.1	17.1	45.4	21	9.6
Botryococcus braunii, 0.5M NaCl	15.0	>0.1	13.3	46.3	25.4	59.6
Dunaliella salina, 2M NaCl	35.9	27.7	12.5	18.5	5.4	21.7
Isochrysis sp. UTEX #2307, 0.5M NaCl	37.0	>0.1	11.2	7.1	44.7	12.0
*Nitzschia sp.	16.8	>0.1	9.2	12.1	61.9	20.4

Growth conditions. Algae were cultivated in growth medium containing NaCl as indicated, 5mM $MgSO_4$. 0.3mM $CaCl_2$. 5mM KNO_3. 0.4mM KH_2PO_4. 1.5μM $FeCl_3$. 30μM EDTA, 50mM $NaHCO_3$. 0.1mM Na_2SiO_3. 0.1mM H_3BO_3, 1μg/l Vitamin-B12, 0.2 μg/l thiamine, 1.0 μg/l biotin, and 20mM Tris, pH 8.0. Temperature, 25°C. Illumination, Cool-white and Agro-lite fluorescent lamps.

*Algae from W. H. Thomas.

close to 400 mg organic weight per liter. The same alga grown on 0.5M NaCl medium divided even slower with a growth rate of about 15 days/division, reaching a cell concentration at harvest of 150 mg organic weight per liter. The composition of both cultures of B. braunii was about the same exhibiting on the order of 50% lipids, 16% protein and 15% carbohydrates. No glycerol was detected in B. braunii cultivated in media containing different salt concentrations.

All other algae divided at a rate of about 1 division/day and were harvested at concentration of around 800 mg organic weight/liter. D. salina and Isochrysis contained about 36% protein and 12% carbohydrates. Lipid content in D. salina exceeded by about 10% that of Isochrysis and reached 18%. Glycerol content in D. salina was about 30% of the organic weight. Nitzschia sp., an alga grown at 1.38 M NaCl, contained 17% protein, 9.2% carbohydrates and 12% lipids. A major cellular fraction of around 62% of the organic weight of Nitzschia has not been identified by the routine procedures employed. The unaccounted weight difference must be further examined. Possibilities could be either certain complex carbohydrate derivatives or highly non-polar type waxes that are insoluble in solvent system employed. Table 2 illustrates the proximate chemistry of D. bardawil and Isochrysis grown on nitrogen deficient medium. The cellular carbohydrate content increased and the protein content decreased. The lipid content increased in Isochrysis while it did not change in D. bardawil.

TABLE 2

Proximate Cellular Composition of Two Unicellular Algae Grown Under NO_3^- - Deficient Conditions

Species	% Organic Weight					% Dry Weight
	% Protein	% Glycerol	% Carbohydrate	% Lipid	% Unknown	% Ash
Dunaliella bardawil 2.0M NaCl	9.7	16.4	40.4	10.4	23.1	14.7*
Isochrysis sp. UTEX #2307, 0.5M NaCl	28.3	>0.1	20.5	26.0	21.2	52.0

Algae were grown in complete medium containing 0.5mM NO_3^- and the indicated concentration of NaCl.

Total Chlorophyll and Carotenoids

The contents of chlorophyll and carotenoid in the algae are illustrated in Table 3. All algae contained chlorophyll a; the green algae contained chlorophyll b in addition. The chrysophyte Isochrysis and the diatom Nitzschia contained chlorophyll c. The major carotenoids, β-carotene and fucoxanthin were analyzed spectrophotometrically and the carotenoid-to-chlorophyll ratios are given in Table 3. Dunaliella bardawil grown on nitrogen deficient medium was the only species with high content of β-carotene. Other species did not show significant pigment response to salt stress or nitrogen starvation.

TABLE 3

Pigment Content in a Few Unicellular Algae

Species	% Organic Weight		
	% Chlorophyll	% Carotenoid	Carotenoid / Chlorophyll
Botryococcus braunii, Fresh water, 5mM NO_3^-	1.76	0.53	0.29
Botryococcus braunii 0.5M NaCl, 5mM NO_3^-	0.69	0.22	0.32
Dunaliella bardawil, 2M NaCl, 0.5-M NO_3^-	0.46	2.71	5.89
Dunaliella salina, 2M NaCl, 5-M NO_3^-	4.34	0.95	0.22
Isochrysis sp, 0.5M NaCl, 5mM NO_3^-	1.83	0.64	0.35
Isochrysis sp. 0.5M NaCl, 0.5mM NO_3^-	0.49	0.25	0.51
Nitzschia sp. 1.38M NaCl	1.44	0.48	0.33

Lipid Extraction

Lipids were extracted by the Bligh-Dyer technique and were fractionated on Unisil silicic acid column with hexane, benzene, chloroform, acetone and methanol. Samples of each solvent were collected, dried and weighed gravimetrically for determining the lipid distribution along the polarity neutrality spectrum (Table 4). Neutral lipids are those found in the hexane, benzene, chloroform eluates while the polar lipids occur in the acetone,methanol eluates. B. braunii contained at least 50% of its total lipid weight as neutral lipids, most of it in the benzene fraction. D. salina and Nitzschia contained relatively low content of neutral lipids. Isochrysis contained about 30% of neutral lipids most of it in the benzene fraction. D. bardawil yielded β-carotene as the predominant fraction of its total neutral lipids [2]. Chlamydomonas and Scenedesmus comprised about 15% and 10% neutral lipids respectively (Table 5). Neutral lipid fractions of nitrogen sufficient or deficient

TABLE 4

Extraction and Fractionation of Lipids

of Unicellular Algae

Species	Total Lipids	% Total Lipid Weight				
	% Organic Weight	% Hexane	% Benzene	% Chloroform	% Acetone	% Methanol
Botryococcus braunii Fresh water, 5mM NO_3^-	45.4	10.5	50.1	19.4	12.3	7.6
Botryococcus braunii 0.5M NaCl, 5mM NO_3^-	46.3	5.2	46.0	28.5	9.3	9.7
Dunaliella bardawil, 2M NaCl, 0.5mM NO_3^-	10.4	0.4	49.8	14.8	24.1	10.8
Dunaliella salina, 2M NaCl, 5mM NO_3^-	18.5	0.2	2.1	28.2	55.9	13.6
Isochrysis sp., 0.5M NaCl, 5mM NO_3^-	7.1	1.4	27.4	32.1	26.3	12.6
Isochrysis sp., 0.5M NaCl, 0.5mM NO_3	26.0	2.2	28.4	18	26	25.3
Nitzschia sp. 1.38M NaCl	12.1	0.9	1.7	51.2	22.0	24.6

Proportions of lipid fractions recovered from Unisil column with the indicated solvents and determined gravimetrically.

TABLE 5

Analysis and Fractionation of Lipids Extracted

from Algae During the SERI Short-term Course

December 1982

Species	Total Lipids	% Total Lipid Weight				
	% Organic Weight	% Hexane	% Benzene	% Chloroform	% Acetone	% Methanol
Chlamydomonas, P202	19.4	0.1	15.5	16.0	60.5	7.9
Scenedesmus	9.3	0.2	9.5	10.4	72.6	7.3
Gracilaria, NE	1.8	.8	1.3	18.3	46.0	33.5
Gracilaria, ND	0.8	0.3	1.9	4.8	62.3	30.8

Proportions of lipid fractions recovered from Unisil column with the indicated solvent and determined by gas chromatograph for the Hexane samples or gravimetrically for the other samples.

grown Gracilaria were quite low, not exceeding 2.5% of the total lipid fraction. After analyzing the data, it was clear that B. braunii and Isochrysis contained the highest fraction of neutral lipids among the algae tested. Conversely, Gracilaria, Chlamydomonas P202 and D. salina contained the higher fraction of polar lipids.

Hexane and Benzene Eluates

The components of the hexane eluates were further analyzed on a OV-17 stainless steel capillary column. B. braunii contained long chain aliphatic hydrocarbons that were in accordance with previous observations [3]. The predominant hydrocarbons of B. braunii in the hexane eluates were C29:0, C30:0 and C31:0 (Fig. 1). Isochrysis contained predominantly C31:0 hydrocarbons (Fig. 2).

Relatively short aliphatic hydrocarbons were detected in D. salina (Fig. 3), in Scenedesmus (not shown) and in Gracilaria(Fig. 4). When the same algae were grown on nitrogen deficient conditions, a shift in the distribution of the hydrocarbon toward longer chain length was observed. Most of the neutral lipid fraction appeared, however, in the benzene fractions and not in the hexane fractions. Thin layer chromatography of the benzene fractions showed a variety of different compounds (Fig. 5) with major spots observed in B. braunii, Isochrysis and D. bardawil. The major spot in D. bardawil was clearly identified as β-carotene. The major spot of B. braunii was tentatively identified as C-34 botryococcene. The major spot of Isochrysis which accounted for about 3% of the nitrogen deficient algal organic weight has been isolated from the chromatogram and purified by preparative TLC. The purified component showing one spot by TLC was dissolved in chloroform and analyzed by MS, IR and NMR. Preliminary results indicate that it is a C-37 isoprenoid chain with one substituted six-membered ring, two double bonds and one tertiary hydroxyl group.

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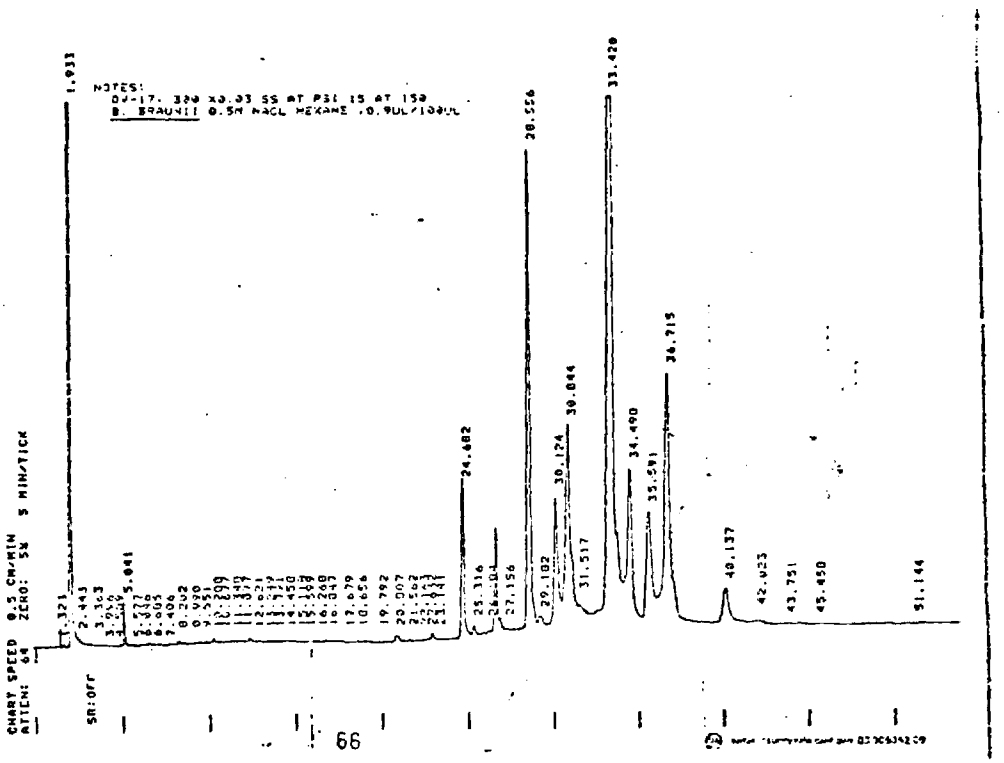


FIGURE 1. GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS IN THE HEXANE ELUATE OF B. BRAUNII GROWN ON 0.5M NaCL MEDIUM

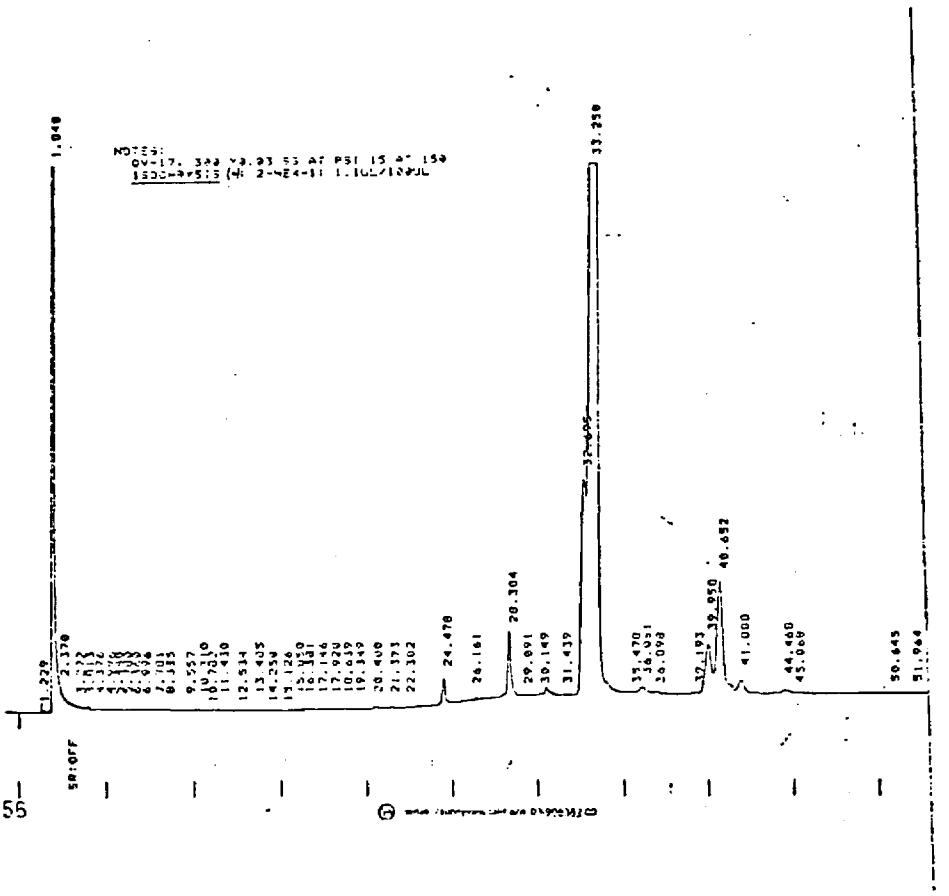


FIGURE 2. GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS IN THE HEXANE ELUATE OF ISOCHRYSIS

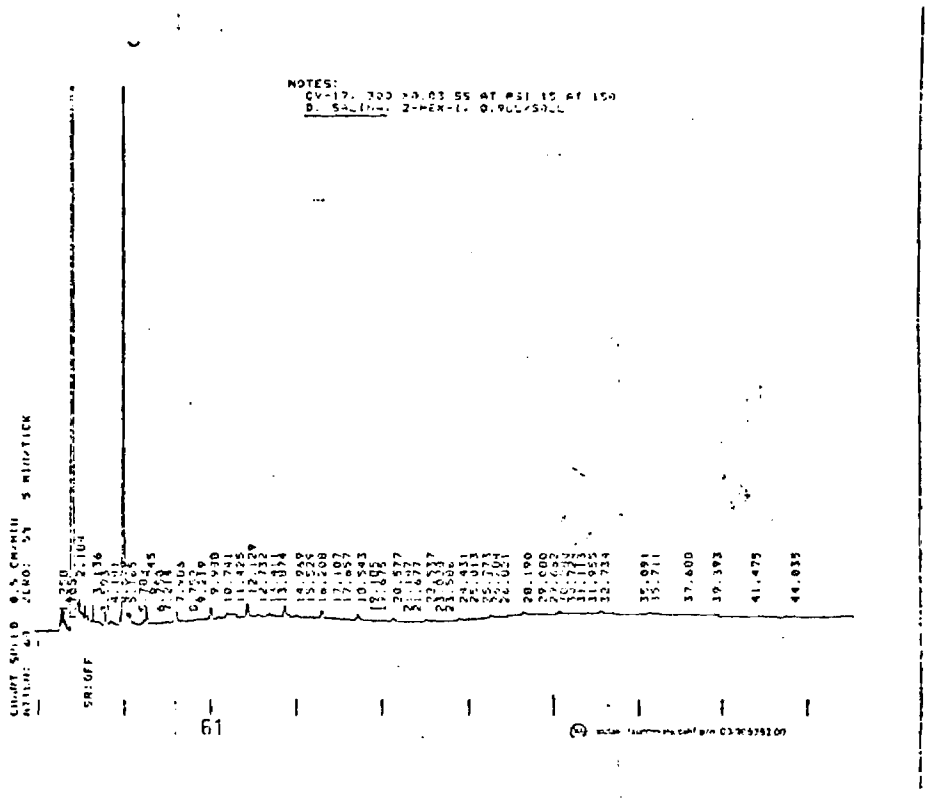


FIGURE 3. GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS IN THE HEXANE ELUATE OF D. SALINA

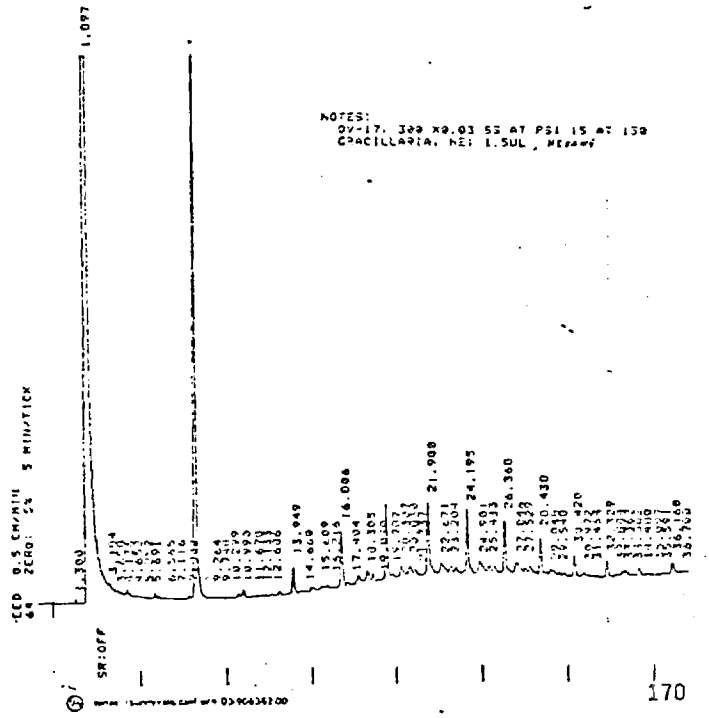


FIGURE 4. GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS IN THE HEXANE ELUATE OF GRACILARIA GROWN ON NITROGEN SUFFICIENT MEDIUM

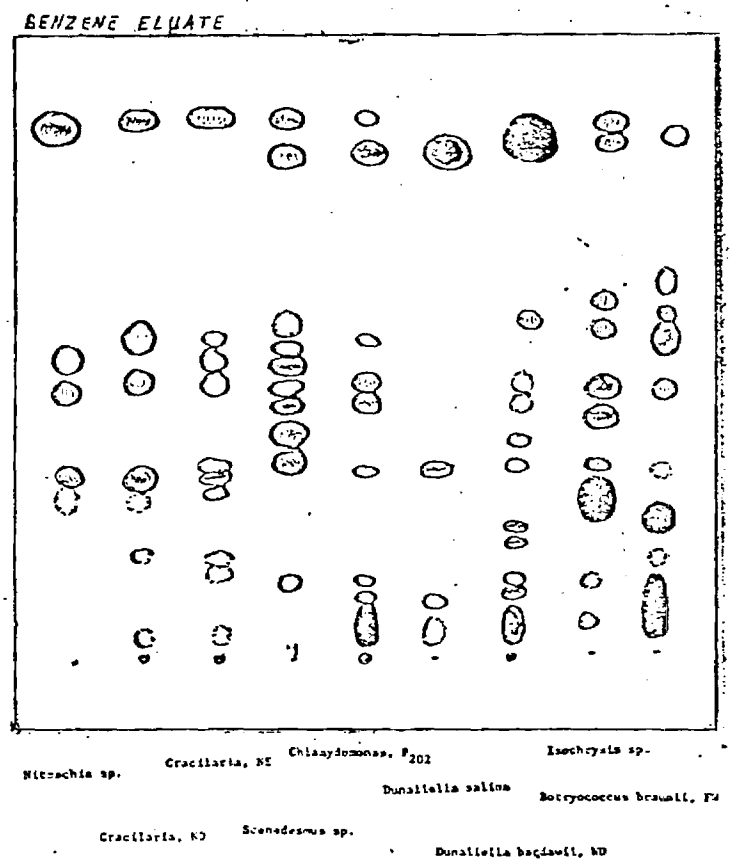


FIGURE 5. TLC OF THE FRACTIONS ELUATED FROM UNISIL COLUMN WITH BENZENE. SOLVENT SYSTEM: HEXANE: DIETHYLETHER (96:4). SPOTS WERE DETECTED BY IODINE VAPOR

TABLE 6

RELATIVE PERCENTAGES OF MAJOR ALIPHATIC HYDROCARBONS IN THE HEXANE ELUATES OF A FEW ALGAE

ro-bon	Botryococcus braunii		Chlamy-domonas P202	Dunaliella salina	Gracilaria sp.		Isochrysis sp.		Nitzschia sp.	Scenedesmus sp.
	Fresh water NE	0.5M NaCl NE	Freshwater ND	2M NaCl NE	Seawater NE	ND	0.5M NaCl NE	ND	1.38M NaCl NE	Freshwater NE
1									23.6	
1										4.3
0										
:1										1.3
:0	0.7	0.4	3.1	2.3	~90	~90				0.6
:1			54.0	90.0						72.9
:0										
:1			16.0						35.4	1.3
:0										
:1										2.3
:0										
:1									11.1	
:2										
:0										
:0										
:0										
:0										
:0								1.2		
3:0	3.3	3.4								
9:0	12.1	12.0					8.1	4.7		
0:0	23.6	8.6						1.4		
1:0	32.3	32.6					41.4	63.2	2.3	
1:1	4.8		21.5				4.4	9.4		1.8
2:0	2.7	13.4					9.0	0.3	4.8	
2:1										
3:0										
3:1	8.7	12.0					4.0	3.4		
3:2	1.8	3.2					5.0	7.2		
nident.	10.7	14.8	5.4	7.7	~10	~10	28.1	9.3	22.8	17.8

bbreviations: NE = nitrogen sufficient, ND = nitrogen deficient

TABLE 7

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS
IN METHANOL ELUATE OF A FEW UNICELLULAR ALGAE

Fatty Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp		Nitzschia sp
	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NaCl NE	ND	1.38M NaCl NE
12:0							
14:0,14:1			1.5	1.6	7.8	18.4	22.8
15:0							
16:0	5.6	1.9	3.4	5.4	16.2	17.6	15.0
16:1	27.0	13.2	30.8	27.8	44.5		
16:2							
16:3				1.4			
17:0					0.4		
18:0,18:2	10.9	1.9	24.1	30.7	5.5	5.4	1.1
18:1	20.7	12.2	12.4	6.3	0.4	8.8	13.0
18:3	14.4	6.2	14.8	20.4	1.1	10.0	12.0
18:4		0.9					
20:0,20:1	3.4	2.3			5.2		
20:2							
20:3	9.5	6.5					1.2
20:4					1.2	1.3	
20:5					0.9		4.4
20:6					0.7		22.2
22:0					2.7		
22:1							
22:2						4.8	0.1
22:3		26.0				30.6	
22:4	1.2		5.6			0.2	0.3
22:5							0.3
22:6			0.9				
Unidentified	7.3	28.9	6.5	6.4	13.4	2.9	7.6

Abbreviations: NE = nitrogen sufficient, ND = nitrogen deficient

TABLE 8

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS IN ACETONE ELUATE OF A FEW UNICELLULAR ALGAE

ty Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater	0.5M NaCl	2M NaCl	2M NaCl	0.5M	NaCl	1.38M NaCl
	NE	NE	ND	NE	NE	ND	NE
0							
0,14:1			0.4	0.6	20.0	17.0	4.2
0							
0	9.5	13.4	22.3	26.2	11.0	9.8	16.0
1	6.9	6.2	25.3	18.6			13.2
2	1.1					0.8	
3							
0							
0,18:2	13.3	5.2	11.0	14.3	3.4	5.0	5.2
:1	14.6	27.0	5.9	2.5	6.6	12.4	14.3
:3	41.7	27.8	33.8	35.4	32.4	33.2	6.1
:4						5.0	
:0,20:1	0.6	1.5				2.4	2.0
:2							
:3	3.2	3.7					3.6
:4							2.4
:5						0.9	16.7
:6					8.6	8.5	
:0							
:1							
:2							
:3						1.4	
:4	0.5		0.2				
:5							
:6							
identified	8.6	15.2	1.3	2.4	18.4	3.6	16.3

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

TABLE 9

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS
IN CHLOROFORM ELUATE OF A FEW UNICELLULAR ALGAE

ty Acid	Botryococcus braunii		Dunaliella bardawil	salina	Isochrysis sp.		Nitzschia sp.
	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NE	NaCl ND	1.38M NaCl NE
0	6.1						
0,14:1	7.7		3.6	44.0	15.1	16.7	8.6
0	1.6			7.4			
0	10.8	8.5	27.8	20.1	9.2	10.8	21.0
1	4.3	1.7		5.0	4.0	2.7	4.1
.2	4.1						
:3	10.8						
:0							
	28.1	50.0	35.2	11.3	23.1	28.1	9.4
				2.6			
.5							12.5
:4							
:0,20:1	2.2	2.5	20.7	7.8	6.0	5.7	
:2					15.9	14.3	
:3	2.3						
:4							
:5							5.0
:6					17.8	9.4	17.7
:0							
:1							
:2							
:3		12.1					
:4							
:5		12.4					
:6							
identified	2.2	12.8	12.7	1.8	8.9	12.3	21.7

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGES OF TOTAL FATTY ACID COMPOSITION OF A FEW SELECTED ALGAE

Fatty Acid	Botryococcus braunii		Chlamydomonas P202	Dunaliella bardawil		Gracilaria sp.		Isochrysis sp.		Nitzschia sp.	Scenedesmus
	Freshwater	0.5M NaCl	Freshwater	2M NaCl		Seawater		0.5M NaCl		1.38M NaCl	Freshwater
	NE	NE	ND	ND	NE	NE	ND	NE	ND	NE	NE
12:0	2.0										
14:0, 14:1	2.6		1.5	1.8	15.4	8.7	5.5	14.0	17.4	11.9	1.1
15:0					2.5						
16:0	9.2	7.9		17.8	17.2	54.6	39.7	12.1	12.7	17.3	
16:1	12.7	7.0	16.6	18.7	17.1			16.2	0.9	5.8	17.2
16:2	1.7		15.4						0.3		15.2
16:3	3.6				0.5						
17:0								0.1			
18:0, 18:2	17.5	19.0	17.5	23.4	18.8	12.0	16.0	10.6	12.8	5.2	7.8
18:1	11.8	13.1	4.9	6.1	3.8			2.3	7.1	9.1	17.4
18:3	18.7	11.3	34.1	16.2	18.6			11.0	14.4	10.2	30.9
18:4		0.3							1.7		
20:0, 20:1	2.0	2.1	0.6	6.9	2.6			3.7	2.7	0.6	
20:2								5.3	4.8		0.4
20:3	4.9	3.4								1.6	
20:4			3.2			11.1	32.3	0.4	0.4	0.8	1.1
20:5								0.3	0.3	8.7	
20:6								9.0	6.0	13.3	
22:0						0.5	0.5	0.9			
22:1			0.3								
22:2									1.6		
22:3		12.7							10.6		
22:4	0.6			1.9					0.1	0.1	
22:5											
22:6		0.3		0.3		0.7	0.6				
Unidentified	12.7	22.9	9.0	6.9	3.5	12.4	5.4	14.0	6.2	15.4	8.9

Identification of fatty acid methyl esters was determined on 99m x 0.75mm stainless-steel capillary column coated with 3% OV-17; temperature programmed at 4°C/min for 150 to 250°C and held isothermally. Relative percentage compositions were determined with CDS 401 Varian computer. Identities of fatty acids were determined by comparing retention times to those of established standards. NE, nitrogen sufficient; ND, nitrogen deficient.

TABLE 11

PROXIMATE CHEMICAL COMPOSITION OF SARGASSUM FILIPENDUA AND ASCOPHYLUM NODOSUM

Relative Percent Dry Cell Weight

	Ascophylum	Sargassum
Ash	20.0	39.3
Protein	9.1	8.6
Lipid	3.6	2.8
Carbohydrate	65.3*	48.3*
Nucleic Acid	2.0*	2.0*

*Values determined by extrapolation

TABLE 12

TOTAL LIPIDS FRACTIONATED ON UNISIL SILICIC ACID

Eluting Solvent	Ascophylum	Sargassum	Probable Identity
Hexane	0.3	0.4	Acyclic hydrocarbon
Benzene	15.7	1.5	Isoprenoids
$ChCl_3$	15.4	1.0	Tri & Diglycerides
Acetone	50.0	74.3	Glycolipid
MeOH	19.5	22.8	Phospholipids

2. Polysaccharides of the Gracilaria Species

Isolation, Purification and Characterizations.

Gracilaria grown in nitrogen sufficient and nitrogen deficient medium were investigated in detail for characterizations of polysaccharides since the lipid contents were undistinguished.

Polysaccharides were extracted from these two species by the procedure of Jabbar Mian and E. Percival (Carbohyd. Res. 26 (1973) 133-146).

1. Preliminary extraction of two species of Gracilaria with hot ethanol (80%) at 70°C three times resulted in isolation of Mannitol, a major sugar in this fraction. Mannitol was further purified by passing through Sephadex-G-50 Column Chromatography.

A second extraction of Gracilaria with cold ethanol (80%) also showed the presence of Mannitol, a major sugar in ethanol extract. Traces of glucose, rhamnose and other unidentified sugars were also detected in the ethanol extract, which were then separated by Sephadex Column Chromatography to purify Mannitol.

After isolation and purification of Mannitol sugar, the residue was washed several times with H_2O and then immersed in 40% formaldehyde for 18 hrs. They were then washed, with H_2O and dried.

2. A dried powder obtained after extractions with 2% $CaCl_2$ (100 ml) for 4 hrs. at 70°C gave purified agar. In this hot $CaCl_2$ fraction, agar was the major component present. Since hot $CaCl_2$ extract after cooling immediately solidifies, due to the presence of agar, other sugars present were unidentified. However extraction of Gracilaria with cold 2% $CaCl_2$ do not extract agar, and by this extraction method glucose, mannose, ribose, xylose and some unidentified sugars were characterized.

3. $CaCl_2$ extracted residue was further extracted with dil HCl (pH 2.0) for 4 hr. at 70°C. Dil HCl extract was concentrated, dialysed for several days, lyophilized and analyzed by gas liquid chromatography.

The major sugars present in this fraction were glucose mannose and xylose.

4. The residue was further extracted with 3% sodium carbonate for 4 hr. at 50°C. (3 times) filtered, filterate concentrated, dialyzed and lyophilized.

This extract contained the major carbohydrate fraction that was characterized by GLC. A large number of unidentifiable (unusual) sugars were found. These are being identified. This fraction also contains a glucose, xylose, ribose, galactose, mannose, etc., which are generally common.

The detailed data is shown in Table 1.

5. Residue was further extracted with ammonium oxalate-oxalic acid (0.25% with respect to each 100 ml, pH 2.8 for 6 hr. at 70°C) extract was dialysed and lyophilized andanalysed by gas liquid chromatography. 3-4 sugars were present. Some sugars were identifiable (Table 1).

6. Residue was then extracted with H_2O, (100 ml) acetic acid (1 ml) and sodium chloride (1g) at 70°C, 3 times.

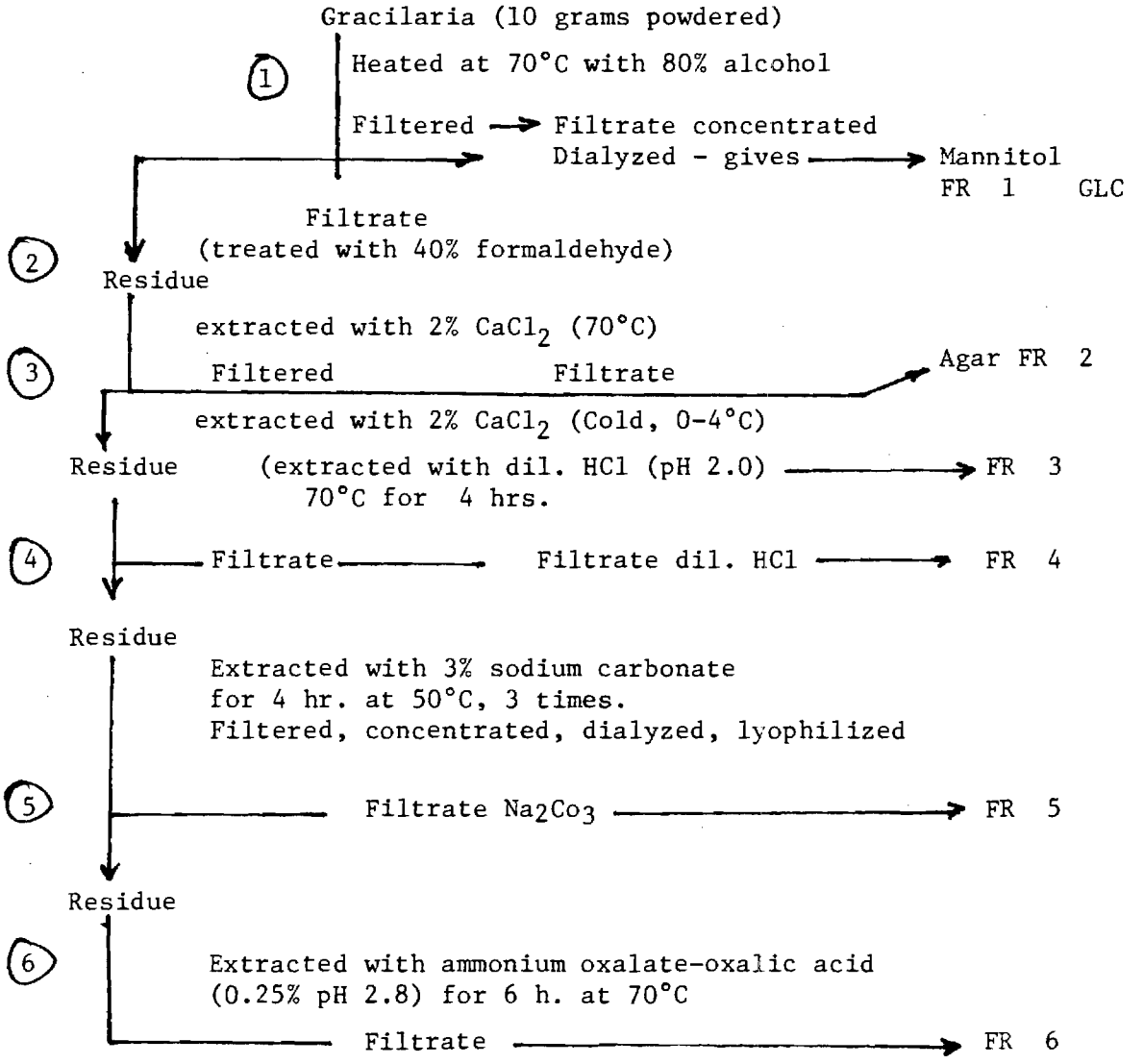
Extracts concentrated, dialyzed extensively, lyophilized and are being analyzed by GLC (Table 1).

7. The residue was further washed with H_2O, and then exhaustively with 6M potassium hydroxide (100 ml) for 48 hr. at room temperature. The residue solid was then washed with dilute HCl, H_2O ethanol, and ether, and was recovered as a white solid.

SUMMARY

Polysaccharides were isolated by sequential extraction of Gracilaria species. They were purified and characterized by gas liquid chromatography. Extraction of the algae by 80% hot and cold ethanol gave mannitol, as a major sugar present in this fraction. This was identified by colorimetric assay and gas liquid chromatography. The resultant material (mannose) was further purified by Sephadex G-50 column chromatography and lyophilized. A second extraction with 2% calcium chloride at 70°C gave agar as the major carbohydrate. However, extraction with cold 2% calcium chloride gave a fraction containing glucose, rhamnose, arabinase, ribose and xylose and other unidentifiable sugars. Extractions with dil hydrochloride, sodium carbonate, ammonium oxalate-oxalic acid, also gave different fractions containing various sugars which were analyzed by gas liquid chromatography. Among all these fractions, sodium carbonate extract contained a large number of sugars.

PROCEDURE OUTLINE FOR EXTRACTION OF POLYSACCHARIDES FROM GRACILARIA SPECIES



GRACILARIA

Isolation and Characterization of Polysaccharides

1. Gracilaria D-1 %

Sugars	Fractions 1	2	3	4	5	6
Glucose	0.36	0	0	0	11.9	5.62
Galactose	0.27	2.45	2.09	6.36	8.5	1.83
Mannose	0.72	0.36	2.27	0.62	2.8	0.56
Ribose	0.42	0	1.37	0	23.6	0
Xylose	0	2.19	0	4.0	5.4	2.1

2. Gracilaria D-2

Sugars	Fractions 1	2	3	4	5	6
Glucose	0	3.46	0	0	14.79	7.39
Galactose	0	0	2.44	9.23	12.25	3.97
Mannose	0	0.37	0.75	1.01	3.1	2.90
Ribose	0	0	2.55	0	30.83	9.19
Xylose	0	2.15	0.89	5.11	5.28	1.71

3. Polysaccharides of the Two Macroalgae, Ascophyllum Nodosum (AN) and Sargassum Filapendum (SF).

Isolation, Purification and Characterizations:

Polysaccharides were extracted from these two macroalgae Ascophyllum Nodussum, and Sargassum filapendum by sequential 2% $CaCl_2$ extractions at 70°C for 3 hrs. and then by 3% Na_2CO_3 extractions at 50°C. Extracts were concentrated, dialyzed extensively, lyophilized, converted into alditol acetates and analyzed by gas liquid chromatography (GLC). Results of analysis are shown in the following table.

POLYSACCHARIDES OF MACROALGAE ASCOPHYLLUM NODUSUM (AN) AND SARGASSUM FILAPENDUM (SF)

	2% $CaCl_2$ Extract		3% Na_2CO_3 Extract	
	AN	SF	AN	SF
Glucose	3.18	8.44	48.38	6.42
Galactose	5.10	14.36	2.55	0
Xylose	11.18	8.15	7.41	6.97
Fucose	40.04	37.16	0	0
Ribose	25.81	5.94	0	0
Mannose	0	15.39	3.01	62.03
Arabinose	0	0	7.40	0
2-Deoxy glucose	0	0	8.82	3.86

% Analyzed by Gas Liquid Chromatography

4. The Fatty Acids Present in the Lipopolysacchardies (Endotoxin) of Cyanobacterium, Microcystis Aeruginosa 006 and NRC-1 Strains

The fatty acids present in the lipopolysaccharides and lipid A moiety of 006, and NRC-1 strains of Microcystis aeruginosa have been analyzed. Oleic acid (C18:1) was the most predominant fatty acid present in these lipopolysaccharides and lipid A preparations. The other major fatty acids identified were myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), nonadecanoic acid (C19:0) and arachidic acid (C20:0). Anteiso-C15:0 and anteiso- C17:0 fatty acids were also present in minute quantities. 2-Hydroxymyristic acid (2-OH-C14:0) was the only hydroxylated fatty acid identified from these lipopolysaccharide and lipid A preparations.

The fatty acid compositions of lipopolysaccharides ane their lipid A moiety from a large variety of Gram-negative bacteria have been extensively examined. Lipopolysaccharides have also been isolated from a few blue green bacteria (Cyanobacteria) and their chemical compositions and biological activities have been studied (10-12). Microcystis aeruginosa, a blue green bacteria oftenly grows in water blooms of eutrophic lakes and reservoirs and are correlated with domestic and wild animal poisoning (2). Recently we have isolated lipopoly-saccharides from 006 and NRC-1 strains of M. aeruginosa and their general gross chemical compositions and biological activities determined (7). As a part of continuing programs of structural and toxicity studies of lipopolysaccharides, we have further analyzed the fatty acids present in lipopolysaccharides and its lipid A moiety from 006 and NRC-1 strains of M. aeruginosa.

The organisms were cultured in 16-1 pyrex bottles with a modified BG-11 medium at 29°C, aerated with 1% CO_2 in air, and illuminated continuously with 500 fc of cool-white fluorescent light as described previously (7). The lipopoly-saccharides were isolated and purified from lyophilized cells essentially by the phenol-water technique of Westphal et. al. (9), and repeated ultra-centrifugation (105,000 xg for 4 hrs) and treatment with RNase (25 ug/ml) in 0.05 M Tris-hydrochloride buffer pH 7.6. Lipopolysaccharides were further purified by Csc1 gradient ultracentrifugation in a SW 50.1 rotor (Beckman Instruments, Fullerton, CA) at 42,000 rpm for 60 hrs as described previously (7,8). The lipid A moiety was isolated after extraction of lipopolysaccharides with chloroform to remove non-covalently bound lipids. The lipopolysaccharides were then hydrolysed with 1% acetic acid at 100°C for 3 hrs. The resulting precipitate (free lipid A) obtained by centrifugation at 27,000 xg for 20 min, was washed with warm water (60°C) and acetone and lyophilized (7).

The purified lipopolysaccharides and lipid A moiety were hydrolysed with 4 M HCl for 5 hrs at 100°C in a sealed tube. The fatty acids obtained were recovered by extraction several times with chloroform, followed by evaporation, quantitation and methylation of residue as described earlier (6).

The fatty acids were analyzed by gas liquid chromatography on a Varian model 3700 gas chromatograph with flame ionization detector and equipped with a Varian CDA-401 data system (Varian Instruments Group, Palo Alto, CA). A stainless steel capillary column 99 m x 0.75 mm coated with 3% OV-17 (Supelco, Bellefonte, PA) with temperature programming at 4°C/min from 150°C to 250°C

held isothermally, and a fused silica capillary column 30 m x 0.252 mm coated with OV-351 (J&W Scientific Inc., Rancho Cordova, CA) with temperature programming at 4°C/min from 140 to 220°C were used. The identities of the fatty acid methyl esters were determined in both columns by comparison of their retention times with those of authentic standards. All standard fatty acid methyl esters were obtained from Supelco (Supelco, Bellefonete, PA). 2-Hydroxy-myristic acid (2-OH-C14:0) and 3-hydroxy-myristic acid (3-OH-C14:0) were the kind gift of Dr. Otto Luderitz (Max-Planck Institut fur Immunbiologie, Freiburg, West Germany).

The total fatty acid content recovered as methyl esters from lipopolysaccharides of 006, and NRC-1 were 18.2%, and 21.4% respectively. The total fatty acid content recovered as methyl esters from lipid A moiety of 006, and NRC-1 were 52.8%, and 67.2% respectively. The results of the analysis of fatty acid pattern of lipopolysaccharide lipid A moiety by gas liquid chromatography of the two strains are given in Table 1. Lipopolysaccharide and lipid A from both strains gave an almost identical fatty acid pattern. The most abundant fatty acid present in both strains was oleic acid (C18:1) accounting more than 55% in lipopoly-saccharide and lipid A preparations. In addition to oleic acid, the other major fatty acids present were pentadecanoic acid (C15:0), palmitic acid (C16:0), hepta decanoic acid (C17:0), nonadecanoic aicd (C19:0), and arachidic acid (C20:0). The lipopolysaccharide and lipid A also contains smaller amounts of myristic acid (C14:0), anteiso-C15:0 and anteiso-C17:0 fatty acids. 2-Hydroxy myristic acid was the only hydroxy fatty acid identified in smaller quantities from these lipopolysaccharide and lipid A preparations. However, the amount of 2-hydroxy myristic acid was found in slightly less quantities in NRC-1 strain than 006. Hydroxymyristic acid and more oftenly 3-hydroxylated myristic acid is a common marker of the lipopolysaccharide and lipid A preparations of most gram-negative bacteria (3). However, in lipopolysaccharides and lipid A of M. aeruginosa instead of 3-hydroxy myristic acid, 2-hydroxy myristic acid was present.

The fatty acid compositions of lipopolysaccharides and lipid A of M. aeruginosa were markedly different from that of enterobacterial lipopolysaccharides (3). M. aeruginosa lipopolysaccharides does not contain lauric acid (C12:0) or larger amounts of myristic acid and hydroxylated fatty acids. Although lipo-polysaccharides of some other blue green bacteria (10-12) have been chemically analyzed, a detailed quantitative description of the fatty acids present in those lipopolysaccharides is not available for comparisons with M. aeruginosa strains. We previously identified glucosamine, the only amino sugar from the lipid A preparations of M. aeruginosa 006 and NRC-1 (7). It is, therefore, likely that the fatty acids in M. aeruginosa lipid A like in endotoxins of Gram-negative bacteria are linked to glucosamine residues. In Gram-negative bacteria lipid A represents the endotoxic center of lipopolysaccharides, and the presence of fatty acids within lipid A seems to be essential for endotoxic activities of lipopolysaccharides (4,5). Our earlier studies have shown that the lipopoly-saccharides and lipid A of 006 and NRC-1 strains were slightly less active in Limulus lysate gelation tests and toxicity in mice as compared to Salmonella lipopolysaccharides (7). The biological significance of the presence of very high concentrations of oleic acid (C18:1) in lipopolysaccharides and lipid A of all three strains of M. aeruginosa is not yet assessed.

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THE FATTY ACIDS PATTERN OF LPS AND LIPID A

OF M. AERUGINOSA 006, 027 and NRC-1

Fatty Acids	006		027		NRC-1	
	LPS	Lipid A	LPS	Lipid A	LPS	Lipid A
(14:0	0.70	1.80	2.01	2.22	1.45	2.17
ai(15:0	0.53	1.02	0.71	1.36	1.31	0.77
(15:0	3.74	4.46	3.77	10.02	5.10	3.56
(16:0	10.29	6.59	4.99	11.21	6.69	5.32
0	0.89	0.89	0.68	0.95	1.02	0.74
(17:0	4.34	3.82	2.37	8.05	4.35	3.60
(18:1	58.01	65.94	57.48	36.21	62.60	66.21
(18:0	0	0	8.72	4.06	0	0
(19:0	4.29	4.08	2.49	7.80	4.62	4.03
(20:1	2.97	4.44	0	0	5.50	trace
(20:0	4.29	.447	10.04	10.63	4.28	12.36
2-OH-C14:0	6.80	3.61	0.56	1.2	0.69	2.90
3-OH-C14:0	0	0	0	0	0	0

ai = anteisomethyl branched fatty acids.
OH = hydroxy fatty acids.

Fatty acid methyl esters were determined using a stainless steel capillary column 99 m x 0.75 mm, coated with 3 1/0 OV-17 and temperature programming at 4°C/min from 150 to 250°C, and on fused silica capillary column 30 m x 0.252 mm, coated with OV-351 and temperature programming at 4°C/min 140 to 220°C, on a Varian Model 3700 gas liquid chromatograph. The identities of fatty acid methyl esters were based on retention times compared with those of authentic standards. The relative percentage compositions were determined with CD5-401 Varian Computer.

5. Lipopolysaccharides (LPS) and Lipid A Moiety of M. Aeruginosa 006 and NRC-1 Strains

Lipopolysaccharides were isolated from two strains, NRC-1 and 006.

Purified by Cscl gradient ultracentrifugation	(Fig. 1)
Total Gross chemical compositions	(Table 1)
Fatty acid pattern of LPS and lipid A	(Table 2)
Toxicity of LPS and lipid A in mice	(Table 3)
Limulus assay of LPS and lipid A	(Table 4)

Paper submitted to

European Journal of Biochemistry with incomplete data in Table 1 - (It will be revised with Revised Table 1 enclosed here).

006 and NRC-1

FIGURE 1

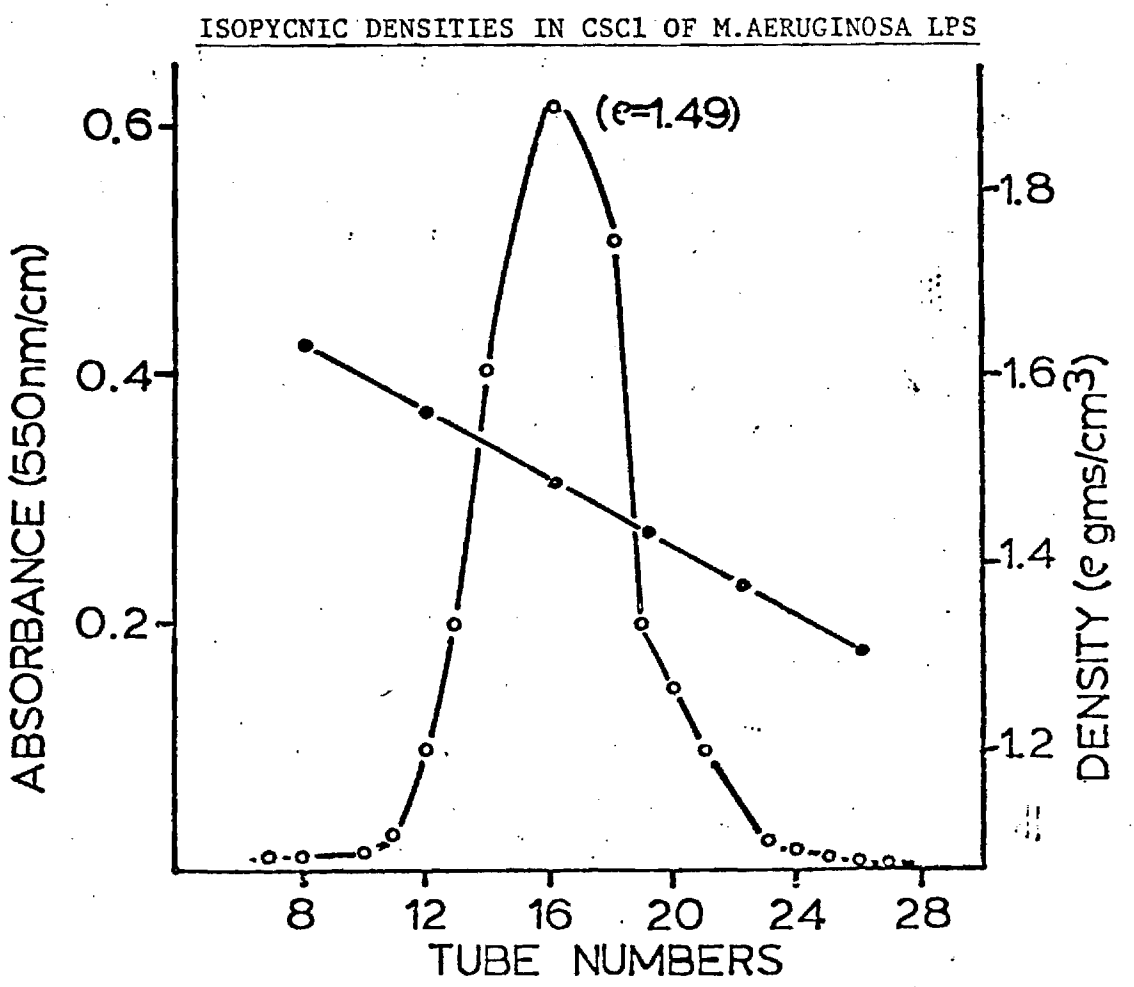

FIGURE 1

LEGEND TO FIGURE-1

Isopycnic density gradient ultracentrifugation of LPS from *M. aeruginosa* isolate 006. Samples containing 1 mg of LPS in 0.1 M Tris-buffer, pH 8.1 (final volume 4.8 ml), were added to 2.8 g of CsCl and centrifuged for 60 hrs. at 142,000 X G. Column fractions were collected from the bottom of the tube and assayed for KDO. Densities were determined from the index of refraction measurements.

Table 1 Chemical compositions of LPS of M.aeruginosa

Components	006	NRC-1
	% of Total	
3-Deoxy-D-manno-octulosonic acid	4.7	5.2
Glucose	11.4	8.1
3-Deoxy sugars	3.1	1.8
Glucosamine	3.7	6.5
D-Glycero-L-mannoheptose	0	0
Total Carbohydrates	36.0	26.5
Total Fatty acids	18.2	21.4
Fatty acid esters	10.8	13.6
Phosphorous	2.7	3.2
Nucleic acids	0	0
Protein	0.4	0.3

TABLE 2

FATTY ACID PATTERN OF LIPOPOLYSACCHARIDES (LPS) AND LIPID A OF M.AERUGINOSA

STRAINS	006		027		NRC-1	
FATTY ACIDS	LPS %	LIPID A	LPS	LIPID A	LPS	LIPID A
C14:0	0.70	1.80	2.01	2.22	1.45	2.17
ai-C15:0	0.53	1.02	0.71	1.36	1.31	0.77
C15:0	3.74	4.46	3.77	10.02	5.10	3.56
C16:0	10.29	6.59	4.99	11.21	6.69	5.32
ai-C17:0	0.89	0.89	0.68	0.95	1.02	0.74
C17:0	4.34	3.82	2.37	8.05	4.35	3.60
C18:1	58.01	65.94	57.48	36.21	62.60	66.21
C18:0	0	0	8.72	4.06	0	0
C19:0	4.29	4.08	2.49	7.80	4.62	4.03
C20:1	2.97	4.44	0	0	5.50	Trace
C20:0	4.29	4.47	10.04	10.63	4.28	12.36
2-OH-C14:0	6.80	3.61	0.56	1.20	0.69	2.90
3-OH-C14:0	0	0	0	0	0	0

ai = anteisomethyl branched fatty acids, OH = hydroxy fatty acids.

Fatty acid methyl esters were determined using a stainless steel capillary column 99 m X 0.75 mm, coated with 3 % OV-17 & temperature programming at 4 C/min from 150 to 250 C, and on fused silica capillary column 30 m X 0.252 mm,coated with OV-351 & temperature programming at 4 C/min from 140 to 220 C, on a gas-liquid chromatograph. The identities of fatty acid methyl esters were based on retention times compared with those of authentic standards. The relative percentage compositions were determined with a Varian computer.

TABLE 3 Toxicity of LPS and Lipid A moiety from *M. aeruginosa* isolates 006 and NRC-1 in C57BL/6 mice[a].

Dose	006		NRC-1		*S. abortus equi*[b]
µg/mouse	LPS	Lipid A[c]	LPS	Lipid A[c]	LPS
400	6/6[d]	6/6	6/6	6/6	6/6
600	6/6	6/6	6/6	6/6	3/6
800	4/6	6/6	3/6	5/6	0/6
1000	2/6	6/6	0/6	2/6	-
1200	0/6	3/6	-	0/6	-
1400	-	0/6	-	-	-

[a]Purified LPS or lipid A moiety were injected into individual mice intraperitoneally in 0.4 ml pyrogen-free saline and survivors/deaths recorded at 48 hrs.

[b]LPS from *S. abortus equi* obtained from Sigma.

[c]Lipid A was complexed to bovine serum albumin.

[d]Number survivors/total injected.

Table 4 Limulus lysate gelation of LPS and lipid A moiety from M. aeruginosa isolates 006 and NRC-1.[a]

µg/ml of LPS or lipid A[b]	006		NRC-1		S. abortus equi[c]
	LPS	Lipid A	LPS	LipidA	LPS
10^{-3}	+++[d]	+++	+++	+++	+++
10^{-4}	+++	++	+++	+++	+++
10^{-5}	++	±	+++	+++	+++
10^{-6}	±	-	+++	++	+++
10^{-7}	-	-	+++	±	+++
10^{-8}	-	-	++	-	+++
10^{-9}	-	-	±	-	++
10^{-10}	-	-	-	-	±
10^{-11}	-	-	-	-	-

[a]The activity of different concentrations of LPS or lipid A was detected by incubating 0.1 ml of the test sample at 37°C with an equal volume of Limulus lysate, in a stoppered 10 X 75 mm test tube.

[b]Lipid A was complexed to bovine serum albumin.

[c]LPS standard obtained from Sigma.

[d]+++ solid gel formed in less than 60 min; ++ solid gel formed after 60 min; ± viscous or granular gel formed after 60 min; - no gel formed after 24 hr.

6. Lipopolysaccharides of M. aeruginosa 027

1. Lipopolysaccharides (LPS) were isolated from 027 strain by phenol/H_2O method.

2. Purified by Cscl gradient ultracentrifugations.

3. Gross chemical compositions determined. Data shown in Table I.

4. LPS were cleaved into polysaccharide (PS) and lipid A moiety by 1% acetic acid hydrolysis at 100°C for 3 hr.

   Lipid A moiety was purified.

   Fatty acid pattern of LPS and lipid A moiety determined.

   Data shown in Table 2.

5. PS moiety was fractionated on Sephadex G-50 column chromatography using pyridine/acetic acid/H_2O eluate. 65 eluates were collected and analyzed for total polysaccharides and Po4. Two major peaks and a minor peak was obtained.

   Data shown in Tables 3 and 4.

6. Two major peaks of PS were pooled, dialysed and lyophilized.

   Different sugars were determined by gas liquid chromatography.

   Data shown in Table 5.

7. From these two major peaks of PS one may be O-specific side chain and the other core polysaccharide. Which could be easily judged by simple hemagglutination assay using sheep red blood cells or by crossed immunoelectrophoresis.

8. Toxicity and limulus assay of this LPS and lipid A moiety will also be examined.

9. Graph (art work) to be done on Sephadex-G-50 fractions to show two major peaks in PS. (Tables 3 & 4).

TABLE 1

CHEMICAL COMPOSITIONS OF THE LIPOPOLYSACCHARIDES

MICROCYSTIS AERUGINOSA 027 STRAIN

	%
2-Keto-3-deoxyoctonate (KDO)	3.8
3-Deoxy sugars	2.9
Glucosamine	5.3
D-Glycero-L-mannoheptose	0
Total carbohydrates	28.6
Total fatty acids	14.0
Fatty acid esters	9.3
Phosphorous	1.8
Nucleic acids	0
Protein	0.8

TABLE 2

FATTY ACID PATTERN OF LIPOPOLYSACCHARIDES AND LIPID A MOIETY OF M. AERUGINOSA 027

027

Fatty Acids	LPS	Lipid A
C14:0	2.01	2.22
ai-C15:0	0.71	1.36
C15:0	3.77	10.02
C16:0	4.99	11.21
ai-C17:0	0.68	0.95
C17:0	2.37	8.05
C18:1	57.48	36.21
C18:0	8.72	4.02
C19:0	2.49	7.80
C20:0	10.04	10.63
2-OH-C14:0	0.56	1.20
3-OH-C14:0	0	0

Column 1. Stainless steel capillary 99 m x 0.75 mm. Coated with 3% OV-17 and temp. 4°C/min from 150°C to 250°C.

Column 2. Fused silica capillary column. 30 m x 0.252 mm. Coated with OV-351, and temp. 4°C/min. from 120°C to 220°C.

Gas liquid chromatography.

TABLE 3

CHARACTERIZATIONS OF POLYSACCHARIDE MOEITY OF
LIPOPOLYSACCHARIDES OF M. AERUGINOSA 027 STRAIN

Fractionated on Sephadex-G-50 (PS-I & PS-II) Analyzed by GLC

Sugars	PS I	PS II (Relative % Compositions)
Glucose	12.07	8.35
Rhamnose	1.0	0
Arabinose	11.71	0.49
Xylose	17.33	12.70
Mannose	0	8.62
Ribose	1.51	0.64
Galactose	6.37	6.40
2-Deoxy glucose	17.86	16.84
Inositol	0	8.31
Heptose	0	0
D-Glucosamine	8.3*	2.8*
Phosphorous	3.2*	0

* D-Glucosamine and phosphorous were determined colorimetrically and represents mg/100 mg of polysaccharides.

7. Analyses of Spirulina

The lipopolysaccharide of Spirulina, a cyanobacterium of commercial importance is now being analyzed for endotoxic properties. This work is being compared to the highly toxic properties of Microcystis. The lipid content of these blue green algae are relatively undistinguished and similar to that previously described by Tornabene in "Hydrocarbon of Bacteria and Algae" in Trends in the Biology of Fermentation for Fuel and Chemicals, ed. by A. Hollaender et al.,Plenum Press, N.Y. 421-438.

M-P-29 ORNL

8. LIPID COMPOSITION OF THE NITROGEN STARVED GREEN ALGA, NEOCHLORIS OLEOABUNDANS

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Key word index - Neochloris oleoabundans; chlorophyceae, green algae; lipids; hydrocarbons, triglycerides; sterols; glycolipids; phospholipids; fatty acids.

Running Title: Lipids of Neochloris oleoabundans

IN PRESS IN ENZYME AND MICROBIAL TECHNOLOGY

ABSTRACT

Neochloris oleoabundans was cultivated in mineral medium deficient in nitrogen. The yield of lipids was 35-54% of cell dry weight. Triglycerides comprised 80% of the total lipids. Aliphatic hydrocarbons, sterols, pigments, glycolipids and phospholipids comprised the remaining lipid fraction. Saturated, monounsaturated and diunsaturated octadecanoic acid represented approximately one-half of the total fatty acids.

GEORGIA INSTITUTE OF TECHNOLOGY
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April 28, 1983

Mike Lowenstein
SERI
1617 Cole Blvd.
Golden, Colorado 80401

Dear Mike:

Enclosed is the original and two copies of an addendum to the progress report on Task V of Subcontract XK-2-02149-01. I will heretofore see to it that this task will be completed.

Sincerely,

Thomas G. Tornabene
Director

TGT:sce

Progress Report for Task V

Appropriate methods of harvesting microorganisms and extracting oils from the cells are not well defined except for bench scale laboratory work and standard laboratory procedures. Simple systems such as centrifugation or standard filtration on microfilters for harvesting cells is unfeasible in any reasonable upscaled system. Only a few algal systems are "settlers" or "floaters" and foaming techniques for recovery are not always applicable. It then follows that standard extraction of cells by refluxing or liquid- liquid partitioning phases as currently practiced in industry for higher plants becomes impractical. If centrifugation was used for harvesting, the cost could easily surpass $1,000 per ton. This alone is a significant economic barrier. The extraction procedures for lipids will not be a significant problem if it were not coupled to harvesting procedures and the cells could be delivered in concentrated solutions, wet packs or dry form.

In essence, the problem is not with extraction of oils, but instead with harvesting of microorganisms. We have had in house demonstrations of cross-flow filtration systems developed by Milipore Corporation and Amicon. These companies each claimed that they had established procedures that could be used at any scale level that could effectively harvest any quantity of cells. Their demonstrations were conducted on Dunaliella cultures cultivated in our laboratory. We judged their application to pilot scale systems as very questionable. There are several important factors or limitations to these membrane systems. At present they are 1) very expensive; small bench models for 1-2 l batches cost minimally $5,000; 2) considerable maintenance involved to maintain the membranes (the replacements are major cost factors running $300-$500 each); 3) limited life of membranes; and 4) very delicate flow rate and concentration parameters required. We do believe here in this laboratory that someday the membrane harvesting system will be developed for our needs, but that its development is sometime in the future.

We have reviewed the potential of a shallow depth settler composed of inclined parallel plates, flocculation, electroflotation, microstraining, filtration and centrifugation. All of them suffer from certain difficulties. Microstaining appears promising for the filamentous and larger microalgae but only the more expensive centrifugation and flocculation have been shown to work with the smaller microalgae. Combination of the various harvesting procedures may be feasible, for example, flocculation followed by sedimentation or flotation. This method has proved marginally successful for sanitation type engineering processes. Limitations of the approach are that they can not be used for all cells of different sizes and it is unlikely to be effective in salt water. The other feasible approaches which are untested are most notably counter-current separation, separation by locomotor behavior and concentration by chemo- and phototactic responses. None of these are new ideas and they can be promoted and demoted depending on which variable or conditional operation parameter one wishes to emphasize.

The bottom line is a simple one, we do not have established harvesting procedures for extracting large scale microbial cultures. Dr. Lucien Maury, Vice President of Hercules Inc. (world's largest extractor of fine chemical from biomass) told me that extract ion is the lesser of the problem; harvesting will be the critical economical consideration (assuming we find the cell lines that can make the compound we want. He said that a lower limit of 4% concentration per cell dry organic weight is acceptable. Dr. Maury and two plant managers are arranging schedules to be here at Tech the first week of June to discuss these matters and potential future endeavors.

It is still my personal opinion that filtration systems are the ideal system to develop. My original ideas and approach described in my original research proposal were not developed because a well recognized hydrologist in civil engineering simply discouraged such a mundane approach. Now that we have reviewed the process, I am excited about the prospects of filtration

with extraction potential. The construction of the system for filtration will be conducted over the next two months and tested with actual cultures by mid-summer.

I wish to again point out that Dr. Maury from Hercules Corp. will be here in June. The details of commercial extraction and processing will be formulated at that time.

Chemical Profiles of Microalgae with Emphasis on Lipids

Progress Report

for period March 15, 1983 - April 15, 1983

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

April 15, 1983

Prepared for
Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose:

The research is directed towards identifying algae that have the highest producing potentials.

Progress Report:

The chemical evaluation of all of the species reported in our quarter report to SERI has been completed with the exception of Isochrysis. Additional data and reports are being compiled for the investigators that supplied us the organisms. We are still attempting to definitely identify the major component in Isochrysis. This component is about 90% characterized at this time. Much time was spent in purifying the component for definitive analysis. We are now is the process of obtaining mass spectral data on the sugar components freed from acid hydrolysis of the glycolipids and their bound fatty acid forms. These analyses were delayed until we could get time on the mass spectrometer in our building. Much of our delay in getting the over-all data out on these organisms is the fact that we have many samples and only one gas chromatograph (GLC). Once we get the GLC set up, for example sygar analyses, we try to run all of the sugars, then we convert it over for acid analyses and so forth.

We obtained new algal species from Bill Thomas and from John Ryther. The analyses on these organisms has begun.

We are also doing cloning experiments on algae on agar surfaces seeded with bacteria to select algal strains that may produce chemicals that are inhibitory or stimulatory to bacterial growth. Lateral experiments are searching for the identifications of the DNA entities in the algae (chloroplast/mitochondria/nucleus) and viruses of algae that may act as algae genetic vectors. The latter work in conjunction with the SERI supported project is being funded by Department seed funds. These are essential preliminary steps in establishing a sound algal genetic program for the ultimate maximization of specific products.

At the present time, the algal species under investigation are not demonstrating production capacities that are encouraging for applied productions of lipids. We must step up our coordinated screening programs and to select "appropriate" strains for study.

At this time, β- carotene, the unknown component in Isochyrsis, and the prolific agar producing Gracillaria, are our only promising compounds. We have not yet tapped into the prolific lipid-producing class of algae that are amendable to pilot scale systems. I am confident that the high lipid-producing algae exists somewhere in our immediate environment. Are we looking in the right places?

Enclosed are tables of the fatty acid contents that were not included in our report to SERI. One interesting aspect is that there is an increase in the proportion of longer chain fatty acids in nitrogen deficient cultures. Another interesting aspect is that the degree of unsaturation in fatty acids is increased markedly when the cells are cultivated in higher salt concentrations. This is demonstrated in Botryococcus. Previously,

polyunsaturation was reserved for marine organisms. We now demonstrate that freshwater organisms will do the same at high concentration of salts demonstrating environment influence on lipid biosynthesis.

With respect to research progress on process and recovery technique, there is none. The reason is simply that we do not have any test systems that are worthy of development. In a more pragmatic sense, however, we have been studying the problem from a design approach. We are currently trying to assemble a research scheme for conducting experiments in this area for unspecified species of algae. A report will be submitted when it is completed. The program is in rough copy at the present time.

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS

IN CHLOROFORM ELUATE OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NaCl NE	NaCl ND	1.38M NaCl NE
)	6.1						
),14:1	7.7		3.6	44.0	15.1	16.7	8.6
)	1.6			7.4			
)	10.8	8.5	27.8	20.1	9.2	10.8	21.0
1	4.3	1.7		5.0	4.0	2.7	4.1
2	4.1						
3	10.8						
0							
0,18:2	28.1	50.0	35.2	11.3	23.1	28.1	9.4
1				2.6			
3							12.5
4							
0,20:1	2.2	2.5	20.7	7.8	6.0	5.7	
2					15.9	14.3	
3	2.3						
4							
5							5.0
6					17.8	9.4	17.7
:0							
:1							
:2							
:3		12.1					
:4							
:5		12.4					
:6							
identified	2.2	12.8	12.7	1.8	8.9	12.3	21.7

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGES OF TOTAL FATTY ACID

COMPOSITION OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater	0.5M NaCl	2M NaCl	2M NaCl	0.5M NaCl		1.38M NaCl
	NE	NE	ND	NE	NE	ND	NE
)	2.0						
),14:1	2.6		1.8	15.4	14.0	17.4	11.9
)				2.5			
)	9.2	7.9	17.8	17.2	12.1	12.7	17.3
1	12.7	7.0	18.7	17.1	16.2	0.9	5.8
2	1.7					0.3	
3	3.6			0.5			
0					0.1		
0,18:2	17.5	19.0	23.4	18.8	10.6	12.8	5.2
1	11.8	13.1	6.1	3.8	2.3	7.1	9.1
3	18.7	11.3	16.2	18.6	11.0	14.4	10.2
4		0.3				1.7	
0,20:1	2.0	2.1	6.9	2.6	3.7	2.7	0.6
2					5.3	4.8	
3	4.9	3.4					1.6
4					0.4	0.4	0.8
5					0.3	0.3	8.7
:6					9.0	6.0	13.3
:0					0.9		
:1							
:2						1.6	
:3		12.7				10.6	
:4	0.6		1.9			0.1	0.1
:5							
:6		0.3	0.3				
identified	12.7	22.9	6.8	3.5	14.0	6.3	15.2

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS IN ACETONE ELUATE OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater	0.5M NaCl	2M NaCl	2M NaCl	0.5M	NaCl	1.38M NaCl
	NE	NE	ND	NE	NE	ND	NE
)							
),14:1			0.4	0.6	20.0	17.0	4.2
)							
)	9.5	13.4	22.3	26.2	11.0	9.8	16.0
1	6.9	6.2	25.3	18.6			13.2
2	1.1					0.8	
3							
0							
0,18:2	13.3	5.2	11.0	14.3	3.4	5.0	5.2
1	14.6	27.0	5.9	2.5	6.6	12.4	14.3
3	41.7	27.8	33.8	35.4	32.4	33.2	6.1
4						5.0	
0,20:1	0.6	1.5				2.4	2.0
2							
3	3.2	3.7					3.6
4							2.4
5						0.9	16.7
:6					8.6	8.5	
:0							
:1							
:2							
:3						1.4	
:4	0.5		0.2				
:5							
:6							
identified	8.6	15.2	1.3	2.4	18.4	3.6	16.3

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS
IN METHANOL ELUATE OF A FEW UNICELLULAR ALGAE

Fatty Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp		Nitzschia sp
	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NaCl NE	ND	1.38M NaCl NE
12:0							
14:0,14:1			1.5	1.6	7.8	18.4	22.8
15:0							
16:0	5.6	1.9	3.4	5.4	16.2	17.6	15.0
16:1	27.0	13.2	30.8	27.8	44.5		
16:2							
16:3				1.4			
17:0					0.4		
18:0,18:2	10.9	1.9	24.1	30.7	5.5	5.4	1.1
18:1	20.7	12.2	12.4	6.3	0.4	8.8	13.0
18:3	14.4	6.2	14.8	20.4	1.1	10.0	12.0
18:4		0.9					
20:0,20:1	3.4	2.3			5.2		
20:2							
20:3	9.5	6.5					1.2
20:4					1.2	1.3	
20:5					0.9		4.4
20:6					0.7		22.2
22:0					2.7		
22:1							
22:2						4.8	0.1
22:3		26.0				30.6	
22:4	1.2		5.6			0.2	0.3
22:5							0.3
22:6			0.9				
Unidentified	7.3	28.9	6.5	6.4	13.4	2.9	7.6

Abbreviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGES OF MAJOR ALIPHATIC

HYDROCARBONS IN THE HEXANE ELUATES OF A FEW ALGAE

:o-)on	Botryococcus braunii		Chlamy-domonas P202	Dunaliella salina	Gracilaria sp.		Isochrysis sp.		Nitzschia sp.	Scenedesmus sp.
	Fresh water NE	0.5M NaCl NE	Freshwater ND	2M NaCl NE	Seawater NE	ND	0.5M NaCl NE	ND	1.38M NaCl NE	Freshwater NE
L									23.6	
L										4.3
)										
L										1.3
)	0.7	0.4	3.1	2.3	~90	~90				0.6
L			54.0	90.0						72.9
)										
L			16.0						35.4	1.3
0										
1										2.3
0										
1									11.1	
2										
0										
0										
0										
0										
:0								1.2		
:0	3.3	3.4								
:0	12.1	12.0					8.1	4.7		
:0	23.6	8.6						1.4		
:0	32.3	32.6					41.4	63.2	2.3	
:1	4.8		21.5				4.4	9.4		1.8
:0	2.7	13.4					9.0	0.3	4.8	
:1										
:0										
:1	8.7	12.0					4.0	3.4		
:2	1.8	3.2					5.0	7.2		
ident.	10.7	14.8	5.4	7.7	~10	~10	28.1	9.3	22.8	17.8

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

Chemical Profiles of Microalgae with Emphasis on Lipids

Progress Report

for period April 15, 1983 - May 15, 1983

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

May 15, 1983

Prepared for

Solar Energy Research Institute

Subcontract No: XK-02149-01

Purpose

The research is directed towards identifying algae that have the highest producing potentials.

Progress report

The analyses of lipid content of test algae are in progress. Mass spectral studies on pure lipid components isolated from the benzene eluate of Botryococcus and Isochrysis are currently being obtained. IR spectra of the purer isolates are also being obtained. The current data demonstrates 50% lipid concentrations in Botryococcus, something not previously reported in laboratory cultured cells. The bulk of this fractions is thought to be a single chemical class of compounds derived from the isoprenoid pathway. Botryococcene is a relatively minor component. Similar type generalizations are also being formulated from other species under study. It now appears that we are focusing in on the fraction of potential commercial importance that are synthesized by most algae. Nitrogen sufficient and deficient cultures of Botryococcus in fresh and saline media have now been harvested and a better comparison of these effects on lipid synthesis can now be accomplished.

Three algal strains reported in the literature as high lipid producers have been obtained and cultivation of these strains has been initiated.

Studies on the harvesting procedure of algae is being evaluated at this time.

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A Fisher Isotemp Muffle Furnace Model 184A, Ser. No. 738 was purchased for $627.77 on 4/22/83. A copy of the purchase requisition is attached.

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Chemical Profiles of Microalgae

with Emphasis on Lipids

Progress Report

for period June 15, 1983 - July 15, 1983

Thomas G. Tornabene
Georgia Institute of Technology
School of Applied Biology
Atlanta, Georgia

July 13, 1983

Prepared for
Solar Energy Research Institute
Subcontract No: XK-02149-01

Purpose

The research is directed towards identifying algae that have the highest lipid producing potential.

Progress Report

1. Seven algal species are being analyzed: Nannochloris, Isochysis, Botryococcus Ankistrodesmus, Spirulina (Bl. gr. bacterium), and 2 Dunaliella spp. The Isochrysis were grown in 1M and 1.5M NaCl; and Botryococcus and D. bardawil in nitrogen sufficient and deficient media. The Botryococcus is being recultivated since there is evidence that the organism had not completely reached nitrogen stravation at time of harvest. The evidence is that the chlorophyll content dropped but the protein concentration remained about the same.

2. The major changes in the lipid contents due to saline or nitrogen concentrations is reflected in the neutral lipids; the polar lipids are relatively constant in relative compositions and only the total amounts are reduced. The individual phospholipids of all green algae, etc. remain fairly constant and we can chemotype the algae by the nature of the phospholipids. The nature of the neutral lipids are more eratic and posing some problems in identifying previously undefined components that have no functional groups. We still do not have the absolute structure of the major neutral lipid in Isochrysis until we position the double bonds, which we are now doing.

The neutral lipid contents (oily hydrocarbons) of Botryococcus are consistently 40-50% of cell dry weight and we have not yet reached the dormancy state. This is the best production yet recorded for laboratory grown strains. The best growth rate in continuous culture are 3 x longer than Isochrysis and demands patience in studying this organism.

The hexane, benzene eluates from unisil columns are being analyzed by GLC, TLC and spectrophotometry; the polar lipids are undergoing characterization by TLC, derivatization and GLC, TLC and paper chromatography with a variety differentiating colorimetric procedures.

3. Several different strains are in cultivation, particularly Monolantis; this organism has been reported to be a prolific producer of lipids.

4. Endotoxin shock analyses are being performed on the lipopolysaccharides of commercially produced spirulina. Preliminary evidence suggests that this "health food" contains endotoxin contents on the order of that found in pathogenic Salmonella bacteria. The lipid contents are relatively unique to photosynthetic organisms but somewhat similar to pathogenic bacteria.

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS

IN CHLOROFORM ELUATE OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NaCl NE	NaCl ND	1.38M NaCl NE
)	6.1						
),14:1	7.7		3.6	44.0	15.1	16.7	8.6
)	1.6			7.4			
)	10.8	8.5	27.8	20.1	9.2	10.8	21.0
1	4.3	1.7		5.0	4.0	2.7	4.1
2	4.1						
3	10.8						
0							
0,18:2	28.1	50.0	35.2	11.3	23.1	28.1	9.4
1				2.6			
3							12.5
4							
0,20:1	2.2	2.5	20.7	7.8	6.0	5.7	
2					15.9	14.3	
3	2.3						
4							
5							5.0
6					17.8	9.4	17.7
:0							
:1							
:2							
:3		12.1					
:4							
:5		12.4					
:6							
identified	2.2	12.8	12.7	1.8	8.9	12.3	21.7

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGES OF TOTAL FATTY ACID

COMPOSITION OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater	0.5M NaCl	2M NaCl	2M NaCl	0.5M NaCl		1.38M NaCl
	NE	NE	ND	NE	NE	ND	NE
)	2.0						
),14:1	2.6		1.8	15.4	14.0	17.4	11.9
)				2.5			
)	9.2	7.9	17.8	17.2	12.1	12.7	17.3
1	12.7	7.0	18.7	17.1	16.2	0.9	5.8
2	1.7					0.3	
3	3.6			0.5			
0					0.1		
0,18:2	17.5	19.0	23.4	18.8	10.6	12.8	5.2
1	11.8	13.1	6.1	3.8	2.3	7.1	9.1
3	18.7	11.3	16.2	18.6	11.0	14.4	10.2
4		0.3				1.7	
0,20:1	2.0	2.1	6.9	2.6	3.7	2.7	0.6
2					5.3	4.8	
3	4.9	3.4					1.6
4					0.4	0.4	0.8
5					0.3	0.3	8.7
:6					9.0	6.0	13.3
:0					0.9		
:1							
:2						1.6	
:3		12.7				10.6	
:4	0.6		1.9			0.1	0.1
:5							
:6		0.3	0.3				
identified	12.7	22.9	6.8	3.5	14.0	6.3	15.2

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS
IN METHANOL ELUATE OF A FEW UNICELLULAR ALGAE

	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp		Nitzschia sp
Fatty Acid	Freshwater NE	0.5M NaCl NE	2M NaCl ND	2M NaCl NE	0.5M NaCl NE	ND	1.38M NaCl NE
12:0							
14:0,14:1			1.5	1.6	7.8	18.4	22.8
15:0							
16:0	5.6	1.9	3.4	5.4	16.2	17.6	15.0
16:1	27.0	13.2	30.8	27.8	44.5		
16:2							
16:3				1.4			
17:0					0.4		
18:0,18:2	10.9	1.9	24.1	30.7	5.5	5.4	1.1
18:1	20.7	12.2	12.4	6.3	0.4	8.8	13.0
18:3	14.4	6.2	14.8	20.4	1.1	10.0	12.0
18:4		0.9					
20:0,20:1	3.4	2.3			5.2		
20:2							
20:3	9.5	6.5					1.2
20:4					1.2	1.3	
20:5					0.9		4.4
20:6					0.7		22.2
22:0					2.7		
22:1							
22:2						4.8	0.1
22:3		26.0				30.6	
22:4	1.2		5.6			0.2	0.3
22:5							0.3
22:6			0.9				
Unidentified	7.3	28.9	6.5	6.4	13.4	2.9	7.6

Abbreviations: NE = nitrogen sufficient, ND = nitrogen deficient

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS IN ACETONE ELUATE OF A FEW UNICELLULAR ALGAE

:y Acid	Botryococcus braunii		Dunaliella bardawil	Dunaliella salina	Isochrysis sp.		Nitzschia sp.
	Freshwater	0.5M NaCl	2M NaCl	2M NaCl	0.5M	NaCl	1.38M NaCl
	NE	NE	ND	NE	NE	ND	NE
)							
),14:1			0.4	0.6	20.0	17.0	4.2
)							
)	9.5	13.4	22.3	26.2	11.0	9.8	16.0
1	6.9	6.2	25.3	18.6			13.2
2	1.1					0.8	
3							
0							
0,18:2	13.3	5.2	11.0	14.3	3.4	5.0	5.2
1	14.6	27.0	5.9	2.5	6.6	12.4	14.3
3	41.7	27.8	33.8	35.4	32.4	33.2	6.1
4						5.0	
0,20:1	0.6	1.5				2.4	2.0
2							
3	3.2	3.7					3.6
4							2.4
5						0.9	16.7
:6					8.6	8.5	
:0							
:1							
:2							
:3						1.4	
:4	0.5		0.2				
:5							
:6							
identified	8.6	15.2	1.3	2.4	18.4	3.6	16.3

breviations: NE = nitrogen sufficient, ND = nitrogen deficient

CHEMICAL PROFILES OF MICROALGAE WITH EMPHASIS ON LIPIDS

A Subcontract Report

T. G. Tornabene
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Prepared under Subcontract No. XK-2-02149-01

Sept. 1983

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SUMMARY

1. The proximate chemical composition of seven (7) species of fresh water, desert and marine unicellular eukaryotic microalgae grown under controlled conditions was measured with emphasis on the lipids. Ankistrodesmus, Dunaliella spp., Isochrysis, Nannochloris and Nitzschia contained a range of 16 to 38% proteins, 9 to 56% carbohydrates, and 7 to 55% lipids of organic cell weight. Botryococcus braunii contained about 45% lipids. The effect of cultivations on proximate chemical composition of Botryococcus braunii, Dunaliella salina and Isochrysis in nitrogen deficiency medium was a decrease in protein content and an increase in carbohydrate content, of Dunaliella salina, a decrease in lipids, and of Isochrysis, an increase in lipid content. Cultivation of Dunaliella salina in medium with increased concentration of sodium chloride induced the accumulation of osmotic glycerol with minimal effect on the other constituents. In Botryococcus and Isochrysis, sodium chloride stress was mainly expressed via reduction in the protein content. The lipids of Botryococcus braunii, Isochrysis sp. and nitrogen deficient cultivated Dunaliella bardawil consisted of relatively high concentrations of neutral lipids with multiple branched hydrocarbons predominating. The polar lipid composition of glycolipids and phospholipids of all species investigated was fairly typical of photosynthetic eukaryotic algae in general. The fatty acid compositions were species specific with changes occurring in the relative intensities of individual acid chains of cells cultivated under different cultivation conditions and growth phases. All species synthesized C14:0, C16:0, C18:1, C18:2, and C18:3 fatty acids, C16:4 and C18:4 in Ankistrodesmus sp.; C18:4 and C22:6 in Isochrysis sp.; C16:2, C16:3 and C20:5 in Nannochloris sp.; C16:2, C16:3 and C20:5 in Nitzschia. Nitrogen deficiency and salt stress induced the accumulation of C18:1 in all treated species and to some extent C20:5 in Botryococcus braunii. The data presented provides additional evidence to the concept of biochemical classification and its control by environmental factors.

2. The nature and quantity of lipids and lipopolysaccharides of a commercial preparation of Spirulina platensis was examined. The organic cell weight consisted of 50% protein, 8.8% carbohydrate and 16.6% lipids. The chlorophyll to carotenoid ratio was 0.3. The lipids consisted of a diverse assortment of pigments, hydrocarbons, glycolipids, and phosphatidyl glycerol. The fatty acid contents were predominantly even-numbered chains with a relatively high proportion of polyunsaturated fatty acids. The fatty acids of the lipopolysaccharide preparation were similar but dissimilar from those of bacterial LPS by the absence of hydroxy fatty acids and the presence of polyunsaturated acids. The carbohydrate content of LPS consisted of hexoses, heptose, octulosonic acid and glucosamine. Assays of the LPS preparation by the Limulus amoebocyte lysate test and by the lethal toxicity test in mice demonstrated that the LPS of Spirulina was less endotoxic than the LPS fraction of Salmonella typhi.

3. Lipopolysaccharides (LPS) of isolates of the cyanophyte Microcystis were extracted with phenol/water and purified. Cesium chloride gradient ultracentrifugation of these preparations yielded only one fraction. The LPS contained significant amounts of 3-deoxy-D-manno-octulosonic acid, glucose, 3-deoxy sugars, glucosamine, fatty acids, fatty acid esters, hexoses, and phosphate. Heptose, a characteristic sugar component of the polysaccharide moiety of LPS of most Gram-negative bacteria was absent. Lipopolysaccharides and lipid A hydrolysate of LPS preparations were active in mouse lethality and Limulus lysate gelation. The lipid A moiety was slightly less active in toxicity and Limulus lysate gelation assays

than the intact LPS. The LPS and lipid A moiety of the two isolates of M. aeruginosa were less active in toxicity in mice and Limulus test than LPS of Salmonella abortus equi.

4. Lipid of macroalgae represented from 3.6 to 0.3% of the cellular composition. The nature and quantity of the lipids were the minimum required for maintaining biological integrity of the cellular membranes. The carbohydrate content of cells ranged from 48 to 73%. Multiple extractions of the cells for carbohydrate were performed and the general chemical nature of the carbohydrate compositions was determined.

5. Efficient separators for harvesting algae were evaluated. There are at present no low-cost effective techniques for algal cultivation. Initial studies with ceramic pipes with controlled porosity were tested. New apparati are being constructed to test the feasibility of their use in algal harvest.

1.0 CHEMICAL PROFILES OF SELECTED MICROALGAE

1.1 Introduction

The proximate chemical composition of eukaryotic algae is generally regarded as species specific and that it is usually regulated by environmental factors. Spoehr and Milner (1949) first studied the cellular composition of Chlorella grown under different physiological conditions and indicated a general trend of protein decrease and lipid increase on nitrogen starvation. Subsequently, Milner (1953) confirmed the same phenomenon in other algae but with concurrent accumulations of cellular carbohydrates in response to nitrogen stress. In the last thirty years additional studies were conducted on the effect of various environmental conditions on the cellular chemistry of many diatoms and a few other algae with similar type responses (Parson et al., 1961; Opute, 1974a,; Handa, 1969; see Lewin, 1962; Stewart, 1974; Aaronson et al., 1980).

Lipids and fatty acid compositions in eukaryotic algae and the effects of environmental factors thereof have been studied with relation to light intensity, temperature, nitrate concentration and various other nutrients (see reviews by Wood, 1974; and Pohl and Zurheide, 1979). Recently, the effect of nitrate and silicate stress and light-dark cycles on the lipid content in a variety of phytoplankton have been reported (Shifrin and Chisholm, 1981). In most algae, enhancement of lipid accumulation occurred during nitrogen deficient conditions. The highest concentration of lipids was reported in Monollantus salina that was deprived of nitrogen for nine days. At low concentrations of nitrogen, Chlorella and Euglena synthesized saturated (16:0) and monounsaturated (18:1) fatty acids, whereas at high nitrogen concentrations the 16:2, 16:3, 16:4 and 18:2 fatty acids predominated. High light intensity and low temperatures caused increases and decreases in total lipids depending on the species. In Chlorella and Euglena the increase in total lipids coincided with the formation of polyunsaturated fatty acids (16:2, 16:3, 16:4, 18:2, 18:3). Neutral hydrocarbons were detected in many algae (Pohl and Zurheide, 1979; Tornabene, 1981) but usually in small concentrations not exceeding 1% of the algal dry weight. The notable exception is the green algae Botryococcus braunii which contains about 15% hydrocarbons per dry weight in its green expoential growth stage and up to 75% hydrocarbons in its brown resting stage (Brown et al., 1969; Maxwell et al., 1968; Knight et al., 1970; Wake and Hillen, 1981; Largeau et al., 1980).

In light of the many influences on the proximate chemistry of eukaryotic algae and the scarcity of data on the specific chemical nature of the components comprising the cellular compositions, a few selected algae were cultivated under controlled conditions. The effects of nitrogen and salt stress on the proximate chemistry and specific lipids were measured. The focus was on the lipids of fresh water, desert and marine microalgal species in an attempt to follow species specific variations or general response of the cellular chemistry to environmental stress.

1.2 Materials and Methods

Organism: Ankistrodesmus sp., W. H. Thomas, Pyramid Lake; Botryococcus braunii, Kutz, UTEX #572; Dunaliella salina, Teod., UTEX #200; Dunaliella bardawil, Ben-Amotz and Avron, ATCC #30861; Isochrysis sp., UTEX #2307; Nannochloris sp.,

W. H. Thomas, Pyramid Lake; Nitzschia sp., W. H. Thomas, Mono Lake.

Growth Conditions: Botryococcus braunii, Dunaliella species and Isochrysis sp. were cultivated in artificial mediums containing NaCl as indicated, 5 mM $MgSO_4$, 0.3 mM, $CaCl_2$, 5 mM KNO_3 or as indicated, 5 mM KCL, 0.4 mM KH_2PO_4, 1.5 M $FeCl_3$, 30 M EDTA, 50 mM $NaHCO_3$, 0.1 mM Na_2SiO_3, 0.1 mM H_3BO_3, 0.1 mg/liter thiamine-HCl, 0.5 mg/liter biotin, 0.5 mg/liter B_{12} and trace metal mix as reported by Guillard (1975). Final pH 8.0. Algae were grown in a temperature controlled growth room (ca 25°C) under continuous illumination with Cool White and Agro-Lite Westinghouse fluorescent lamps (light intensity of about 8 Wm^{-2}).

Nitzschia sp. was cultivated in Mono Lake's artificial medium containing 0.45 M NaCl, 0.24 M Na_2CO_3, 0.18 M $NaHCO_3$, 0.1 M Na_2SO_4, 39 mM KCl, 31 mM H_3BO_3, 10 mM KNO_3, 1 mM KH_2PO_4, 0.14 mM $MgSO_4$, 0.7 mM Na_2SiO_3, 0.21 mM $Ca(NO_3)_2$, 18.5 µM FeEDTA, 0.28 µM $ZnSO_4$, 0.63 µM $CoCl_2$, 0.23 µM $CuSO_4$, 3.17 µM $MnCl_2$, 0.32 µM $(NH_4)_6Mo_7O_{24}$. Final pH 9.3 - 9.7. The cultures were maintained on a 12:12 hrs fluorescent light:dark cycle (light intensity of about 39 Wm^{-2}) at temperature of 20°C.

Ankistrodesmus sp. and Nannochloris sp. were grown in Pyramid Lake's artificial medium containing 20 mM KNO_3, 2 mM K_2HPO_4, 56 mM NaCl, 14 mM $NaHCO_3$, 3.7 mM Na_2CO_3, 0.2 mM $CaCl_2$, 1 mM KCl, 1.5 mM Na_2SO_4, 2.5 mM $MgCl_2$, 0.26 mM NaF, 0.025 mM $Na_2B_4O_7$, vitamin mix and trace metal mix as described above. The culture were grown at 20°C on a 12:12 hrs fluorescent light:dark cycle (light intensity of 39 Wm^{-2}).

Daily sampling for growth measurements was done on aliquots of the culture suspension and included cell counting in a Thomas blood-cell counter, chlorophyll content by extraction with acetone or methanol (Jensen, 1978) and organic weight determination by drying the samples at 60°C and ashing at 540°C.

Unicellular algae were harvested by centrifugation at the end of the logarithmic phase. Wet algal pellets and the seaweed were lyophylized for chemical analysis. Nitrogen deficient unicellular algae were treated similarly but the algae were grown on 0.5 mM KNO_3 to the end of the logarithmic phase, left for about 10 more days at the steady state phase and finally harvested by centrifugation.

Extraction & Fractionation of Lipids

Total lipids were assayed by repeated extraction with methanol-chloroform-water (10:5:4, v/v) (Bligh and Dyer, 1959) modified as previously described (Kates et al., 1964) to complete visual extraction of chlorophyll and other pigments. The lipids were then phase separated by adjustment of the solvent ratios to 10:10:9 (methanol-chloroform-water, v/v). The chloroform phase was evaporated to dryness under a stream of N_2, dried under vacuum and then the weight determined gravimetrically.

Total lipid extracts were then fractionated on heat activated silicic acid columns (Unisil, Clarkson Chemical Company, Williamsport, PA) with hexane, benzene, chloroform, acetone, methanol to improve the resolution of the lipid components by thin-layer and paper chromatography (Tornabene, et al., 1969; Morrison et

al., 1971). The following types of components were eluted: acyclic hydrocarbons (hexane); cyclic hydrocarbons, polyunsaturated acyclic hydrocarbons, fatty acid methyl esters, sterols, and carotenoids (benzene); tri-, di- and mono-glycerides, free fatty acids, and carotenoids (chloroform); glycolipids, chlorophylls a and b and carotenoids (acetone); phospholipids, and chlorophyll c (methanol). The fractions were reduced in volume by flash evaporation and taken to dryness undera stream of N_2 and further dried under vacuum over KOH, or P_2O_5.

Mild Alkaline Methanolysis

Lipid components were deacylated by mild alkaline methanolysis according to the procedure described by Tornabene and Ogg (1971). Fatty acids were recovered from the chloroform layer.

Acid Hydrolysis of Deacylated Water-Soluble Products

Water-soluble products obtained from alkaline hydrolysis of lipid components were hydrolyzed with 2 M HCl at 100°C for 1 hr. The hydrolysates were taken just to dryness in a stream of N_2 and then dissolved in methanol-water (10:9, v/v).

Paper Chromatography

The acid hydrolysates were chromatographed on Whatman No. 1 paper with pyridine-ethyl acetate-water (4:10:10, by vol., upper phase). Compounds were detected by Ninhydrin or alkaline $AgNO_3$ (Trevelyan et al., 1950).

Thin-Layer Chromatography

Total and column-fractionated lipids, as well as hydrolyzed lipids, were studied by thin-layer chromatography on 20 cm x 20 cm glass plates coated (0.6-1-mm layers) with silica gel G or precoated hard-layered commercial TLC silica gel plates (Supelco, Inc.). Chromatography was carried out in lined jars by the ascending method using solvent mixtures: a) hexane-benzene (9:1, by vol); b) petroleum ether-diethyl ether-acetic acid (90:10:1, by vol); c) diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, by vol) as first solvent and hexane-diethyl ether (96:4, by vol) as second solvent for separating non-polar lipids; d) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by vol) for separation of polar lipids; and e) technical chloroform (0.75% ethanol) for separation of alkyl-lipid chains (Tornabene et al. 1982). Spots were visualized by exposure to I_2 vapors, acid charring, ninhydrin for amino acids, molybdate for phosphates, Draggendorff for quartenary amines, α-napthol solution for glycolipids, and sulfuric and acetic acid for sterols and sterol esters as previously described (Kates, 1972).

The deacylated water-soluble products were separated on cellulose thin-layer chromatographic plates (Eastman chromatograms 6064, Rochester, N.Y.) with solvents of 3.8 mM EDTA and 0.7 M NH_4HCO_3 in 90 mM NH_4OH containing 67%, by vol, ethyl alcohol in the first dimension and isobutyric acid-water-concentrated NH_4OH (66:33:1, by vol) in the second dimension (Short et al. 1969). The compounds were detected by o-tolidine method over stained with acidic ammonium molybdate solution as previously described (Burrow et al., 1952).

Analytical Method

Glycerol was determined by periodic oxidation followed by treatment with acetylacetone as previously described (Ben-Amotz and Avron, 1978). Carotenoids were extracted according to Jensen (1978) and assayed by using $E^{1\%}_{1cm}$ of 2273 at 480 nm. Protein was assayed as previously described by Lowery et al., (1951) or by Kochert (1978a) after hydrolysis in NH_4OH for 1 hr at 100°C. Total carbohydrates were analyzed by the phenol-sulfuric acid method following acid hydrolysis in 2N HCl for 1 hr at 100°C (Kochert, 1978b). Extended hydrolysis of up to 8 hrs did not produce detectable increase in the carbohydrate concentration.

Fatty acid methyl esters were prepared by esterification with 2.5% methanolic-hydrochloride (Kates, 1964). Sugars freed from the lipids by acid hydrolysis were converted to alditol acetates (Albersheim et al., 1967). Derivatized lipids and sugars were analyzed on a Varian 3700 gas-liquid chromatograph equipped with flame ionization detectors and a Varian CDS 401 data system. The following columns were used: (a) 99 m x 0.75 mm stainless steel capillary column coated with 3% OV-17 running at 15 psi of He from 150°C to 250°C at 4°C/min and held isothermally, (b) 32 m x 0.25 mm fused quartz capillary column, coated with 0.25 mm OV 351 (J. & W. Scientific, Inc.) operated at 8 psi of He and 125°C to 220°C at 4°C/min and held isothermally, (c) 30 m x 0.249 mm fused quartz silica capillary column, coated with 0.25 nm DB-5 (J. & W. Scientific, Inc.) operated at 8 psi of He from 125°C to 230°C at 4°C/min and held isothermally, (d) 2 M x 0.31 cm glass column packed with 10% SP2330 on gas chrom W AW and operated at 27 psi of He and 4°C/min from 110°C to 250°C and held isothermally.

1.3 Results

Growth and Yield of Algae

The growth conditions, specific growth rates and cellular yields are summarized in Table 1. Under non-limited nutrient conditions Dunaliella salina was the fastest grower of all species studied while Botryococcus braunii was the slowest in growth. Salt stress inhibited growth rate of all species tested but it did not affect the total cell yield in mg organic weight per liter (Table 1). Nitrate deficiency did not inhibit the logarithmic growth rate until the culture reached nitrogen deprivation. The growth rate was then in the steady state phase with the resulting total yield being lower than that of the control.

Most algae, grown under optimal nitrogen sufficient conditions contained about 4% chlorophyll and 1% carotenoids per organic cell weight with a carotenoid to chlorophyll ratio of about 0.25 (Table 2). Growth under stress conditions of nitrogen deficiency or high salt concentration decreased the chlorophyll concentration more markedly than the carotenoids producing yellowish-whiteish cells. Dunaliella bardawil was the only species which synthesized β-carotene under stress condition producing reddish cells with a very high carotenoid to chlorophyll ratio (Table 2) as previously described by Ben-Amotz and Avron, (1983).

Proximate Cellular Composition

The results of the analyses of the proximate chemistry of the algae are summarized in Table 3. Cells grown on nitrate limitation generally contained less protein.

In Dunaliella, the decrease in the protein content under nitrate starvation coincides with a decrease in lipid content and an increase in carbohydrates. Both Dunaliella bardawil and Dunaliella salina contained over 40% carbohydrates. Botryococcus braunii contained 44.5% lipids which increased to 54.2% under nitrate limitation. Nitrogen starvation induced lipid and carbohydrate accumulations in Isochrysis from 7% to 26% and from 11.2% to 20.5% of the organic cell weight, respectively. These increases were apparently at the expense of cellular protein which decreased from 37 to 23.3%. Glycerol was observed only in Dunaliella and its concentration varied with the external salt concentration. The proximate composition of Botryococcus braunii and Isochrysis was somewhat affected by salt stress with the major response being in the reduction of the protein content. Protein biosynthesis in Dunaliella, however, was relatively insensitive to extracellular salt concentrations. The proximate compositions of Ankistrodesmus and Nannochloris cultivated on nitrate sufficient medium contained 31.4% and 33.1% protein, respectively, and about 20% lipids. Nitzschia grown in a high Na^+ medium contained smaller quantities of protein, lipid and carbohydrate. A major fraction of the Nitzschia composition was not identified (Table 3).

Lipid Composition

The total lipid extracts of each alga were fractionated on silicic acid columns with hexane, benzene, chloroform, acetone and methanol. The distribution of the lipids are summarized in Table 4. The composition of each fraction is described below.

Neutral Lipids

Hexane eluates. The quantity of components isolated in the hexane eluates was the greatest in Botryococcus braunii lipids and the least in the Dunaliella spp. The hexane fraction of Botryococcus represented about 15% of the total lipids (Table 4). The hexane fraction of Isochrysis grown in 0.5 m NaCl medium under nitrogen depletion, comprised about 2.2% of total lipid while under nitrogen sufficient conditions it represented 1.4%. The concentration of acyclic hydrocarbons in the other species was lower than 1%. The hexane fraction of Botryococcus contained long chain aliphatic hydrocarbons identified by GLC as C29:0, C30:0 and C31:0 (Table 5) as previously reported (Wake and Hillen, 1981). Similarly the hydrocarbons in the hexane eluate of Isochrysis (Table 5) were identified as those ranging from C27:0 to C34:0 with C31:0 as the predominant component. The qualitative composition of the hydrocarbon components in Isochrysis grown at different salinities and on different nitrate concentration were closely comparable, however, there were variations in the relative percentages of the components. The hexane eluate of Dunaliella salina contained C17:0 and C17:1 hydrocarbons (Table 5), similar to that previously reported (Tornabene et al., 1980; Fried et al., 1982). The hexane eluates of Ankistrodesmus contained predominantly C23:0 and C27:0 hydrocarbons. Nitzschia grown on high salt medium contained C21:0, C25:0 and C27:0 as the major hydrocarbon components.

Benzene eluate. The benzene eluates were the major fraction of the neutral lipids of all the algae as well as the major fraction of the total lipids of most of the algae. The benzene eluate comprised 46-53% in Botryococcus braunii, 49.8% in nitrogen deficient Dunaliella bardawil, 0.4 to 24.8% in nitrogen deficient Dunaliella salina, and 13.6 to 32% in Isochrysis lipids (Table 4). The TLC distributions

of the components comprising the benzene eluates is shown in the composite tracings in Figs. 1A and 1B. The chromatogram contained pigmented components and various components of which only a few have been identified. The major component of Dunaliella bardawil was β-carotene (R_f 0.89). The major component of Isochrysis sp. was a oxygenated cyclic C-37 isoprenoid chain (R_f 0.27) the identity of which has not been fully illucidated. The major components of the benzene eluate of the lipids of Botryococcus and nitrate deficient Dunaliella salina (Fig. 1A) were neutral lipids with relative high polarity (R_f 0.05-0.08). These components and a large number of apparently branched, unsaturated components in the range of C_{15}-C_{37} exists in the benzene eluate of all the algae. These components have not been identified but preliminary studies by various chromatographic and spectral procedures indicate they are a complex mixture of high molecular weight isoprenoid compounds and isoprenoid derivatives.

Chloroform eluate. The chloroform eluates of the microalgae comprised from 3.4% to greater than 50% of the total lipids (Table 4). The variation in the concentrations of the components in the chloroform eluates of cells cultivated in media of different nitrogen and salt levles were significant but without a correlated pattern (Table 4). In Botryococcus the increase in contents of chloroform eluates correlated with increases in saline solution concentrations, while in Dunaliella salina it correlated with either saline or nitrogen concentrations. Conversely, in Isochrysis, the contents of the chloroform eluates decreased in nitrogen deficient cells but increased in medium with higher salt concentration. Identification of the components by TLC was made difficult by the presence of most of the carotenoid pigments which exhibited a wide range of polarities. Specific components did cochromatograph with authentic standards and were tentatively identified as tri-, di- and mono-glycerides, sterols and free fatty acids. Triglycerides and free fatty acids were the principal components of these fractions.

Polar Lipids

Acetone eluate. The acetone eluates collected from the silicic acid columns comprised different concentrations of lipids from a minimum of about 9% in Botryococcus braunii to 77% in D. salina when grown on 0.5 m NaCl and enriched with nitrate (Table 4). The TLC separation of the acetone eluates are shown in Figs. 2A and 2B. The major spot in all eluates was digalactosyldiglyceride (R_f 0.39) and monogalactosyldiglyceride (R_f 0.87). A relatively small amount of phosphatic acid (R_f 0.58), phosphatidyl ethanolamine (R_f 0.48), phosphatidyl glycerol (R_f 0.32), phosphatidyl choline (R_f 0.23), and phosphatidyl inositol (R_f 0.17) exist in most of the eluates. The remaining component on the chromatogram are chlorophyll a and b and an assortment of yellow to orange pigments.

Methanol eluate. The methanol eluate contained most all (about 98%) of the phospholipids (Table 4 and Figs. 3A and 3B). The components were identified by cochromatography with standards and differential colorimetric stains for primary amines, phosphates and sugars. The phospholipids detected in most samples were phosphatidylinositol (R_f 0.11), phosphatidylcholine (R_f 0.22), phosphatidylglycerol (R_f 0.29), phosphatidylethanolamine (R_f 0.36) and diphosphatidylglycerol (R_f 0.41). Chlorophyll c (determined also by spectrophotometrical procedures) and yellowish-orange pigments were also visible in the methanol eluates. Spots also cochromatographed with a Sulfoguinovosyl standard (R_f 0.06). Whether or not glycerol trimethylhomoserine (Evans et al., 1982) was present was not determined in the analytical systems employed. The identities tentatively assigned to the phospholipids

and glycolipids were supported by further studies by two dimensional cellulose-TLC of their deacylated derivatives as described by Tornabene et al., (1971, 1973, 1980). The deacylation of the methanol eluates resolved two additional phospholipids that were not identified by one dimension TLC of the total fraction because of interference of the pigments. These two components were phosphatidylserine and phosphatidic acid. The major phospholipid in all the algae was phosphatidylcholine and phosphatidylglycerol. Phosphatidic acid was not detected in the methanolic eluates of Isochrysis sp. but appeared in trace amounts in the acetone eluate. Relatively small quantities of galactosyl diglyceride and monogalactosyl diglycerides were detected in the methanol eluates as well.

Acid Hydrolysate

Acid hydrolysis of the deacylated methanol-water soluble fractions of the acetone and methanol eluates in 2N HCl for two hours at 100°C and then at 4N HCl for six hours at 100°C released sugars and an amino compound, respectively. The sugars analyzed by paper chromatography and by GLC as acetylated alditols revealed glycerol and galactose in all preparations. Relatively small quantities of mannose and glucose were also detected. No amino sugars were detected, however, most samples contained a nihydrin positive component that cochromatographed on paper with a serine standard.

Fatty Acids

The fatty acid composition of the total lipids is given in Table 6. The fatty acid composition of the lipids eluted with chloroform and methanol from a silicic acid column differed only by moderate changes in the relative intensities of the component. The major fatty acids present in Ankisterodesmus sp. were 16:0, 16:4, 18:1, 18:2, 18:3, and 18:4; in Botryococcus braunii, 16:0, 18:1, 18:3 and 18:3; in Dunaliella bardawil, 16:0, 18:1, 18:2 and 18:3; in Dunaliella salina 16:0, 16:3, 18:1, 18:2 and 18:3; in Isochrysis, 14:0 (14:1), 16:0, 18:1, 18:2, 18:3, 18:4 and 22:6; in Nannochloris, 14:0 (14:1), 16:0, 16:1, 16:2, 16:3 and 22:5; and in Nitzschia,14:0 (14:1) 16:0, 16:1, 16:2, 16:3 and 20:6. Nitrogen starvation increased the percentage of 18:1 in Botryococcus and Dunaliella salina, but only slightly affected is fatty acid in Isochrysis. The effect of nitrogen starvation was expressed to some degree on the relative intensities of the long chain unsaturated fatty acids. The percentage of 20:5 in Botryococcus and Dunaliella bardawi increased. Similar observations were observed in Dunaliella salina except that 20:6 instead of 20:5 was synthesized. The effect of salt stress was significant on the production of 18:1 in Isochrysis and Botryococcus.

1.4 Discussion

All species investigated in this study were photosynthetic algae grown autotrophically on mineral medium containing carbon dioxide (and bicarbonate) as a carbon source and nitrate as a nitrogen source. The principal effort of this study was to determine the effect of nitrogen as well as salt stress on the proximate chemical compositions of the algae. The general conclusion is that the species tested differed in their basic cellular composition when they were exposed to environmental stress. On growth under non-stressed conditions, Botryococcus contained the highest concentration of lipids with the greatest proportion of which was hydrocarbon in nature. All other algae contained lower levels of lipids with an average of 23% per organic weight for the green algae, 12% for Nitzschia sp.

and 7% for Isochrysis sp.

The effect of nitrogen stress on the lipid fraction in the algae cannot be summarized as a single trend. In Botryococcus, the neutral lipids comprised a major proportion of the total lipids (Tables 3 and 4); however, the greatest neutral lipid production occurred in the resting stage and that the greatest amount formed in the conversion of the alga from the green to the brown growth phase, Maxwell et al., 1968; Largeau et al., 1980; Wake and Hillen, 1981; Wolf and Cox, 1981). Cultivation under nitrogen deficient conditions did indeed increase the neutral lipid contents however, the brown growth phase did not occur in this laboratory until storage for 6-9 months. This time period precluded any attempt at a systematic comparative study. In contrast to the 10% increase in the lipids in Botryococcus, there was a drop in the lipid fraction in Dunaliella bardawil and Dunaliella salina to about 10% of the organic weight. These halotolerant green algae shifted towards carbohydrate storage under nitrogen stress. Werner (1977) and Shifrin and Chisholm (1981) indicated a similar observation of carbohydrate storage in nitrogen deficient diatoms and in Dunaliella tertiolecta. On the other hand, Isochrysis accumulated higher fractions of lipids and carbohydrates under nitrogen deficiency, with the lipids comprising about one-fourth of the algal organic cell weight following 10 days of nitrogen starvation. In general, the effects of nitrate deficiency were that the protein content and the chlorophyll level decreased while carbohydrate and lipids exhibited a species specific change.

High salt concentration clearly induced glycerol accumulation in Dunaliella as part of the algal osmotic adaptation (Ben Amotz and Avron, 1973; Brown and Borowitzka, 1979) with only a slight effect on the other cell constituents. The osmotic regulator in Botryococcus, Isochrysis and Nitzschia has not yet been identified. The concurrent reduction in protein synthesis under salt stress suggests protein synthesis inhibition allowing accumulation of free amino acids (Brown and Hellebust, 1980).

An interesting observations in this study was the distribution of the lipid fraction along the neutrality-polarity spectrum in relation to species and growth conditions. The neutral lipid content is expressed in the algae that shifts to lipid storage when under environmental stress. These neutral lipids are not predominantly straight chain saturated hydrocarbons but multibranched and/or polyunsaturated components. This was observed for Botryococcus, Isochrysis and Dunaliella species grown under nitrogen stress. The benzene eluates collected from silicic acid columns contained complex mixtures of a very large number of components which ranged from C_{15} to C_{37} carbons. A more detailed study will have to be conducted to isolate and identify all of the lipid components in this fraction. A few of the components occurring in the benzene eluate were isolated from preparative TLC plates and analyzed. β-carotene was a major component in the Dunaliella as previously reported (Ben-Amotz et al., 1983) A C_{37} oxygenated cyclic isoprenoid in Isochrysis that comprised 4% of organic cell weight was isolated but whose identity has not yet been fully elucidated. This component is currently being characterized. It may be speculated that the components comprising the benzene components are intermediates, derivatives or homologues of the biosynthetic pathway of carotenoids (Goodwin, 1980).

The major polar lipids of the algae are those commonly found in photosynthetic algae (Kates, 1970; Pohl and Zurheide, 1979; Evans et al., 1982). Although the concentration of the total phospholipids and glycolipids changed when the cells were

cultivated under nutritional or salt stress, the relative proportion of the individual polar lipid compounds remained fairly constant.

The fatty acid composition of the algae investigated were generally in agreement with the distribution of fatty acid in eukaryotic algae and its differentiation between the algae classes as previously reported (Wood, 1976; Pohl and Zurheide, 1979). Dunaliella salina and Dunaliella bardawil grown under optimal conditions had similar fatty acid patterns with high degrees of unsaturation in the C_{16} and C_{18} range like those previously reported (Tornabene et al., 1980; Evans et al., 1982; Fried et al., 1982). When the same species were grown under nitrogen deficient conditions, however, C_{20} unsaturated fatty acids were present. In contrast to the effect of nitrogen stress on the fatty acids of Dunaliella, salt stress did not change the fatty acid distribution. A notable feature of Dunaliella bardawil relative to other species of Dunaliella, was the high percentage of palmitic acid (C_{16}) irrespective of the growth conditions.

The chrysophyte Isochrysis contained relatively high contents of 22:6 and 22:4 like that reported for Isochrysis galbana (Chuceas and Rilley, 1969). Isochrysis used in the study was the UTEX species #2307, originally a Thaitian strain acclimated to warm water and used as food for shrimp aquaculture (Starr, 1977 in 1982 suppl). The importance of dietry long chain polyunsaturated fatty acids in mariculture (Scott and Middleton, 1979; Takeuchi and Watanabe, 1982) correlated with the high percentages of these essential fatty acids in Isochrysis. It was interesting to note that neither nitrogen stress nor salt stress influenced the production of C_{22} indicating that this long chain polyunsaturated fatty acid is species specific.

The predominant fatty acids in Botryococcus was 16:0, 18:1, 18:3 with only the 18:1 increasing in response to salt stress and nitrogen stress. A similar type of increase was observed in the 18:1 production by Isochrysis, Dunaliella bardawil and Dunaliella salina. Fatty acids of fresh water algae were influenced in a similar way when exposed to low nitrogen levels (Pohl and Zurheide, 1979).

The fresh water algae Ankistrodesmus sp. was capable of synthesizing the 16:4 and 18:4 fatty acids but not the polyunsaturated C20 fatty acids that are common in Nannochloris sp.and in Nitzschia sp. These findings were quite similar to that previously reported (Wood 1974; Pohl and Zunheide, 1979). The predominance of the 20:0 fatty acid in Nannochloris sp. in comparison to 20:5 of Nitzschia closterium and N. angularis (Kates and Volcani, 1966) may reflect the high concentration of Na^+ used to grow the Nitzschia sp. in this study.

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TABLE 1

Growth and Yield of Species Investigated

No.	Species	Conditions	Specific Growth Rate[b] ($.d^{-1}$)	Yield[c] (mg Organic Weight $.l^{-1}$)
1	Botryococcus braunii	FW, NE	1.25	911
2		FW, ND	1.25	623
3		0.5M NaCl, NE	1.12	862
4	Dunaliella bardawil	2M NaCl, ND	1.8	795
5	Dunaliella salina	0.5M NaCl, NE	2.5	823
6		0.5M NaCl, ND	2.5	541
7		2M NaCl, NE	1.9	789
8	Ankistrodesmus sp.	FW, NE	-	-
9	Isochrysis sp.	0.5M NaCl, NE	1.6	843
10		0.5M NaCl, ND	1.6	563
11		1M NaCl, NE	1.4	828
12	Nannochloris sp.	FW, NE	-	-
13	Nitzschia sp.	1.4M Na^+, NE	-	-

[a]FW, freshwater; NE, nitrogen sufficient; ND, nitrogen deficient.

[b]Specific growth rates of 2 and 1.25 represent doubling time of 24 hrs and 72 hrs, respectively.

[c]Ash free dry weight determined at the end of the logarithmic phase of nitrogen sufficient cultures, and about 10 days more following the end of the logarithmic phase for nitrogen deficient cultures.

TABLE 2

Chlorophyll and Carotenoid Content in Unicellular Algae Grown Under Optimal, Nitrate Deficiency, or Salt Stress Conditions

NO.	Species	Growth Conditions	% Organic Weight		Carotenoids to Chlorophyll Ratio
			Total Chlorophyll	Carotenoids	
1	Botryococcus braunii	FW, NE	1.64	0.48	0.29
2		FW, ND	0.46	0.26	0.56
3		0.5M NaCl, NE	0.69	0.22	0.32
4	Dunaliella bardawil	2M NaCl, ND	0.46	2.71	5.89
5	Dunaliella salina	0.5M NaCl, NE	4.27	1.17	0.27
6		0.5M NaCl, ND	0.59	0.39	0.67
7		2M NaCl, NE	4.34	0.95	0.22
8	Ankistro-desmus sp.	FW, NE	5.50	1.00	0.18
9	Isochrysis sp.	0.5M NaCl, NE	1.83	0.64	0.35
10		0.5M NaCl, ND	0.49	0.25	0.51
11		1M NaCl, NE	0.41	0.33	0.80
12	Nanno-chloris sp.	FW, NE	3.88	0.97	0.25
13	Nitzschia sp.	1.4M Na^+, NE	1.44	0.48	0.33

Abbreviations as in Table 1.

TABLE 3

Proximate Cellular Composition of Unicellular Algae Grown Under Optimal, Nitrate Deficiency or Salt Stress Conditions

No.	Species	Growth Conditions	% Dry Weight	% Organic Weight				
			Ash	Protein	Carbohydrate	Lipid	Glycerol	Unknown
1	*Botryococcus braunii*	FW, NE	5.6	22.0	14.1	44.5	< 0.1	19.3
2		FW, ND	7.8	20.6	14.3	54.2	< 0.1	10.8
3		0.5M NaCl, NE	59.6	15.0	13.3	46.3	< 0.1	25.3
4	*Dunaliella bardawil*	2M NaCl, ND	14.7	9.7	40.4	10.4	16.4	23.1
5	*Dunaliella salina*	0.5M NaCl, NE	8.6	29.3	16.3	25.3	9.4	19.7
6		0.5M NaCl, ND	7.7	12.5	55.5	9.2	4.7	18.1
7		2M NaCl, NE	21.7	35.9	12.5	18.5	27.7	5.4
8	*Ankistrodesmus sp.*	FW, NE	4.5	31.1	10.8	24.5	< 0.1	33.5
9	*Isochrysis sp.*	0.5M NaCl,NE	12.0	37.0	11.2	7.1	< 0.1	44.6
10		0.5M NaCl, ND	52.0	23.3	20.5	26.0	< 0.1	30.1
11		1M NaCl, NE	65.9	34.7	15.5	15.3	< 0.1	34.4
12	*Nannochloris sp.*	FW, NE	13.6	33.1	13.2	20.8	< 0.1	32.8
13	*Nitzschia sp.*	1.4M Na^+, NE	20.4	16.8	9.2	12.1	< 0.1	61.8

Analysis was conducted on 5 different algal cultures of each species; statistical analysis showed no standard error of more than 10%. Abbreviations as in Table 1.

TABLE 4

Fractionation of Algal Lipids on Unisil Columns

No.	Species	Growth Conditions	% Total Lipid Weight				
			Hexane	Benzene	Chloroform	Acetone	Methanol
1	Botryococcus braunii	FW, NE	4.6	51.4	4.5	30.0	9.4
2		FW, ND	14.9	52.7	3.4	21.6	7.4
3		0.5M NaCl, NE	5.2	46.0	28.5	9.3	9.7
4	Dunaliella bardawil	2M NaCl, ND	0.1	49.9	14.8	24.1	10.8
5	Dunaliella salina	0.5M NaCl, NE	0.1	0.4	6.3	76.8	16.5
6		0.5M NaCl, ND	0.1	24.8	20.6	31.7	22.8
7		2M NaCl, NE	0.2	2.1	28.2	55.9	13.6
8	Ankistrodesmus sp.	FW, NE	0.7	1.8	39.6	40.9	17.0
9	Isochrysis sp.	0.5M NaCl, NE	1.4	27.4	32.1	26.3	12.6
10		0.5M NaCl, ND	2.2	28.4	18.0	26.0	25.3
11		1M NaCl, NE	1.4	23.6	13.6	43.0	18.4
12	Nannochloris sp.	FW, NE	0.2	5.9	32.5	35.9	25.5
13	Nitzschia sp.	1.4M Na^+, NE	0.2	1.7	51.2	22.0	24.6

Proportions of lipid eluates were determined by GLC for the hexane eluate and gravimetrically for the others. Abbreviations as in Table 1.

TABLE 5

Relative Percentages of Major Aliphatic Hydrocarbons in the Hexane Eluate of Unicellular Algae Grown Under Optimal, Nitrate Deficiency and Salt Stress Conditions

Hydrocarbon	Ankistrodesmus sp.	Botryococcus braunii			D. salina	Isochrysis sp.			Nannochloris sp.	Nitzschia sp.
	FW	FW	FW	0.5M NaCl	2.0M NaCl	0.5M NaCl	0.5M NaCl	0.1M NaCl	FW	1.4M Na^+
	NE	NE	ND	NE	NE	NE	ND	NE	NE	NE
% Organic Weight	0.17	2.05	8.07	2.4	0.04	0.1	0.57	0.21	0.04	0.02
17:0					2.3					
17:1					90.0					
21:0	6.3								18.0	23.6
22:0	3.3									
23:0	18.0									
24:0										
25:0									29.0	35.4
27:0	40.3					1.2	1.3	1.1	9.7	11.1
28:0		4.2	3.0	3.4						
29:0		13.2	13.5	12.0		4.7	8.1	4.1	4.7	2.3
30:0	5.0	22.3	25.2	8.6		1.4			2.9	
31:0		31.0	33.9	32.6		63.2	50.3	66.2	2.9	4.8
31:1		5.4	5.7			9.4	4.4	1.2		
32:0		5.9	8.0	13.4						
32:1		4.2	2.3							
32:2		3.4	2.5							
33:0										
33:1		2.4	2.2	12.0		7.2	5.0	4.8		
33:2		1.8		3.2		3.4	4.0	1.2		
34:0						0.9	1.6	2.0		
Unidentified	27.1	5.3	2.7	14.4	7.7	8.6	25.3	19.4	32.8	22.8

Hydrocarbons were analyzed by GLC on a 99, x 0.75mm OV-17 stainless steel capillary column. D. bardawil ND and NE and D. salina, ND produced none or insufficient quantities to be assayed. Abbr. as in Table 1.

TABLE 6

Relative Percentages of Total Fatty Acid Composition of Unicellular Algae Grown Under Optimal, Nitrate Deficient and Salt Stress Conditions

Fatty* Acid	Ankistrodesmus sp.	Botryococcus braunii			Dunaliella bardawil	Dunaliella salina			Isochrysis sp.			Nannochloris sp.	Nitzschia sp.
	FW	FW	FW	0.5M NaCl	2.0M NaCl	0.5M NaCl	0.5M NaCl	0.2M naCl	0.5M NaCl	0.5M NaCl	1.0M NaCl	FW	1.4M Na^+
	NE	NE	ND	NE	ND	NE	ND	NE	NE	ND	NE	NE	NE
12:0	3.1	3.4	1.1	1.1	5.6	4.0	1.5	3.8				2.8	1.5
14:0,14:1	4.7	4.6	0.8	1.2	7.1	5.4	1.2	5.6	13.3	18.6	11.5	6.3	9.0
16:0	13.1	18.1	13.3	12.3	46.2	14.7	21.0	11.7	11.7	1.4	8.5	9.2	10.4
16:1	3.4	2.0	0.9	1.3	0.9	0.7	0.9	1.9	6.3	2.9	3.8	19.8	20.7
16:2	1.4											6.6	7.9
16:3	1.2				2.6	16.6	10.3	16.2				8.6	5.9
16:4	13.8					7.4	3.3	6.2					
17:0		1.5	2.9	2.8	0.7								
18:0												2.1	1.3
18:1	8.6	27.3	46.3	45.3	17.2	2.2	7.1	4.1	15.0	17.2	21.6	3.6	3.6
18:2	5.4	12.3	5.7	2.4	7.9	10.9	6.2	11.3	3.7	5.0	4.3	1.1	1.7
18:3	28.6	18.1	19.6	10.7	8.1	30.5	38.0	28.4	5.6	6.3	4.4	0.5	0.9
18:4	9.8	1.2							16.6	14.0	17.7		1.1
19:0						1.9	1.7	1.0			0.9		
20:1				1.2	1.2	1.2				1.1			
20:3		1.7	1.1	3.4								2.5	4.4
20:5	1.3	2.7	5.6	7.6	2.1				1.6			26.5	
20:6							1.6						18.2
22:4									1.3	2.7	6.0		
22:6									12.8	14.9	14.6		
Unidentified	6.5	7.1	2.7	10.8	0.4	5.7	7.2	5.0	10.0	5.9	6.7	11.4	13.4

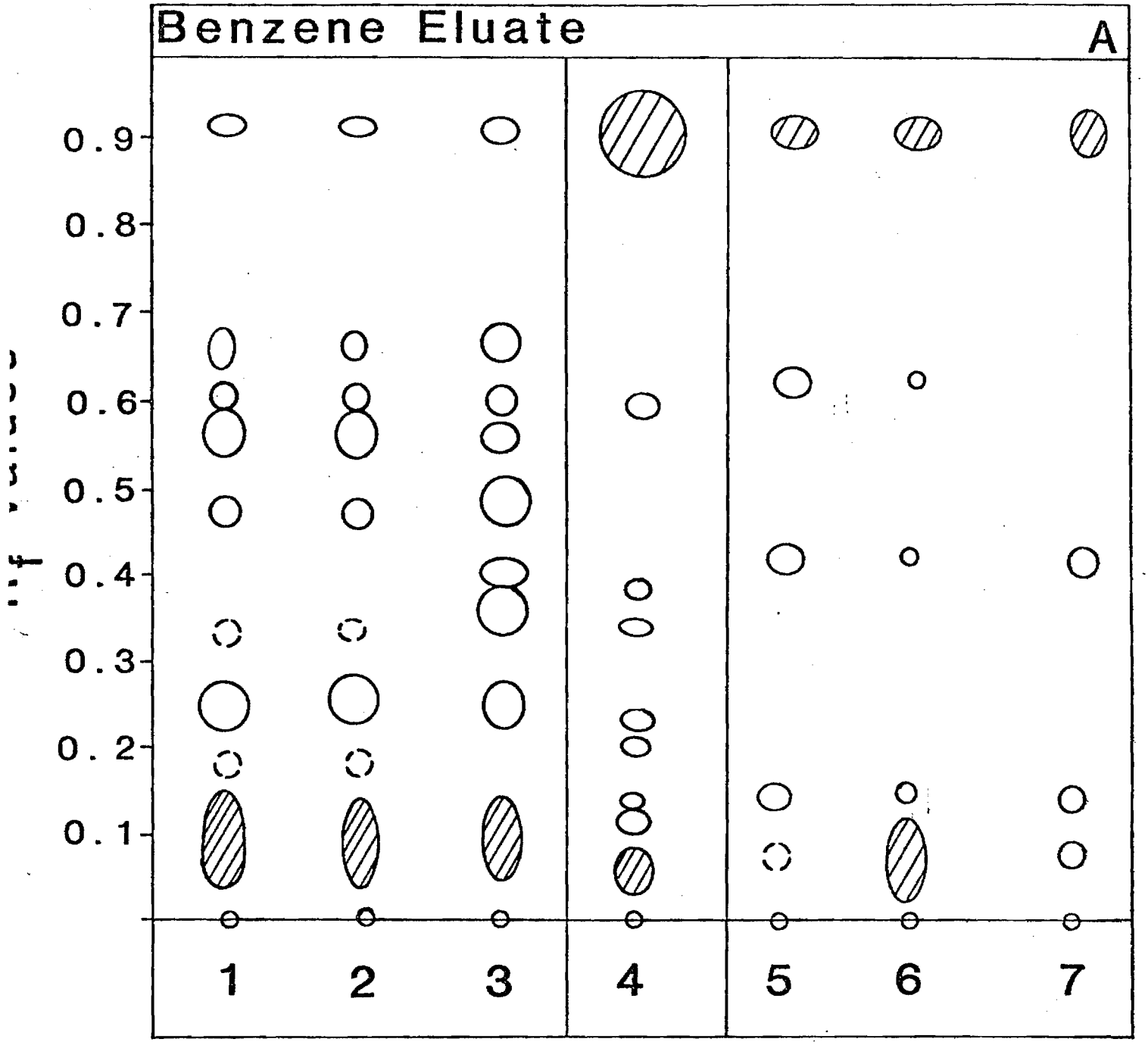
*The values were determined by GLC on a 32m x 0.25mm OV 351 fused quartz capillary column. The first number indicates the number of carbons; the second number indicates the number of unsaturated bonds. Identities were obtained by comparing retention time values to those of standards and by comparing patterns on OV 351 and OV 17 coated capillary columns.

Legends to Figures

Figure 1A and 1B. TLC separation of the benzene eluates collected from silicic acid columns in solvent system (a). See Tables for identification of numbers correlated to the organisms. Standards and their R_f values were: β-carotene, 0.89; squalene, 0.79; cholesteryl palmitate, 0.58; trioleiate, 0.21; myristic acid, 0.13; cholesterol, 0.03.

Figure 2A and 2B. TLC of acetone eluates collected from silicic acid columns in solvent system (d). Abbrev: as in Fig. 1; P = phosphate positive; N = ninhydrin positive. Standards and their R_f values were: monogalactosgyldiacylglycerol (0.87); digalactosyldiacylglycerol (0.39); phosphatidylinositol (0.17); phosphatidylcholine (0.23); phosphatidylglycerol (0.39); phosphatidylethanolamine (0.48); diphosphatidylglycerol (0.54).

Figure 3A and 3B. TLC of methanol eluates in solvent system (d). Conditions and abbreviations are as described in Figure 2. Standards and their R_f values were: phosphatidyl inositol (0.11); phosphatidyl choline (0.22); phosphatidylglycerol (0.29); phosphatidylethanolamine (0.36); diphosphatidylglycerol (0.41).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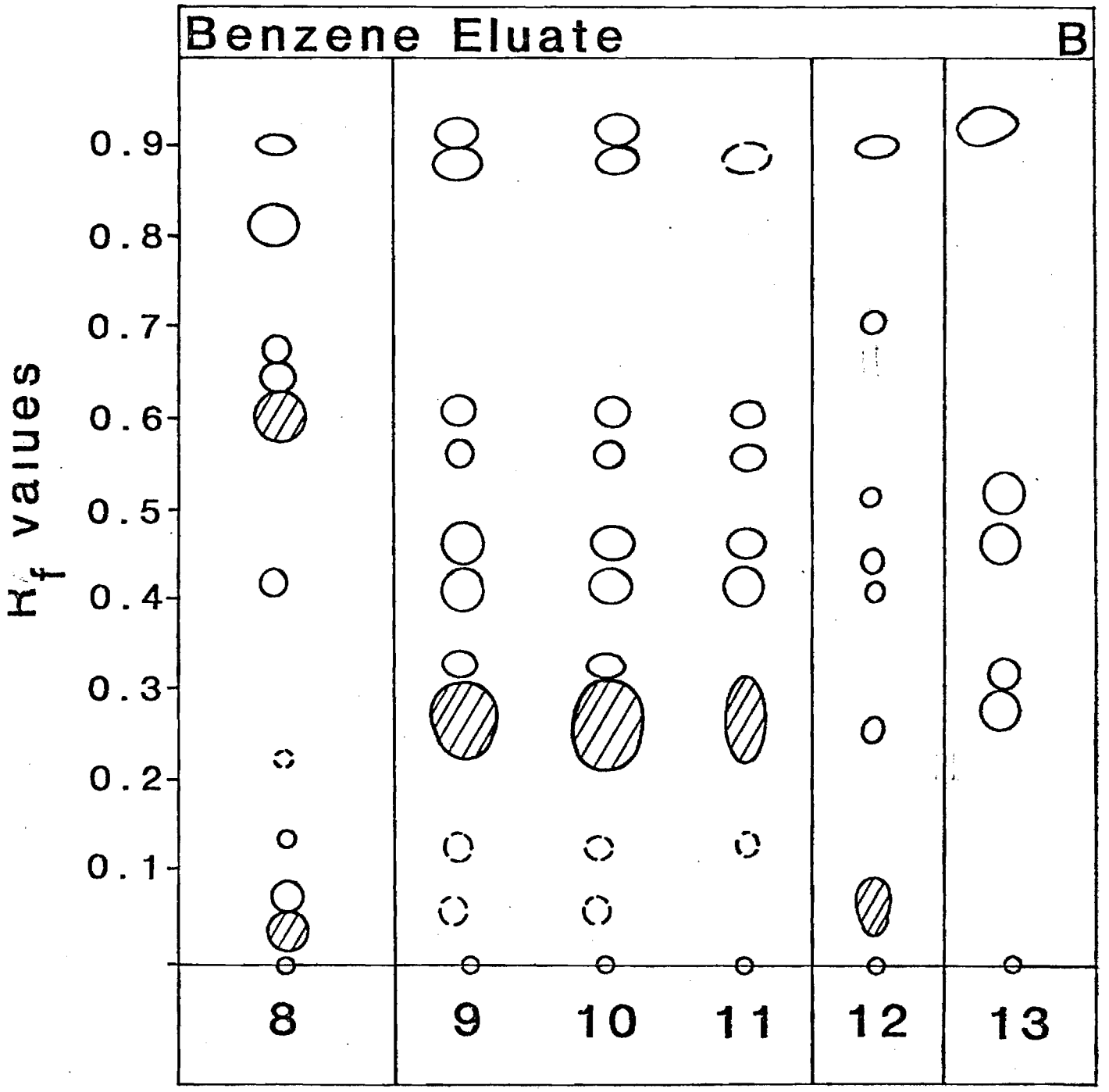
Benzene Eluate
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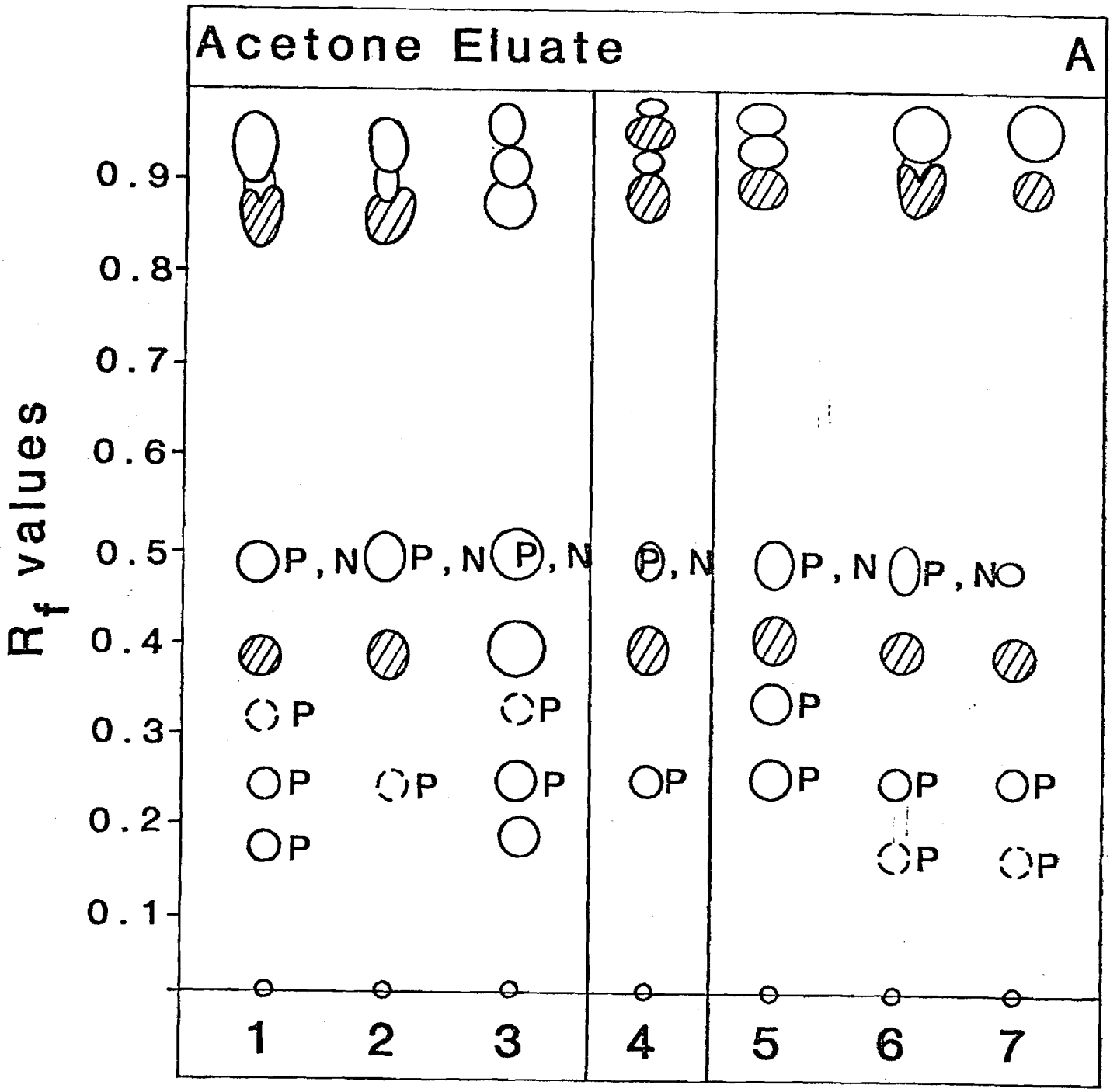
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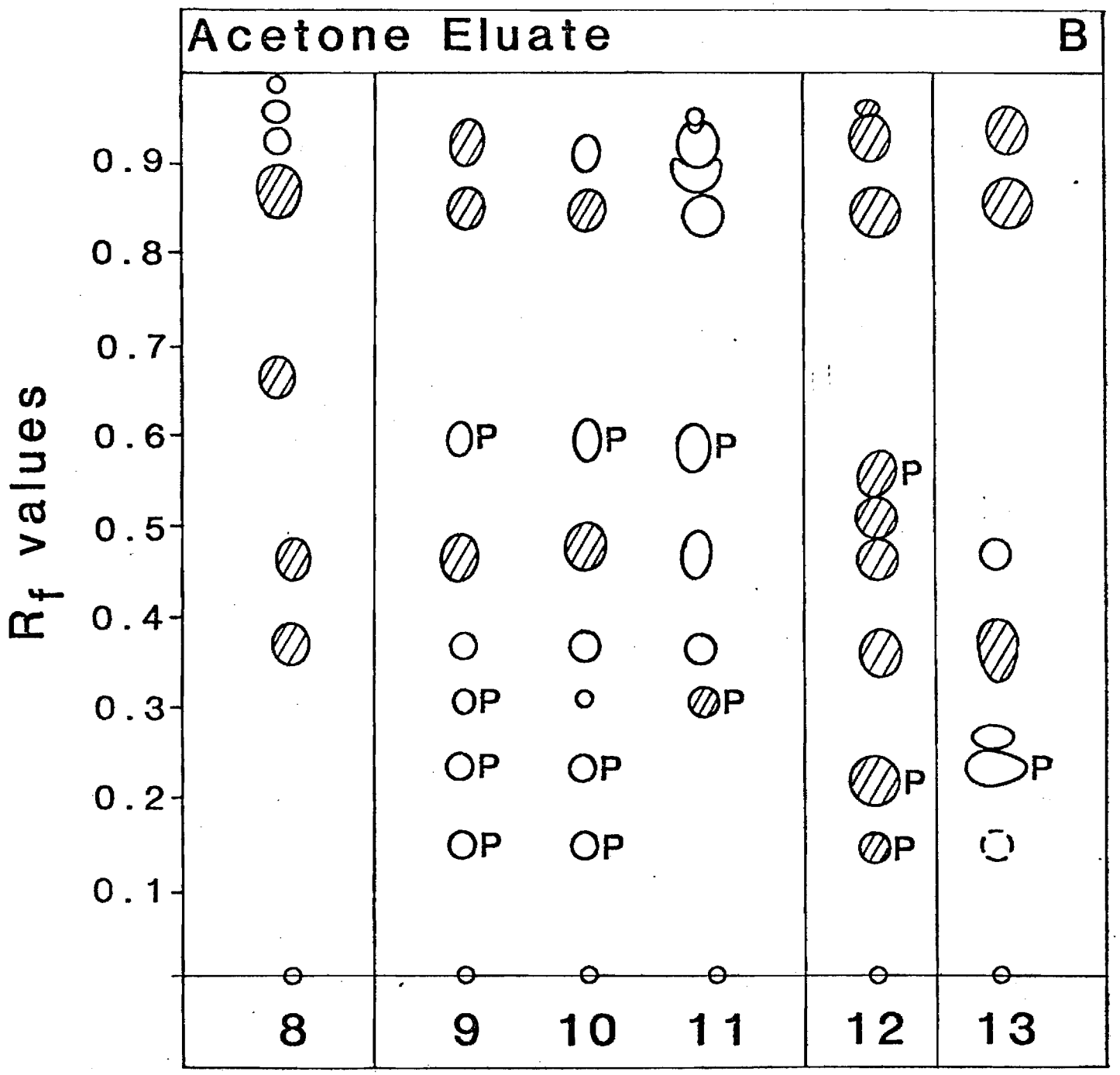
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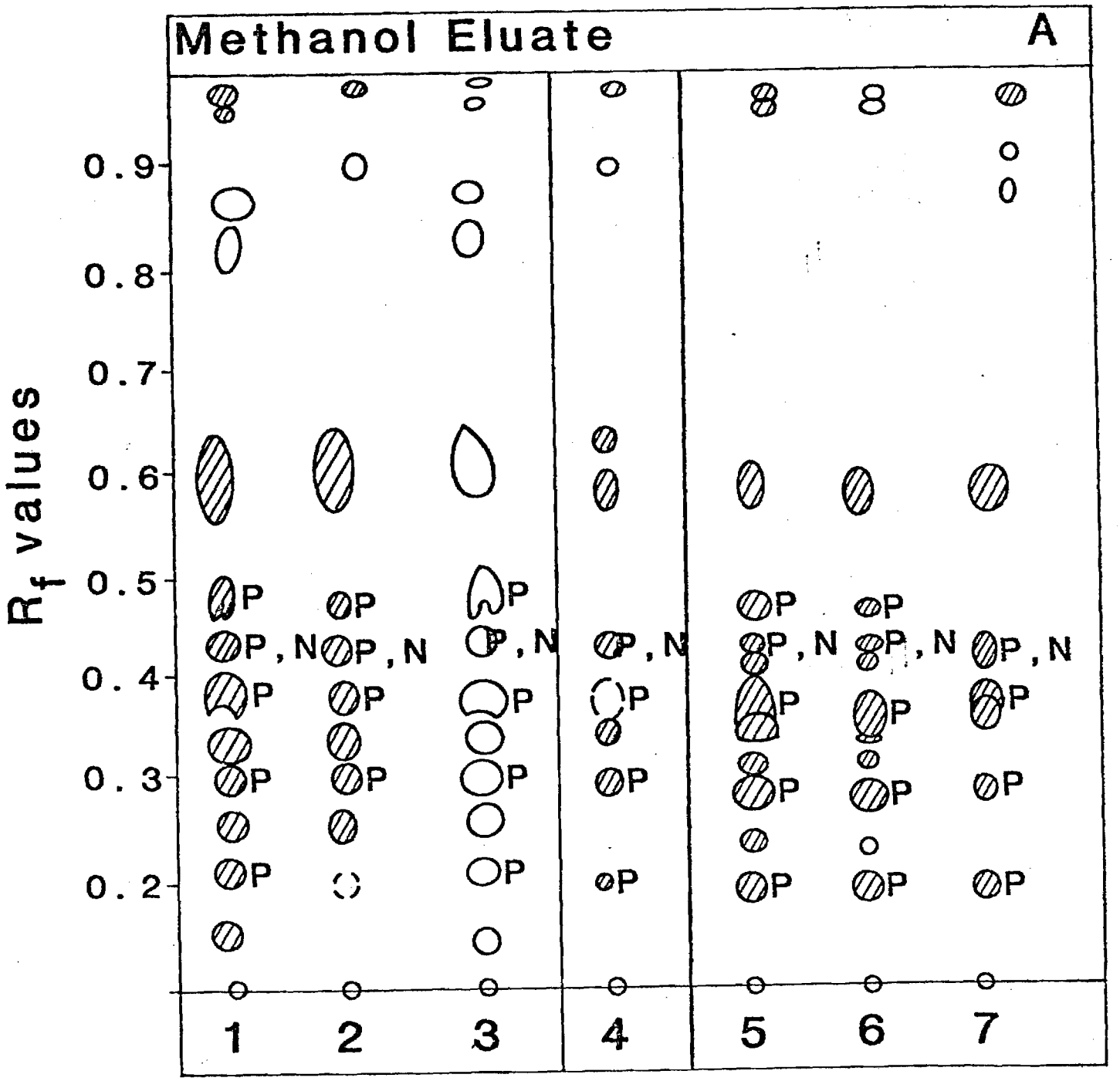
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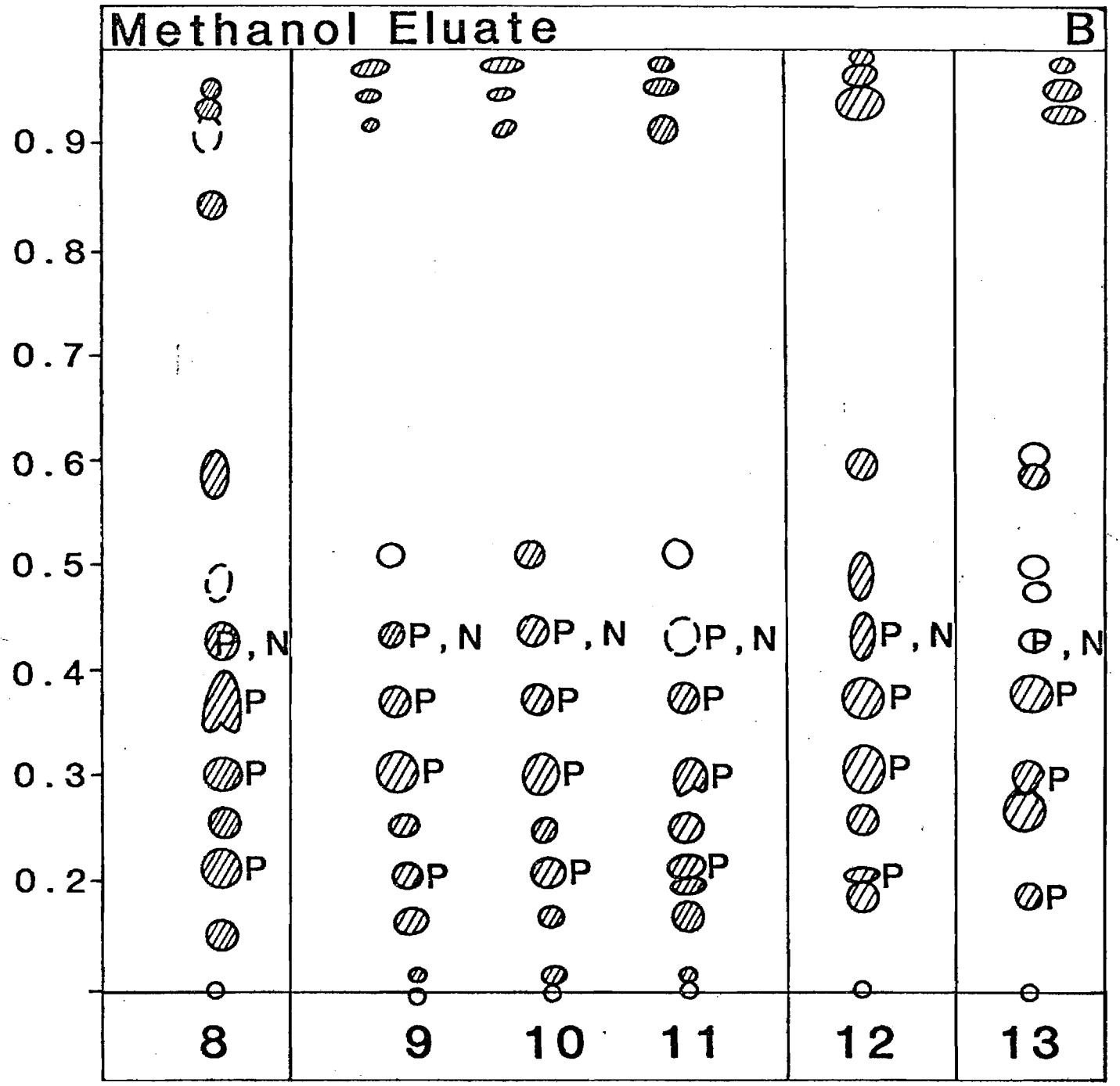
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2.0 LIPIDS AND ENDOTOXIC COMPONENTS OF COMMERCIAL BLUE-GREEN BACTERIUM SPIRULINA PLATENSIS

2.1 Introduction

Spirulina platensis is a cyanobacterium (blue-green bacterium) that often grows in salty lakes and ponds. It contains more than 50% protein and is currently recognized as a protein source for the future. Studies have been done on mass cultivation and extraction of proteins from Spirulina spp (25,,38,39,47) and this cyanophyte is now commercially available.

Biologically, the cyanophytes are curious organisms since they are photosynthetic prokaryotes that contain the basic structure and chemical composition of the cell wall of Gram-negative bacterial while possessing a photosynthetic apparatus like that found in eukaryote. The cyanophytes contain lipopolysaccharides (LPS) (19,29,41-43) that are characteristic components of most Gram-negative bacteria and well recognized as an endotoxic and antigenic component of the cell wall. The lipid compositions are reported to be relatively simple containing principally mono- and digalactosyldiacylglycerides, phosphatidyl diacylglycerol and sulfoquinovosyl (2,26,27). Only a few of the cyanophyte species are recognized as producers of toxic strains (7,12) that cause animal poisonings with the majority of the species accepted as non-toxic strains (7). There are numerous reviews on the properties of cyanobacteria, many of which have been summarized by Stanier and Cohen-Bazere (29).

While there appears to have been solid information acquired on the cyanophytes, it also appears that much more is needed before they can be considered as a source of protein or a feed stock. A major concern is that the cyanobacteria constitutes a large, diverse and widely distributed group of photosynthetic bacteria and that only a few of them have been studied for their proximate chemical composition and properties (7,19,29,41). In spite of the developments in the commercial production of Spirulina, no detailed studies on the lipids and LPS of the commercially available Spirulina have been reported. In this connection, we assayed the lipids and LPS properties of commercially prepared S. platensis.

2.2 Materials and Methods

Organisms and Growth Conditions

S. platensis Lb 1475/4a obtained from the Cambridge Culture Collection was grown in Zarouk's (48) medium modified as described previously (25). These organisms were cultivated by Koor Food Ltd., Israel, in semi-controlled open ponds as described previously (38), harvested, and spray-dried prior to shipment.

Extraction and Fractionation of Lipids

Cell suspensions were repeatedly extracted until complete visual extraction of chlorophyll by the method of Bligh and Dyer, 1959 (4) modified as described previously (18). The non-polar and polar lipids were separated by column-chromatography (34) with hexane, benzene, chloroform, acetone and methanol to improve the resolution of the lipid components by thin-layer and paper chromatography.

The lipid components were isolated from chromatograms by elution with chloroform-methanol, 9:10.

Isolation and Fractionation of Lipopolysaccharide (LPS)

LPS was extracted from dried cells (10 g) by the phenol-water method for isolating smooth-LPS (44,45). The aqueous phases were dialyzed against distilled water and the volume was reduced. An equal volume of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.7) containing 30 mg of ribonuclease (Sigma type I-A) (44) was added to the LPS solution, and the mixture was stirred at 37°C for 16 h. After dialysis against distilled water, the ribonuclease digest was concentrated by centrifugation at 8000 x g for 20 min. The resulting supernatant fluid was centrifuged at 103,000 x g for 4 h, and LPS was recovered as a gel-like precipitate.

Mild Alkaline Methanolysis

Lipid components were deacylated by mild alkaline methanolysis according to the procedure described by Tornabene and Ogg (33), Deacylated lipids were recovered from the methanol-water phase; the fatty acids were recovered from the chloroform layer.

Acid Hydrolysis of Water-soluble Products

Water-soluble products obtained from mild alkaline hydrolysis of lipid components and the LPS preparation were hydrolyzed with 2 M HCl at 100°C for 2 h for neutral sugars and in 4 M HCl for 6 h at 100°C for amino sugars. For amino acids, hydrolysates were hydrolyzed in 6 N HCl for 12 h at 100°C. Ketodeoxyoctulonic acid (KDO) samples were hydrolyzed with 0.025 N sulfuric acid for 1 h at 100°C. For amide linked fatty acids, samples were hydrolyzed in 2.5% methanolic-HCl for 8 h at 100°C and methyl esters recovered as described (16).

Thin-layer and Paper Chromatography

Total and column-fractionated lipids, as well as hydrolyzed lipids, were studied by TLC on 20 cm x 20 cm glass plates coated (0.6 to 1 mm layer) with silica gel G or 0.25 mm precoated Supelco hard layer silica gel plates. Chromatography was carried out in lined jars by the ascending method using the following solvent mixtures: (A) hexane-benzene (9:1), (B) petroleum ether-diethylether-acetic acid (90:10:1), (C) diethyl ether-benzene-ethanol-acetic acid (45:50:2:0.2) as first solvent and hexane-diethyl ether (96:4) as second solvent for separating nonpolar lipids (14); (D) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) for separation of polar lipids (15), (E) technical chloroform (0.75% ethanol) for separation of alkyl-lipid chains. Spots were visualized by exposure to I_2 vapor, acid charring, phosphate spray (37), ninhydrin, α-napthal for glycolipids (17), Draggendorff stain for quartenary amines (17), or H_2SO_4-acetic acid (1:1) for sterols (17).

The deacylated water-soluble products were separated on cellulose TLC plates (Eastman chromatograms 6064) with solvents of 3.8 mM EDTA and 0.7 M NH_4HCO_3 in 90 mM NH_4OH containing 67% (v/v) ethyl alcohol in the first dimension and isobutyric acid-water-concentrated NH_4OH (66:33:1) in the second dimension as previously described (28). The samples were visualized by the o-tolidine staining method overstained with an acidic ammonium molybdate solution (6), or by ninhydrin.

The acid hydrolysates were chromatographed on Whatman No. 1 paper with pyridine-ethyl acetate-water (4:10:10, upper phase). Compounds were detected by ninhydrin or alkaline $AgNO_3$ (36).

Analytical Methods

Total carbohydrates were estimated after acid hydrolysis by the phenol sulphuric acid method (21), with a mixture of equal amounts of D-glucose, D-mannose and D-galactose as standard. Nucleic acids were estimated by the ratio of absorbance at 280/260 nm after hydrolysis of the material with 0.1 N NaOH, with purified RNA (Sigma) as standard (15). Total protein was determined after hydrolysis in NH_4OH at 100°C for 1 h by the Lowry method (23) or by Kochert (20) with bovine serum albumin as standard. 3-Deoxy sugars were assayed by the thiobarbituric acid method of Cynkin and Ashwell (8). KDO was determined by a modification of the method described by Warren (40) with correction for 2-deoxyribose. Glucosamine was assayed by the method of Boas (5). Heptose was measured by the method of Dische (9) as modified by Wright and Rebers (46) using D-glycero-L-Mannoheptose as the standard. Phosphorous was determined by the method of Bartlett (3). Total fatty acids of LPS were determined gravimetrically after hydrolysis in 4 M HCl at 100°C followed by alkaline hydrolysis (24). Chlorophyll and carotenoids were extracted and assayed as described by Jensen (14).

Free fatty acids were converted to the methyl ester form by hydrolysis in 2.5% anhydrous methanolic-HCl (16). Aliquots of fatty acids were hydrogenated in chloroform-methanol (3:1) with H_2 and 10% Pd on activated charcoal. Free sugars were converted to alditol acetates (1).

Derivatized components were analyzed on a Varian 3700 gas-liquid chromatograph (GLC) equipped with dual flame ionization detectors and a Varian Vista 401 data system. The following columns were used: (a) 99m x 0.75mm stainless steel capillary column coated with 3% OV-17 at 4°C/min from 150°C to 250°C and then held isothermally; (b) 30m x 0.252mm fused quartz capillary column, with 0.25 M of OV 351 at 8 psi of He and 4°C/min from 125°C to 220°C and held isothermally; (c) 30m x 0.249mm fused quartz capillary column, with 0.25 M DB-5 at 8 psi of He and 4°C/min from 150°C to 230°C and held isothermally, 2m x 0.31 cm glass column packed with 10% SP2330 on 100/120 Gas chrom W AW at 27 psi of He and 4°C/min from 110°C to 250°C and held isothermally.

The Limulus Amoebocyte Lysate Test

The Limulus amoebocyte lysate (Associates of Cape Cod, Woods Hole, Mass) test was performed by mixing 0.1 ml of Limulus amoebocyte and 0.1 ml of sample in a sterile pyrogen free 10mm x 75mm glass test tube with an aluminum foil cover and incubating the mixture in a water bath at 37°C. The mixtures were examined after 60 min and 24 hrs of incubation. A negative control of pyrogen-free saline and a positive control of S. abortus equi LPS (Sigma Chemical Co., St. Louis, MO) were processed with the samples of S. platensis. Activity was expressed as the lowest concentration needed to form a gel.

The Lethal Toxicity in Mice

Mouse lethality tests of LPS was carried out in C57BL/6 male mice, six to seven weeks of age. The animals were injected intraperitoneally with various concentrations of LPS in 0.5 ml pyrogen free saline (Abbott Laboratories, Chicago, IL).

Six mice were used for each concentration and deaths were recorded after 48 hrs. Toxicity of the S. platensis was compared to the LPS of S. abortus equi. (Sigma Corp.).

2.3 Results

Lipid Composition

The proximate cellular composition of commercial S. platensis is given in Table 1. The major fraction was protein accounting for 50% of the algal weight. Lipid content was 16.6% and carbohydrate content 8.8%. In agreement with previous observations (47), the carotenoids-to-chlorophyll ratio was about 0.3.

The total lipids were fractionated on a silicic acid column to enhance the resolution of the lipid components by TLC. The proportion of lipid components eluted from a silicic acid column with non-polar and polar solvents is given in Table 2. Most of the lipids eluted with acetone and methanol. The combined hexane, benzene and chloroform eluates comprised only 5% of the total lipids. The hexane fraction comprised only 02.% of the total lipids. Three components were identified in the hexane fraction by GLC and GLC-MS. They were pentadecane (5.5%), hexadecane (4.3%), and heptadecane (90.1%) as previously reported (30). No unsaturated and branched hydrocarbons were detected.

The benzene eluate representing about 3% of total lipids (Table 2) consisted of 5 components when analyzed by TLC (Fig. 1). Four of the components were yellow colored pigments; the fourth component, representing a relatively small percent of the fraction, cochromatographed with a cholesteryl-palmitate standard. The component also stained red with H_2SO_4 and acetic acid indicating a sterol component; however, no sterol was recovered after methanolic HCl hydrolysis and analysis by the chromatographic procedures. The component was not identified.

The chloroform eluate comprised only 1.4% (Table 2) of the total lipids and appeared to consist of 4 minor components, 2 of which were yellow pigmented (Fig. 1). Two relatively small components cochromatographed with a triglyceride standard (R_f 0.50) and a hydrocarbon standard (R_f 0.80).

The acetone eluate contained more than one-half of the total lipids (Table 2). TLC analysis of the fraction is shown in Figs. 1 and 2. In addition to the assortment of green, yellow, orange and rose colored pigments indicated in Figs. 1 and 2, several components cochromatographed with authentic standards. On the basis of their R_f values, and staining behavior, they were tentatively identified. Free fatty acids (R_f 0.27), sterols (R_f 0.26) and triglycerides (R_f 8.52) in Fig. 1 and monogalactosyl diglyceride (R_f 0.81), phosphatidyl glycerol (R_f 0.50) and digalactosyl diglyceride (R_f 0.33) in Fig. 2. Two components (Fig. 2) with R_f values of 0.67 and 0.55 did not differentially stain or cochromatograph with standards and they were not assigned a tentative identification.

The methanol eluate (Table 2 and Fig. 2) contained pigments and components that were also found in the acetone eluate. The concentration of the less-polar component that moved the fastest on TLC was greater in the acetone eluate while conversely the slower moving component by TLC were more concentrated in the methanol eluate. Only two phosphates containing components (R_f 0.42

and R_f 0.15) were detected. The component with R_f of 0.42 cochromatographed with phosphatidyl glycerol and it was isolated by preparative TLC. The component with an R_f of 0.15 did not correlate with any of the available standards and it was also eluted from the TLC plate. Mild alkaline hydrolysates from each isolate, however, produced identical water soluble products. They were identified as glycerolphosphoryl glycerol by comparing R_f values to a standard on 2-dimensional cellulose-TLC as previously described (31,32,35). Quantitation of the fatty acid and glycerol content by GLC after acid hydrolysis and derivatization showed that the component with an R_f value of 0.42 consisted of 2 fatty acids and 2 glycerols where as the component with an R_f value of 0.15 had 1 fatty acid and 1 glycerol. The components were therefore identified as phosphatidyl glycerol and lysophosphatidyl glycerol. This same analytical procedure was repeated for components with R_f value 0.32 (cochromatographing with digalactosyldiglyceride) and the one with an R_f value of 0.12 (Fig. 2). The mild alkaline hydrolysates of both components gave products that migrated identically on two-dimensional cellulose-TLC. The components corresponded to a glycerol-digalactose standard. The fatty acid to glycerol ratio for the lipid component with an R_f of 0.32 was 2:1 and only 1:1 for the components with an R_f of 0.12. The components were, therefore, identified as digalactosyl diglyceride and lysogalactosyl diglyceride. A relatively small amount of a component (R_f of 0.21) cochromatographed with Sulfoquinovosyl. A rose colored component (R_f of 0.5) was detected in both the acetone and methanol eluates. Attempts at purifying the components and identifying it, however, were unsuccessful. The component appeared to be labile in subdued light and the standard chromatographic procedures employed.

GLC analysis of the alditol acetates of the acid hydrolysates of the acetone and methanol eluates revealed only two components with retention time values that were identical to acetyl derivatives of glycerol and galactitol.

The fatty acid composition is summarized in Table 3. The fatty acids are principally even-numbered carbon chains with a relatively large proportion of them being unsaturated. The existence of the polyunsaturated chains (Table 3) were substantiated by hydrogenation of the components with a corresponding increase in the C_{14}, C_{16}, C_{18} and C_{20} chains. The lipids fractionated on a silicic acid column had similar fatty acid compositions although the relative intensities of the individual components were substantially varied.

Lipopolysaccharide (LPS) Compositon

The LPS of S. platensis by the aqueous phenol method comprised 1.6% of the cellular dry weight. No measurable amounts of LPS was extracted by the phenol/chloroform/petroleum ether procedures of Galanos et al (40) or by extraction with aqueous butanol. The LPS fraction obtained was soluble in water and 1% Triton X-100 but not in acetone, ethanol or pyridine. The chemical composition of the LPS is given in Table 1. It was free of nucleic acids but contained traces of proteins (0.6%). The total carbohydrate (31.6%) and fatty acids (14.3%) represented 46% of the LPS composition. Sugar analysis revealed the presence of 3-deoxy-D-manno-octulosonic acid, glucose, rhamnose, fucose, ribose, xylose, mannose, galactose, inositol, D-glycerol-D-mannoheptose, D-glycero-L-mannoheptose and 3- or 4-O-methyl hexose. The O-methyl hexose was identified by its mass spectral fragmentation pattern. Glycerol was also identified in the hydrolysate. About seven components that eluted in the GLC chromatogram in the area where deoxyhexose and pentoses elute accounted for 22% of the total fraction analysed. These components were not identified. Glucosamine was the only amino sugar identified.

The fatty acids as methyl esters derivatives were identified by GLC as principally saturated and unsaturated C_{16} and C_{18} fatty acids (Table 5). No hydroxy fatty acids were detected, although 2-OH-C14:0 and 3-OH-C14:0 fatty acid standards had near identical retention time values to sample components as well as standards of 18:2 and 18:3 fatty acids. The identities of the sample acids were delineated after hydrogenation and the concurrent increase in the relative intensities of the corresponding saturated fatty acids confirming that the sample acids were the polyunsaturated ones.

Toxicity Measurement

The biological activity of the LPS was assayed by the Limulus amoebocyte lysate test and lethal toxicity test in C57BL/6 mice. The Limulus lysate activity of the lipopolysaccharide of S. platensis was compared to a preparation of LPS from S. abortus equi (Sigma Chemical Co., St. Louis, MO). The Limulus lysate activities with the two LPS fractions are shown in Table 6. Gelation of the Limulus lysate assay with S. platensis LPS occurred at a concentration of 10^{-9} ug/ml. Gelation of the Limulus amoebocyte lysate with LPS of S. abortus equi occurred with a concentration of 10^{-12} ug/ml. These results demonstrated that the LPS of S. platensis was considerably less active in the Limulus assay than the LPS of S. abortus equip.

Toxic properties of the LPS preparations of S. platensis were determined by intraperitonial injections into C57BL/6 mice. The results are shown in Table 2. The lethal dose for S. platensis lipopolysaccharide was 8.5 mg per animal as compared to 0.8 mg/animal for S. abortus equi LPS.

2.4 Discussion

Microscopic examination of the commercial preparation of S. platensis indicated that the culture was relatively pure and not obviously contaminated by other algae or protozoans. This observation was important in view of the occurrence of the C-20 polyunsaturated fatty acids which are indicative of eukaryotic algae. The relatively large proportion of C_{18} polyunsaturated fatty acid has been previously recognized in cyanophytes and well reviewed (29) and their existence in cyanophytes remains an exception to the general prokaryotic rule that polyunsaturated fatty acid do not occur in bacteria. Whether the C_{20} polyunsaturates are from an extrinsic source or, indeed, a natural component of the cells remains to be determined. It is not uncommon for marine photosynthetic species to synthesize the polyunsaturated form (32). The fatty acid content of the LPS preparation was quite similar to those in the free lipid fraction with the exception that no C_{20} acids were detected. No hydroxy acids were detected in any of the LPS fractions or supernatants derived from the LPS preparation. These findings differ from the reported existence of OH-C14, OH-C16 and OH-C18 in LPS preparations from other cyanobacteria (41). The absence of the hydroxy acids on polar and non-polar coated capillary column was difficult to establish because of the existence of polyunsaturated acids; however, hydrogenation of the samples clearly revealed the existence of polyunsaturated acids by their conversion to the saturated configuration.

The polar lipid contents are like those previously reported (2,26,27). The existence of free fatty acids and lyso-forms of glycolipids and phosphatidyl glycerol in the actone eluate collected from a silicic acid column indicated that the lipids were

hydrolysing after extraction in spite of the fact that the complete procedure was performed in less than three days and that the samples were stored in the cold under a nitrogen atmosphere.

With the exception of the absence of hydroxy fatty acids and the presence of polyunsaturated ones, the composition of the LPS preparation was fairly typical of many Gram-negative bacteria. The toxicity of the LPS preparation was relatively low which, perhaps supports the suitability of this alga as a feedstock. The existence of an LPS component in S. platensis, however, suggests that more consideration be given to the commercial preparation of Spirulina. The relatively low toxicity of the LPS now becomes a determination of the dose level that an animal will receive. While this study provides no evidence that the commercial preparation of Spirulina is immediately harmful, it does point out the need for further studies on the potential low level toxicity properties of cyanophytes in general.

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TABLE 1

Proximate Cellular Composition of Spirulina platensis

Component	% Organic Cell Weight
Protein	50.0
Carbohydrate	8.8
Lipid	16.6
Chlorophyll	1.7*
Carotenoids	0.5*
Ash	7.0
Unknown	17.6

*The values of chlorophyll and carotenoid determined colorimetrically.

TABLE 2

Fractionation of Spirulina platensis Lipids on Unisil Column

Solvent	% of Total Lipids
Hexane	0.2
Benzene	3.4
Chloroform	1.4
Acetone	58.4
Methanol	36.6

The lipids were fractionated on a Unisil column. The relative percent compositions were determined by GLC for hexane eluate and gravimetrically for the others.

TABLE 3

Relative Retention and Percentage Composition Data of Fatty Acids Composition

Fatty Acid	Ret. time (min)	Eluates from Silicic Acid Column			Total Lipids	
		$CHCl_3$	Acetone	MeOH	unhydro-genated	hydro-genated
14:0	19.0	0.6	-	-	0.8	1.6
14:1	19.6	0.5	-	-	0.6	-
16:0	24.1	5.9	35.6	50.2	26.4	38.9
16:1	24.8	1.0	8.4	2.8	4.6	-
16:2	26.5	0.3	1.2	0.6	1.0	-
16:3	29.2	2.4	1.0	0.1	2.1	-
17:0	26.9	-	0.3	0.1	0.7	0.7
18:0	30.0	0.2	0.5	1.5	0.4	55.5
18:1	30.9	64.5	0.7	0.5	22.8	-
18:2	32.6	2.8	5.1	1.7	9.8	-
18:3	35.3	10.3	43.2	23.2	21.1	-
19:0	34.0	0.6	-	12.7	3.5	0.7
20:0	39.2	-	-	-	-	2.5
20:2	44.6	-	0.3	-	0.3	-
20:3	47.4	1.8	0.7	5.3	0.6	-
20:5	51.4	2.7	-	-	1.7	-
Un-identified	(16-52)	5.9	2.7	1.3	3.6	-

Components were analyzed by GLC on 30m x 0.252mm fused quartz capillary column with 0.25 M OV351 at 5 psi of He and 125 + 4°/m to 220°C and held isothermally. The percentages were determined with a Varian Vista 401 data system.

TABLE 4

Chemical Compositions of the Lipopolysaccharides of Spirulina Platensis

Components	Lipopolysaccharides % of the Total
3-Deoxy-D-manno-octulosonic acid[b]	1.2
Glucose[a]	2.9
Rhamnose[a]	6.8
Fucose[a]	1.3
Ribose[a]	3.2
Xylose[a]	1.8
Mannose[a]	0.7
Galactose[a]	3.2
Inositol[a]	2.4
D-Glycero-L-mannoheptose[a,b]	1.4
D-Glucosamine[b]	2.1
Total carbohydrates[b]	31.6
Total fatty acids[b]	14.3
Phosphate[b]	0.6
Nucleic acids[b]	0
Protein[b]	0.6

[a]Determined by GLC on a 6m x 0.31cm glass column containing 10% SP2330 on 100/120 Gas Chrom W AW. The unidentified represents the sum of 7 components.

[b]Determined colorimetrically.

TABLE 5

Fatty Acids of the Lipopolysaccharides of Spirulina platensis

Fatty Acid	Ret. time (min)	Relative %	
		unhydrogenated	hydrogenated
12:0	12.9	0.1	3.9
16:0	23.9	37.6	54.6
16:1	24.4	5.4	-
18:0	29.0	3.5	34.6
18:1	29.7	2.6	-
18:2	31.3	16.6	-
18:3	32.4	18.1	-
Un-identified	(9-35)	16.1	6.8

Fatty acid methyl esters were determined by GLC as described in Table 3 with the exception that the OV351 column was operated at 8 psi of He from 120 + 4%/m to 250°C. The identities of fatty acid methyl esters were based on retention times compared to those of authentic standards. The retention times of 2-OH-myristate and 3-OH-myristate were 31.3 and 32.2 respectively. The relative percentages were determined with a Varian data system.

TABLE 6

The Limulus Amoebocyte Lysate Activity of the Lipopolysaccharides of Spirulina Platensis and Salmonella Abortus Equi.[a]

Lipopolysaccharides	10^{-6}	10^{-7}	10^{-8}	10^{-9} ug/ml	10^{-10}	10^{-11}	10^{-12}	10^{-13}
S. platensis	+++[c]	+++	++	±	-			
S. abortus equi[b]	+++	+++	+++	+++	+++	++	±	-

[a]The activity of different concentrations of LPS determined by incubating 0.1 ml of the test sample with an equal volume of Limulus amoebocyte lysate at 37°C in a stoppered 10 x 75mm glass test tube.

[b]Lipopolysaccharide standard obtained from Sigma.

[c]+++ Solid gel formed in less than 60 min; ++ solid gel formed after 60 min; ± viscous or granular gel formed after 60 min; - no gel formed after 24 hrs.

TABLE 7

Lethal Toxicity Tests in C57BL/6 Mice with LPS Preparations from Spirulina Platensis and Salmonella Abortus Equi

Lipopolysaccharide	Dose g/mouse	Survival %
S. platensis	8500	0
	8000	60
	7000	100
S. abortus equi	800	0
	600	50
	400	100

The percentage survival was determined with 6 mice per dose after 48 hours.

Fig. 1. TLC of eluates collected from silicic acid column developed in solvent system (c). The plate was sprayed with acid and charred. Abbreviations are: Y = Yellow color, G = Green color, R = Rose color, HC = Hydrocarbon, SE = Sterol ester, TG = Triglyceride, S = Sterol, FFA = Free fatty acid, Hex = Hexane eluate, Benz = Benzene eluate, Acet = Acetone eluate, MeOH = Methanol eluate.

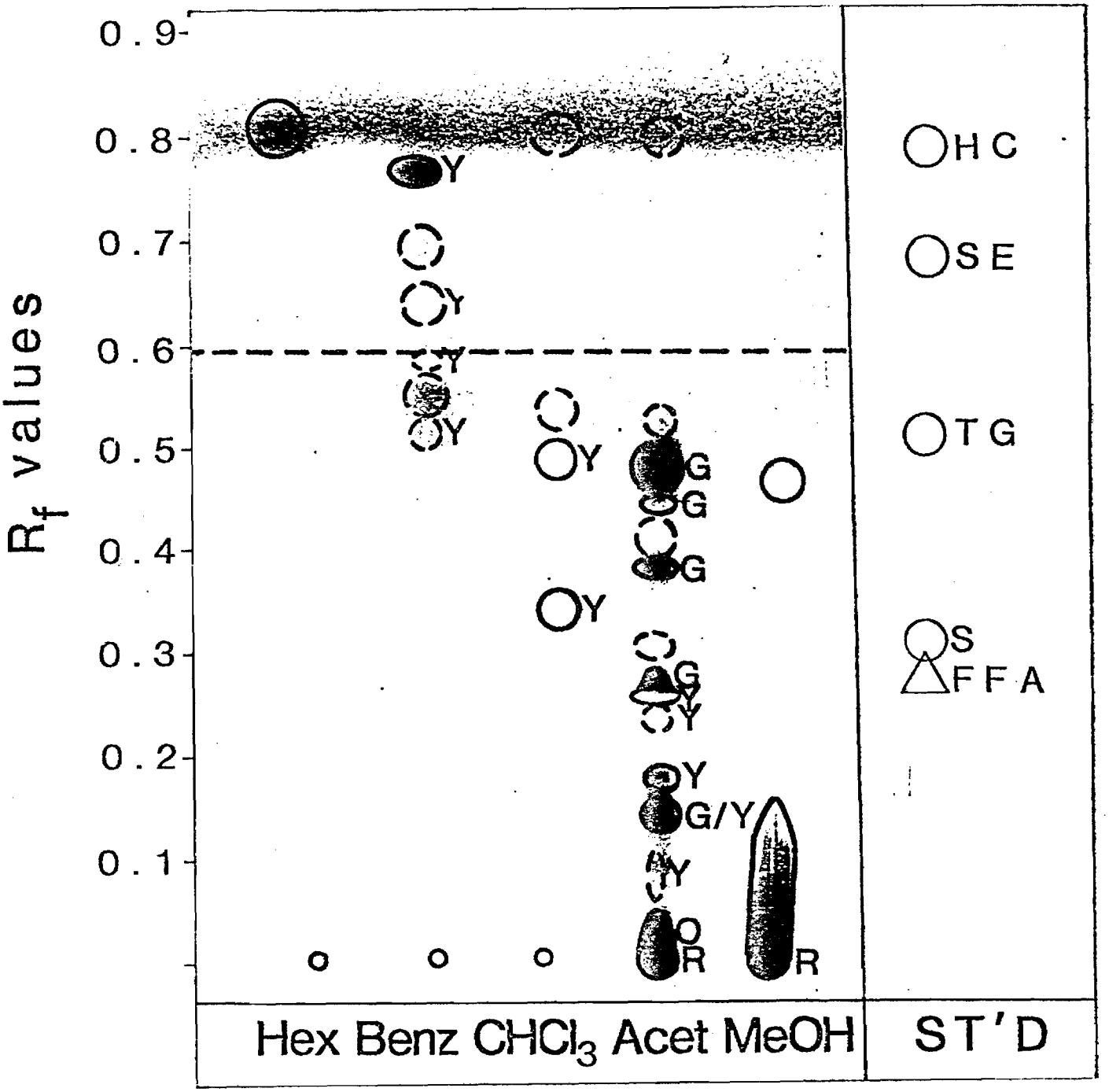
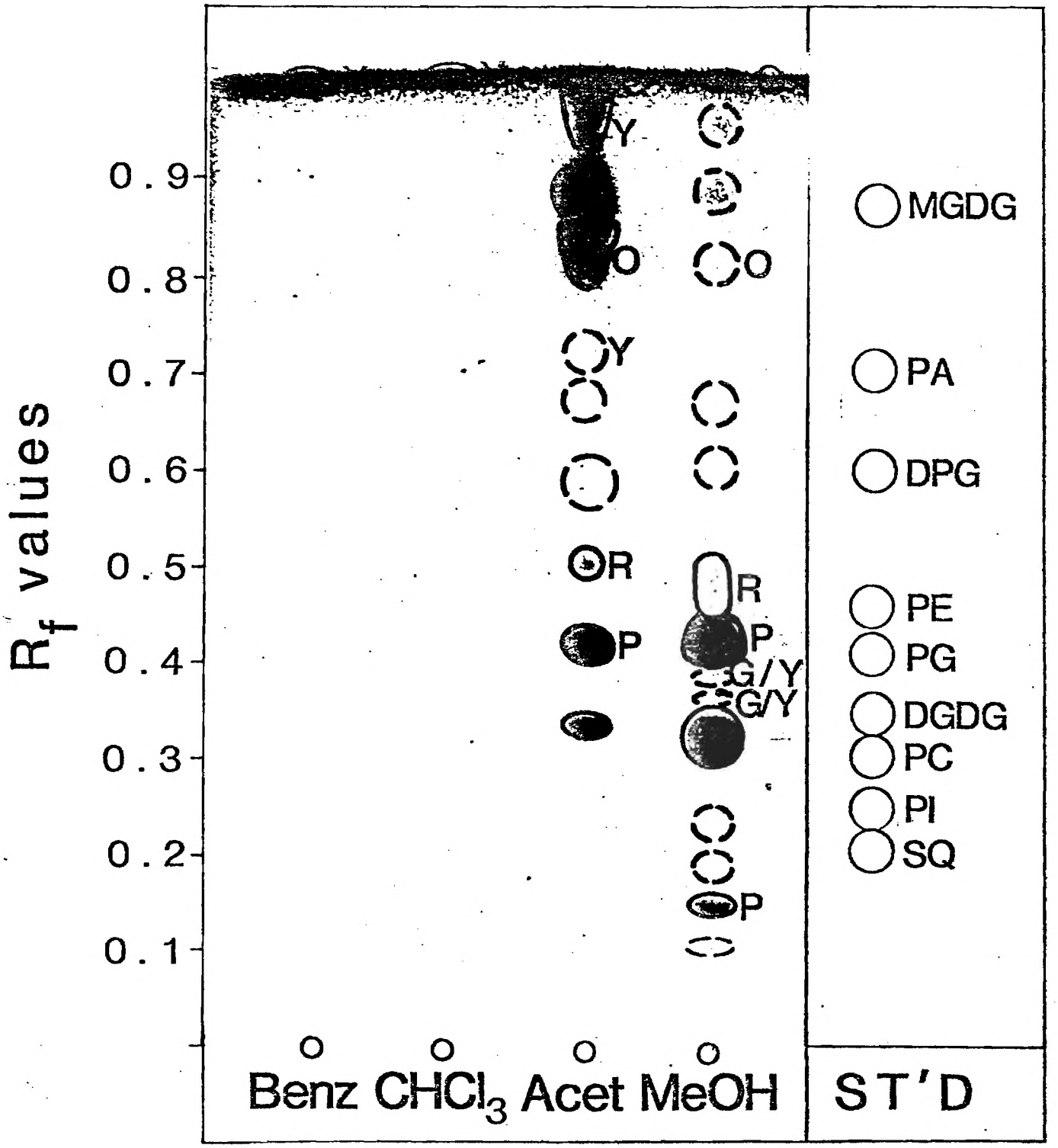

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f values
HC
SE
TG
S
FFA
Hex Benz $CHCl_3$ Acet MeOH
ST'D

Fig . 2 TLC of eluates collected from silicic acid column developed in solvent system (d). The plate was sprayed with phosphate spray and then charred. Abbreviations are as in Fig. 1 and O = Orange color, P = Phosphate positive, MGDG = monogalactosyl acyl diglyceride, PA = Phosphatic acid, DPG = Diphosphatidyl glycerol, PE = Phosphatidyl ethanolamine, PG = Phosphatidy glycerol, DGDG = Digalactosyl acyl diglyceride, PC = Phosphatidyl choline, PI = Phosphatidyl inositol, SQ = Sulfoquinovosyl.


R_f values
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Y
O
Y
R
P
R
P
G/Y
G/Y
O
P
Benz
$CHCl_3$
Acet
MeOH
MGDG
PA
DPG
PE
PG
DGDG
PC
PI
SQ
ST'D

3.0 ENDOTOXIC PROPERTIES OF BLUE-GREEN BACTERIUM SPECIES OF MICRO CYSTIS

3.1 Introduction

Lipopolysaccharides (LPS) are characteristic components of Gram-negative bacteria (1). They are usually toxic, highly inflammatory agents, responsible for the activation of numerous immunological, cellular and humoral mediated systems (2-4). Chemical studies over two decades have established a detailed structure of many bacterial LPS. The LPS are amphiphilic compounds comprised of lipid A and polysaccharide moieties with lipid A being the toxic principle (5).

While a great deal of information exists about LPS and lipid A moieties, there are only a limited number of studies conducted on the LPS compositions of the cyanobacteria (6-9). No studies have been conducted on the LPS of the cyanobacterium Microcystis aeruginosa although it is often the major organism in water blooms of eutrophic lakes and reservoirs (10); occurs world wide; causes serious water management problems; and correlated with domestic and wild animal poisoning (10). To what extent the LPS contributes to the pathogenic or toxic properties of this organism is unknown. Here we report our preliminary findings on the isolation, composition and biological activities of the LPS contents of two isolates of M. aeruginosa.

3.2 Materials and Methods - (see details in Section 2.2)

Organisms and Culture

Unialgal clonal isolates of M. aeruginosa strain UV 006 and 027 were obtained from the culture collection of the Botany Department, University of the Orange Free State, Bloemfontein, South Africa. A unialgal clonal isolate of NRC-1 was furnished by W. W. Carmichael, Wright State University, Dayton, Ohio, U.S.A. The isolates were cultured in 16-l pyrex bottles (11) with a modified BG-11 medium at 29°C (12), aerated with 1% CO_2 in air, and illuminated continuously with 500 fc of cool-white fluorescent light. Cells in late-log phase were harvested by continuous centrifugation and freeze dried.

Isopycnic Density Gradient Ultracentrifugation

Cesium chloride solutions having an average density of 1.40 g/cm were prepared by adding 2.8 g of CsCl and 1 mg of LPS in 0.1 M tris-hydroxymethylaminomethane buffer at pH 8.1 in a final volume of 4.8 ml. The solutions were centrifuged to equilibrium in a SW 50.1 rotor (Beckman Instruments, Fullerton, CA) at 42,000 rev/min for 60 hrs at 4°C as described earlier (14). At the end of the centrifugations, fractions were collected from the bottom of the tubes and assayed for 3-deoxy-D-manno-octulosonic acid (dOclA). Isopycnic densities were determined by refraction index.

3.3 Results

The yield of LPS obtained from M. aeruginosa isolates 006 and NRC-1 were 6.8 mg/gm and 6.2 mg/gm of dried cells, respectively. The isolated LPS were soluble in water, dimethyl sulfoxide, 1% Triton X-100, and 1% sodium deoxycholate, but not in acetone, ethanol or pyridine. LPS could not be extracted from dried cells by the phenol/choloroform/petroleum ether method of Galanos et al. (13). LPS were not released from the cells by incubation of whole cells in ethylenediaminotetraacetic acid or saline.

Isopycnic Density Gradient Ultracentrifugation

LPS purified by CsCl gradient ultracentrifugation gave typical single peak gradient profiles (Fig. 1). The densities of LPS from isolates 006 and NRC-1 were 1.49 g/Cm^3 and 1.46 g/Cm^3, respectively.

Gross Composition of LPS

The chemical compositions of LPS isolated from M. aeruginosa isolates 006 and NRC-1 are given in Table 1. Both preparations of LPS were essentially free of nucleic acids but contained a small amount of protein (0.3 to 0.4%). Differences in the dOclA, 3-deoxy sugars, glucose, total carbohydrates, glucosamine, and total fatty acids were detected in the LPS from the two isolates. The amount of dOclA was 4.7% in 006 and 5.2% in NRC-1. The amounts of dOclA, total fatty acids, glucosamine, and phosphate in LPS of 006 isolate were less than that in the LPS of NRC-1 isolate. NRC-1 LPS contained lesser amounts of 3-deoxy sugar, glucose, and total carbohydrates than the LPS from isolate 006. Fatty acid esters from 006 LPS accounted for 10.8% and NRC-1 13.6% of the total LPS. Using the heptose assay of Dische and the modified procedure of Wright and Rebers, no heptoses could be detected in these LPS preparations. The absence of heptose was also confirmed by gas-liquid chromatography (unpublished work).

Lipid A prepared by acetic acid hydrolysis of LPS from the 006 isolate was 28.7% of the total LPS and contained 6.8% of glucosamine and 3.2% phosphorus, whereas, lipid A from NRC-1 isolates was 36.3% of the total LPS and contained 7.2% glucosamine and 4.3% phosphorus. dOclA was not detected in these lipid A preparations indicating that the lipid A preparations were free of the carbohydrate moiety of the LPS.

Mouse Toxicity

The lethal endotoxic efficacy of LPS and lipid A of 006 and NRC-1 isolates and LPS of S. abortus equi (Sigma) in C57BL/6 mice are given in Table 2. All mice injected with 1200 µg of LPS from isolates 006 and 1000 µg of LPS from isolate NRC-1 were dead within 48 hrs of injection. These results demonstrated that LPS of NRC-1 was slightly more toxic than that of the 006; however, the lipid A moiety obtained from LPS of both 006 and NRC-1 isolates were slightly less toxic than the intact LPS. The LPS of S. abortus equi was more toxic than either M. aeruginosa preparation.

Limulus Lysate Gelation Activity

The Limulus lysate gelation activity of all LPS and lipid A preparations are summarized in Table 3. Limulus lysate tests proved very sensitive for both LPS and lipid A preparations of isolates 006 and NRC-1. LPS from 006 at a concentration of 10^{-6} µg/ml and from NRC-1 at a concentration of 10^{-9} µg/ml created gelation in the Limulus lysate assays. The LPS of 006 and NRC-1 isolates were more active in Limulus lysate gelation than the lipid A moiety, however, there were significant differences between 006 and NRC-1 in the assay. The LPS and lipid A of NRC-1 is more active in gelation of Limulus lysate than 006 strain. However, LPS of S. abortus equi was significantly more active than whole LPS or lipid A of M. aeruginosa strains.

3.4 Discussion

The present study constitutes the first report on the isolation, purification, and biological activities (toxicity in mice and Limulus lysate gelation) of the LPS from two different isolates of the Cyanobacterium M. aeruginosa. The LPS preparations tested gave a single distinct peak in cesium chloride gradient ultracentrifugation and were free of nucleic acids but contained traces of proteins. Even though the detailed chemical compositions of these LPS preparations are not yet completed, sugars of the LPS were identified colorimetrically as dOclA, glucose, 3-deoxy sugars, and glucosamine. Mild acid hydrolysis of these LPS preparations resulted in the release of a carbohydrate moiety and an insoluble precipitate identified as lipid A. Preliminary evidence indicated the absence of heptoses, a sugar common to many LPS of bacteria with the exception of Pseudomonas maltophilia (14,15) and Cyanobacteria (8). The total carbohydrates, total fatty acids, fatty acid esters, and phosphate are relatively typical of Gram-negative bacteria (16). The LPS and lipid A moiety of M. aeruginosa were significantly active in toxicity in C57BL/6 mice and in Limulus lysate gelation assays. Thus, these chemical and biological characteristics of LPS isolated from M. aeruginosa conform to the general properties of endotoxins.

LPS from the blue-green bacteria, Anacystis nidulans (6) and Anabaena variabilis (7) have been described. The LPS specific sugar dOclA was present in Anacystis nidulans, but it was absent from Anabaena variabilis (7). No heptoses were detected in either of the LPS compositions (6-9). Although the phosphorus content of LPS preparations of Gram-negative bacteria are generally higher than 2%, the LPS from a number of bacteria, such as Pseudomonas diminuta (18), Chromatium vinosum (19), Rhodopseudomonas virdis (20), Thiioocapsa roseopersicina (21) as well as some Cyanobacteria (6,22,23) have been reported to contain only very small amounts of phosphate. No phosphate was detected in the LPS of cyanobacterium Anabaena variabilis (7). In contrast, a significant amount of phosphate was detected in the LPS of M. aeruginosa isolates 006 and NRC-1.

The biological activities, toxicity in mice and Limulus lysate gelation activity, of M. aeruginosa LPS and lipid A indicated that lipid A was slightly less active in these tests than their parent LPS. These differences may be due to the solubility, percipitability and physiochemical properties between lipid A and LPS 91). These biological activities, however, are clearly dependent upon the lipid A rather than the carbohydrate components of LPS. The LPS from NRC-1 contains more dOclA, glucosamine, phosphate, fatty acid esters and lipid A than 006, and it correspondingly possesses higher activities in mouse lethality and Limulus tests. The extent to which differences in chemical composition or structure in LPS between NRC-1 and 006 reflect differences in these biological activites can not yet be assessed. The fatty acid and carbohydrate compositions of these LPS and lipid A preparations are under study and the results will be reported elsewhere.

The high level of toxicity recognized in M. aeruginosa (24) is attributed to principally the peptide toxins. The contribution of LPS to the lethality or any possible synergistic effects with the peptide toxins are not yet determined. Apparently the LPS content alone is not a principal factor for the major environmental impacts associated with this organism.

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TABLE 1

The Chemical Composition of the LPS of M. aeruginosa

Components	006	NRC-1	0 2 7
		% of the Total	
dOclA	4.7	5.2	2.9
Glucose	11.4	9.1	5.8
Mannose	-	-	1.7
Xylose	-	-	1.3
3-Deoxy sugars	3.1	1.8	3.8
Glucosamine	3.7	6.5	5.3
D-Glycero-L-mannoheptose	0	0	0
Total Carbohydrates	36.0	26.5	28.6
Total Fatty Acids	18.2	21.4	14.0
Fatty Acid Ester	ND	ND	9.3
Phosphate	0.7	0.8	0.6
Nucleic Acids	0	0	0
Protein	0.4	0.3	0.6

TABLE 2

M. aeruginosa Isolates 006 and NRC-1 in C57BL/6 Mice[a]

	006		NRC-1		S. abortus equi[b]
µg/mouse	LPS	Lipid A[c]	LPS	Lipid A[c]	LPS
400	6/6[d]	6/6	6/6	6/6	6/6
300	6/6	6/6	6/6	6/6	3/6
800	4/6	6/6	3/6	5/6	0/6
1000	2.6	6/6	0/6	2/6	-
1200	0/6	3/6	-	0/6	-
1400	-	0/6	-	-	-

[a]Purified LPS or lipid A moiety were injected into individual mice intraperitoneally in 0.4 ml pyrogen-free saline and survivors/deaths recorded at 48 hrs.

[b]LPS from S. abortus equi obtained from Sigma.

[c]Lipid A was complexed to bovine serum albumin.

[d]Number survivors/total injected.

TABLE 3

Limulus Lysate Gelation of LPS and Lipid A Moiety from M. Aeruginosa Isolates 006 and NRC-1[a]

µg/ml of LPS or lipid A[b]	006		NRC-1		S. abortus equi[c]
	LPS	Lipid A	LPS	Lipid A	LPS
10^{-3}	+++[d]	+++	+++	+++	+++
10^{-4}	+++	++	+++	+++	+++
10^{-5}	++	±	+++	+++	+++
10^{-6}	±	-	+++	++	+++
10^{-7}	-	-	+++	±	+++
10^{-9}	-	-	±	-	++
10^{-10}	-	-	-	-	±
10^{-11}	-	-	-	-	-

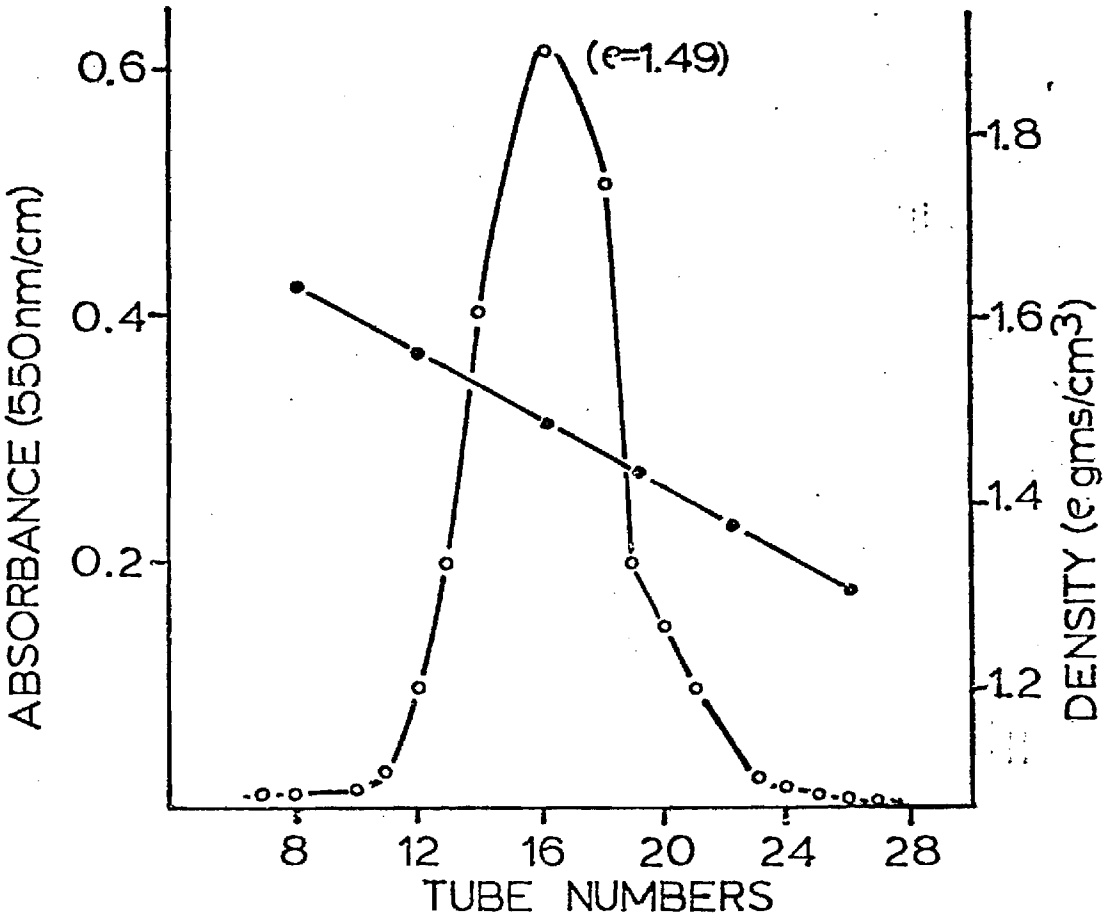
[a]The activity of different concentrations of LPS or lipid A was detected by incubating 0.1 ml of the test sample at 37°C with an equal volume of Limulus lysate, in a stoppered 10 x 75 mm test tube.

[c]LPS standard obtained from Sigma.

[d]+++ solid gel formed in less than 60 min; ++ solid gel formed after 60 min; ± viscous or granular gel formed after 60 min; - no gel formed after 24 hr.

Legend to Figure 1

Isopycnic density gradient ultracentrifugation of LPS from M. aeruginosa isolate 006. Samples containing 1 mg of LPS in 0.1 M Tris-buffer, pH 8.1 (final volume 4.8 ml), were added to 2.8 g of CsCl and centrifuged for 60 hrs. at 142,000 x G. Column fractions were collected from the bottom of the tube and assayed for dOclA. Densities were determined from the index of refraction measurements.


(e=1.49)
0.6
0.4
0.2
ABSORBANCE (550nm/cm)
1.8
1.6
1.4
1.2
DENSITY (e.gms/cm3)
8
12
16
20
24
28
TUBE NUMBERS

4.0 LIPIDS OF MACROALGAE

4.1 Introduction

Controlled cultivation of macroalgae has changed from a curosity to a science in the last ten years. The macroalgae are generally recognized as carbohydrate producers with cellular lipid biosynthesis playing a substantially small role in cellular productivity. It is now possible, however, to produce many different macroalga of different growth stages. Under growth conditions which feature nitrogen deficiencies, the possibilities exist that the cellular biochemical mechanisms could switch from carbohydrate to lipid biosynthesis. In this context, the proximate cellular chemistry of three macroalgae were measured.

4.2 Materials and Methods

Gracilaria tikvahiae, Ascophylum nodosum and Sargassum filipendua were grown outdoors in an open culture system by Dr. J. Ryther et al. (1).

The analytical procedures are so described in Section 1.2.

4.3 Results

The proximate chemical composition of macroalgae is given in Table 1. The cellular organic compositions are principally carbohydrates with lipids representing the smallest amount of the chemical classes. Nitrogen deficient cultivated Gracilaria cultures featured carbohydrate biosynthesis with a reduction in total lipid yield to less than 1%. The distribution of lipids collected from a silicic acid column (Table 2) demonstrated that more than 92% of the total lipids in the form of pigments, glycolipids and phospholipids, were found in the acetone and methanol eluates. This was expected since the lipid concentrations are at absolute minimum required for maintaining biological integrity of the cellular membranes. The fatty acid pattern for total Gracillaria lipids is given in Table 3. The fatty acids are typical of those found in marine algae.

Because of the minute quantities of the lipids found and the fact that the major lipid components are typical lipids that are found in all cells, a detailed presentation of the lipid composition would only be repetitious to what has already been presented. Therefore, no further description of macroalgae lipids are presented.

4.5 References

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TABLE 1

Proximate Chemical Composition of Macroalgae

Species	% Ash	Relative % Organic Composition		
		Proteins	Carbohydrate	Lipids
G. tikvahiae, SW, NE	45.0	9.1	54.0*	1.8
G. tikvahiae, SW, ND	92.6	9.2	72.6*	0.8
S. filipendua, SW	39.3	8.6	48.3*	2.8
A. nodosum, SW	20.0	9.1	65.3*	3.6

*Values determined by extrapolation since colorimetric analysed appeared to be influenced by other factors in the hydrolysates.

TABLE 2

Total Lipids Fractionated on Unisil Silicic Acid

Eluting Solvent	Ascophylum	Sargussum	Gracillaria NE	Gracillaria ND
Hexane	0.3	0.4	0.8	0.3
Benzene	15.7	1.5	1.3	1.9
Chloroform	15.4	1.0	18.3	4.8
Acetone	50.0	74.3	46.0	62.3
Methanol	19.5	22.8	33.5	30.8

The proportions of the eluates were determined by GLC for hexane fractions and gravimetrically for the others. SW, seawater; ND, nitrogen deficient; NE, nitrogen efficient.

TABLE 3

Relative Percentages of Total Fatty Acid Composition of Gracilaria tikvahiae Grown Under Nitrogen Sufficient and Deficient Conditions

Fatty Acid	Gracillaria tikvahiae	
	Nitrogen Enriched Sea Water	Nitrogen Deficient Sea Water
12:0	0.5	0.9
14:0, 14:1	8.1	5.1
16:0	55.0	40.9
18:0	4.0	2.1
18:1	10.1	13.0
18:2	1.1	2.4
20:3	-	2.4
20:4	11.1	31.1
Unidentified	10.1	2.1

Fatty acids as methyl esters were determined as in Table 6.

5.0 POLYSACCHARIDES OF MACROALGAE

5.1 Gracilaria Isolation, Purification and Characterizations

Gracilaria grown in nitrogen sufficient and nitrogen deficient medium were investigated in detail for characterizations of polysaccharides since the lipid contents were undistinguished.

Polysaccharides were extracted from these two species by the procedure of Jabbar Mian and E. Percival (Carbohyd. Res. 26 (1973) 133-146).

1. Preliminary extraction of two species of Gracilaria with hot ethanol (80%) at 70°C three times resulted in isolation of mannitol, a major sugar in this fraction. Mannitol was further purified by passing through Sephadex-G-50 Column Chromatography.

A second extraction of Gracilaria with cold ethanol (80%) also showed the presence of mannitol, a major sugar in ethanol extract. Traces of glucose, rhamnose and other unidentified sugars were also detected in the ethanol extract, which were then separated by Sephadex Column Chromatography to purify mannitol.

After isolation and purification of mannitol sugar, the residue was washed several times with H_2O and then immersed in 40% formaldehyde for 18 hrs. They were then washed, with H_2O and dried.

2. A dried powder obtained after extractions with 2% $CaCl_2$ (100 ml) for 4 hrs. at 70°C gave purified agar. In this hot $CaCl_2$ fraction, agar was the major component present. Since hot $CaCl_2$ extract after cooling immediately solidifies, due to the presence of agar, other sugars present were unidentified. However, extraction of Gracilaria with cold 2% $CaCl_2$ did not extract agar, and by this extraction method glucose, mannose, ribose, xylose and some unidentified sugars were characterized.

3. $CaCl_2$ extracted residue was further extracted with dil HCl (pH 2.0) for 4 hr. at 70°C. Dil HCl extract was concentrated, dialysed for several days, lyophilized and analyzed by gas liquid chromatography.

The major sugars present in this fraction were glucose, mannose and xylose.

4. The residue was further extracted with 3% sodium carbonate for 4 hr. at 50°C (3 times) filtered, filterate concentrated, dialyzed and lyophilized.

This extract contained the major carbohydrate fraction that was characterized by GLC. A large number of unidentifiable (unusual) sugars were found. These were not completely identified. This fraction contained glucose, xylose, ribose, galactose, mannose, etc., which are generally common.

Details of the data are shown in Table I.

5. Residue was further extracted with ammonium oxalate-oxalic acid (0.25% with respect to each 100 ml, pH 2.8 for 6 hr. at 70°C) extract was dialysed and lyophilized and analysed by gas liquid chromatography. 3-4 sugars were present. Some sugars were identifiable (Table I).

6. Residue was then extracted with H_2O, (100 ml) acetic acid (1 ml) and physiological saline at 70°C, 3 times.

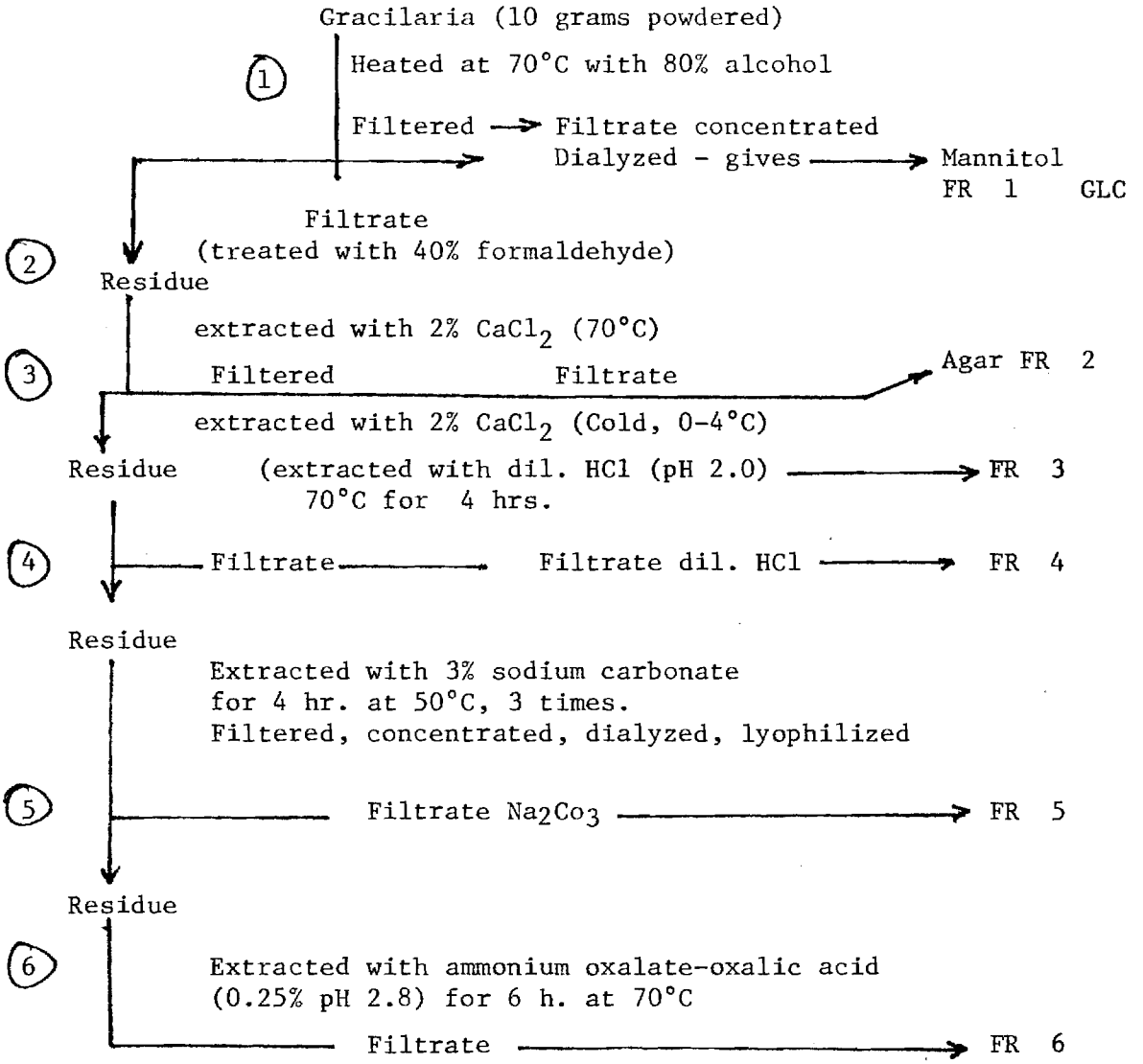
Extracts concentrated, dialyzed extensively, lyophilized and analyzed by GLC (Table 1).

7. The residue was further washed with H_2O, and then exhaustively with 6M potassium hydroxide (100 ml) for 48 hr. at room temperature. The residue solid was then washed with dilute HCl, H_2O ethanol, and ether, and was recovered as a white solid.

Summary

Polysaccharides were isolated by sequential extraction of Gracilaria species. They were purified and characterized by gas liquid chromatography. Extraction of the algae by 80% hot and cold ethanol gave mannitol, as a major sugar present in this fraction. This was identified by colorimetric assay and gas liquid chromatography. The resultant material (mannose) was further purified by Sephadex G-50 column chromatography and lyophilized. A second extraction with 2% calcium chloride at 70°C gave agar as the major carbohydrate. However, extraction with cold 2% calcium chloride gave a fraction containing glucose, rhamnose, arabinose, ribose and xylose and other unidentified sugars. Extractions with dil-hydrochloride, sodium carbonate, ammonium oxalate-oxalic acid, also gave different fractions containing various sugars which were analyzed by gas liquid chromatography. Among all these fractions, sodium carbonate extract contained largest number of sugars.

PROCEDURE OUTLINE FOR EXTRACTION OF POLYSACCHARIDES FROM GRACILARIA SPECIES



GRACILARIA

Isolation and Characterization of Polysaccharides

1. Gracilaria NE

Sugars	Per Cent Fractions 1	2	3	4	5	6
Glucose	0.36	0	0	0	11.9	5.62
Galactose	0.27	2.45	2.09	6.36	8.5	1.83
Mannose	0.72	0.36	2.27	0.62	2.8	0.56
Ribose	0.42	0	1.37	0	23.6	0
Xylose	0	2.19	0	4.0	5.4	2.1

2. Gracilaria ND

Sugars	Per Cent Fractions 1	2	3	4	5	6
Glucose	0	3.46	0	0	14.79	7.39
Galactose	0	0	2.44	9.23	12.25	3.97
Mannose	0	0.37	0.75	1.01	3.1	2.90
Ribose	0	0	2.55	0	30.83	9.19
Xylose	0	2.15	0.89	5.11	5.28	1.71

5.2 Polysaccharides of the Two Macroalgae, Ascophyllum Nodosum (AN) and Sargassum Filapendum (SF)

Isolation, Purification and Characterizations:

Polysaccharides were extracted from these two macroalgae Ascophyllum Nodussum, and Sargassum filapendum by sequential 2% $CaCl_2$ extractions at 70°C for 3 hrs. and then at 3% Na_2CO_3 extractions at 50°C. Extracts were concentrated, dialyzed extensively, lyophilized, converted into alditol acetates and analyzed by gas liquid chromatography (GLC). Results of analysis are shown in the following table.

Polysaccharides of Macroalgae Ascophyllum Nodusum (AN)

and Sargassum Filapendum (SF)

	2% $CaCl_2$ Extract		3% Na_2CO_3 Extract	
	AN	SF	AN	SF
Glucose	3.18	8.44	48.38	6.42
Galactose	5.10	14.36	2.55	0
Xylose	11.18	8.15	7.41	6.97
Fucose	40.04	37.16	0	0
Ribose	25.81	5.94	0	0
Mannose	0	15.39	3.01	62.03
Arabinose	0	0	7.40	0
2-Deoxyglucose	0	0	8.82	3.86

% Analyzed by Gas Liquid Chromatography

6.0 DESIGN OF EFFICIENT SEPARATORS FOR HARVESTING ALGAE

6.1 Background

The purpose of this phase of the investigation was to evaluate the "state of the art" in harvesting algae. A general review of the literature immediately indicated that there is no "state of the art".

Harvesting "is the Achilles heel of algal ponds. There are at present no low-cost effective techniques for algal harvesting" (1). No demonstrated improvements in this situation have been made recently (2). Thus it is necessary that new techniques be developed, if cultures of microalgae are to become useful sources of any but the most expensive products.

Dunaliella is currently being exploited on a small scale in Israel (3) and a detailed economic analysis has been performed on a proposed process that would use Dunaliella for large scale conversion of sunlight and waste CO_2 in stack gas to glycerol (4). The current commercial processes require a great deal of energy to concentrate glycerol from dilute solutions. Since Dunaliella produces high internal concentrations of glycerol, the potential exists for more energy efficient production of glycerol using the alga. In the current petrochemical process, the energy value of the glycerol is only 30% of the energy input in feedstock and processing. The calculated energy yield for glycerol in the proposed process is 190% of the estimated processing energy and the feedstock (CO_2) has no energy value. If the energy value of the protein byproduct of the proposed process is included, the energy yield jumps to 443% (4). Moderating this very optimistic conclusion from the operating energy point of view is the fact that the algal process requires a much larger capital investment. Nonetheless, the estimated costs of production are 15% less for the algal process than the current petrochemical process (4). Thus the proposed process appears to be economically attractive although there are many untested assumptions and extrapolations involved in the estimate.

For example, consider the process proposed by Chen and Chi (4) for production of glycerol. The part of the process that is of interest here is the separation of the algal cells from the saline solution. The proposed method is to use a shallow depth settler composed of inclined parallel plates. This type of settler has proved to be advantageous in wastewater treatment (5,6,7). However, its use for the recovery of microorganisms has only been tested with yeast on a small laboratory scale operating in a batch mode (8). The settling velocity of the yeast was reported as less than 1 cm/hr compared with estimates for D. tertiolecta in seawater of 1.6 cm/hr (9) and for Chorella of 0.1 cm/hr (10). Thus the small-scale tests run with yeast appear to be applicable. However, the settling velocity of alum and iron floc particles, which are typical particles the commercial scale settlers deal with, are in the vicinity of 5 cm/min (6) or several hundred times greater. Thus the proposed settler involves a large extrapolation and uncertainty of scale up.

Economically the separation is important. In the proposed process, the settler represented 11% of the large capital costs using a fairly optimistic assumption about the concentration to which the algae cells can be grown (4). However, the required size of a given design of settler is inversely proprotional to the density to which cells can be grown. If the maximum cell density is substantially less than assumed, the required settler will dominate all other costs even if it works as predicted.

Algae vary a great deal in the ease with which they can be harvested and a large number of different methods has been tested. Relatively large, filamentous algae can be harvested inexpensively by microstraining (11). However, small species are frequently desirable in other respects and may be generally more productive (11).

Centrifugation is a technique that is straightforward, clean, and can work with any species. Unfortunately, it is expensive (11-15). Thus, it can be used only where a product of high value is involved or the algae are very concentrated.

The most generally used alternative to centrifugation is flocculation (11,12) (usually with alum) followed by sedimentation or flotation on air bubbles (15,16). Chemical flocculation has the disadvantage that the product is contaminated by the flocculant but the main problem is that the flocculants are expensive. Algae sometimes flocculate on their own but this has not proven to be a reliable technique (11). Another limitation is that flocculation is not expected to work in saline waters where electrostatic forces are greatly reduced.

Mohn (13,14) has tested a number of different types of industrial filters. He determined that a small alga, Scenedesmus acutus, could be successfully harvested by a precoated vacuum drum-filter. Unfortunately the algae must be preconcentrated and the product is about 1/3 potato starch. Nonetheless the least expensive method for harvesting Scenedesmus was preconcentration by a nozzle centrifuge followed by the precoated vacuum drum-filter. This is a good example of the fact that multistage separation processes are likely to be optimal. Relatively inexpensive methods can be used to concentrate the algae from large volumes of water. More expensive techniques can then be applied to the smaller volumes to remove the remaining water.

The cross-flow fiber and membrane filtration systems that are commercially available by Amicon and Millipore Corps. were also reviewed. Demonstrations of these two systems at Georgia Tech using algal cultures as the test organism were substantially less than impressive. The fibers and membranes are relatively expensive and require substantial maintenance. It is one estimate that these factors are prohibitive for application to the diverse situation required in algal pond harvesting.

6.2 Filtration Studies

It is our judgement that the cross-flow fiber filtration principal is the most suitable design for application to harvesting algae from diverse pond environments. Since the fibers and membranes currently available are unacceptable because of maintenance requirements and expense, new techniques must be developed. The approach to this problem is to find a substitute for cross-flow fibers that is inexpensive, requires minimal maintenance and is sufficiently durable to be handled in the field by the average unspecialized worker.

The Manville Corporation, Denver, CO, has a pipe division that excells in constructing a ceramic pipe that has uniform and controlled porosity with supersaturated water properties. The pipes are reasonably strong, threadable, and temperature independent (pipes were fired overnight at 500°C with no deleterious effects).

Pipes with porosities of 2μ, 4μ, 10μ or 20μ in 30cm lengths were prepared with a sealed inlet. The porosity was the exit port. Ten liters of Escherichia coli and

ten liters of Dunaliella salina represented the test samples. The 20μ pipe filtered 100% of the D. salina fed by gravity flow in less than 10 min. In the laboratory situation, we did not have sufficient sized culture to adequately challenge the system and were unable to determine plugging rate, desired pressure states, etc.

With regards to filtration of E. coli at 10^9 cells/ml, the 10μ sized pipe filtered only 50-60% of the cells and had to be recycled twice for effective concentration. The time required to accomplish this was 1.25 hrs. The 4μ pipe removed most all of the E. coli but the 30cm pipe showed plugging rates that were directly proportional to amount of cells filters. To pass 10 liters of E. coli through the 4μ pipe we had to back-flush the pipe and empty the concentrated E. coli slurry.

There were several things that became obvious from our experiments: 1) contract a system with the pipe as an insert with compression fitting a circulating pump and pressure gauge assembly; 2) build a pipe with intermediate size porosity; 3) arrange for larger batch cultures of cells for testing.

At this writing, the pipe and the pipe assembly have been constructed. We are now approaching the second test system.

It is my understanding, if our experiments are successful, the Manville Corp. interest is simply to be able to supply the ceramic pipes to commercial outfits such as Millipore or Amicon for their use in constructing and marketing of culture concentration systems.

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7.0 APPENDIX

7.1 Papers Presented

1. Biotechnology Applied to Naval Needs. National Academy of Science Lecture, American Academy of Arts and Sciences, Cambridge, MA, Sept. 19-20, 1983.

2. Algal Cultivations, Phycology Meeting, Nova Scotia, Canada, Sept. 1982.

7.2 Papers in Press

1. Lipid composition of the nitrogen starved green alga Neochloris oleoabundans. Eng. Microbial Technol. Oct. 1983 issue.

2. Lipopolysaccharides of the cyanobacterium, Microcystis. Fed. Europ. J. Biochem. Dec. 1983 issue.

7.3 Papers in Preparation

1. Chemical profiles of selected species of algae with emphasis on lipids.

2. Lipid and lipopolysaccharide constituents of cyanobacterium Spirulina platensis.

7.4 Study Guide

Isolation, Analysis and Identification of Lipids. Study guide prepared for the Aquatic Species Program of Solar Energy Research Institute. Oct. 1982.