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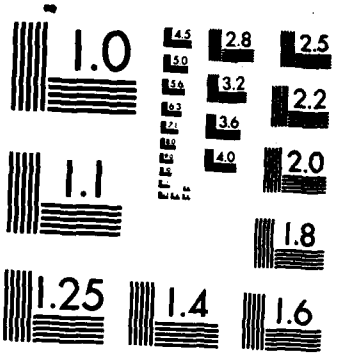

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Biodeuterated Materials: High-Temperature Lubricants from Algae

REX A. NEIHOF

Combustion and Fuels Branch
Chemistry Division

and

MARK M. ROSS AND JOSEPH E. CAMPANA

Chemical Diagnostics Branch
Chemistry Division

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NAVAL RESEARCH LABORATORY
Washington, D.C.

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Substitution of deuterium for hydrogen imparts an enhanced resistance to oxidation in materials such as lubricants. The exploitation of this and other novel properties of deuterated materials in applications of naval interest is only beginning to be realized. As an alternative to the conventional chemical methods of synthesizing deuterated compounds employing high temperature and reducing conditions, the present work explores the possible advantages of using algae grown in deuterium oxide (D_2O) to provide completely deuterated precursors that are suitable for synthesizing a variety of lubricants. By proper choice of organism and growth conditions, it was possible to obtain dense cultures of algae containing 50 to 60% of their dry weight as long-chain fatty acids with different degrees of unsaturation. This report outlines strategies for synthesizing lubricant materials from these fatty acids and discusses the extension of biosynthesis to other deuterated materials such as polymers.

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Mark M. Ross	(202) 767-3551	6110

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EXECUTIVE SUMMARY

Substitution of deuterium for hydrogen can change the chemical and physical properties of molecules in ways that can be used advantageously to produce new materials. For example, researchers at the Naval Air Development Center and at the Naval Research Laboratory have shown that the oxidative stability of lubricant-base stocks is increased remarkably (five- to tenfold) on complete or selected deuteration, and they have initiated exploratory development programs to demonstrate the payoff of such deuterated materials in selected oil-lubricated mechanical systems.

In an effort to decrease the potential cost and to improve the quality of deuterated materials, we have been investigating techniques of biotechnology to produce deuterated materials. In the present investigation, algae grown in heavy water (D_2O) and provided only with mineral salts, carbon dioxide, and light have synthesized lipids containing fatty acids that were over 98% deuterated. These fatty acids constituted as much as 50 to 60% of the dry weight of the organism. By the appropriate choices of organism and growth conditions, it was possible to obtain fatty acids with suitable deuterocarbon chain length and double-bond density for the synthesis of a variety of lubricants. Biosynthetic production of other novel deuterated materials such as polymers also appears feasible.

BIODEUTERATED MATERIALS: HIGH-TEMPERATURE LUBRICANTS FROM ALGAE

INTRODUCTION

Soon after the discovery of the heavy hydrogen isotope, deuterium, in 1931 by Urey and coworkers [1,2], it was predicted that reaction rates involving cleavage of bonds to deuterium would be lower than those involving cleavage of bonds to hydrogen because of the difference in the zero-point energies [3,4]. This means that a reaction rate limited by H-C bond breakage would in theory be faster than for the D-C analog. The experimental values for the deuterium isotope effect depend on a variety of factors as discussed by Wiberg [5], and large effects have been observed in some cases [6-8].

In 1975 Rebuck and Conte at the Naval Air Development Center (NADC) reported that the substitution of deuterium for hydrogen in synthetic hydrocarbon lubricants resulted in increased oxidative stability (fivefold as determined by a chemical method) and a corresponding increase in bearing life-performance in laboratory-controlled spindle tests [9-11]. This demonstration of the deuterium isotope effect in materials applications led researchers at the Naval Research Laboratory (NRL) to investigate the effect with a different chemical class of lubricant base stock. The pentapolyol esters were chosen for study because of their excellent high-temperature properties. A deuterated tetraester lubricant, pentaerythritol perdeuterotetrahexanoate, exhibited a much higher oxidative stability than the hydrogen analog (more than tenfold) [7,12,13].

A preliminary cost and benefit analysis of the impact of deuterated fluids on a gyro bearing was impressive but very conservative [14]. In 1985 dollars, to remove a system (such as a missile) from service, refurbish it, and put it back into service would cost from $30,000 to $50,000. Typical application of a deuterated lubricant would cost about one dollar more than application of an undeuterated lubricant. Therefore, a modest 50% increase in bearing life would result in a $1.5- to $3-million savings per year, assuming 100 units are refurbished per year.

A follow-up applied program is in progress at NRL with the objective of extending the life of selected high-failure critical bearings in Fleet auxiliary machinery through the use of deuterated fluids. This program will represent the first application of the deuterated lubricant technology. The cost of the lubricant is an insignificant factor where it is used in small amounts and the costs of servicing and refurbishing the bearing systems are high. However, the range of applications for deuterated lubricants could be enlarged if the present high cost of synthesis were lowered.

An alternative to the conventional chemical synthesis of deuterated materials is to employ microorganisms adapted to growth in pure deuterium oxide (D_2O) [15]. Possible advantages are lower costs, greater ease of production, and assurance of complete deuteration compared to conventional catalytic hydrogen/deuterium exchange employing high temperatures, prolonged reaction times, and proprietary catalysts. The biosynthetic approach may also make possible the synthesis of deuterated compounds too labile for chemical synthetic methods.

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Algae are particularly attractive organisms for this because they can be induced to grow in essentially 100% D_2O media containing simple inorganic salts if light and carbon dioxide are supplied [15]. No perdeuterated carbon source is necessary as would be required if a nonphotosynthetic organism were used. Because no hydrogen is present, the algal products are completely deuterated.

This report presents the results of experimental investigations to find algal species that would grow and produce appropriate lipids in 100% heavy water media, and to optimize growth conditions to obtain particular fatty acids most suitable for synthesis of deuterated lubricants. Discussion is also devoted to the possibility of using microorganisms to produce other novel deuterated compounds with materials applications.

EXPERIMENTAL

Organisms

The unicellular algae listed below were used in experiments to obtain significant growth in D_2O media.

1. *Chlorella vulgaris* (UTEX 397)
2. *Chlorella vulgaris* (Arg)
3. *Scenedesmus obliquus* (Arg)
4. *Scenedesmus obliquus* (UTEX 393)
5. *Scenedesmus obliquus* (UTEX 417)
6. *Scenedesmus obliquus* (UTEX 746)
7. *Scenedesmus obliquus* (UTEX 1237)
8. *Ochramonas danica* (UTEX L1298)
9. *Chrysochromolina strobilis* (UTEX LB981)
10. *Chlamydomonas reinhardtii* (F60)

Organisms 2 and 3 (Arg) were supplied by H.L. Crespi of Argonne National Laboratory, and they were already adapted to growth in D_2O. Because no difficulty was encountered in growing dense cultures of these organisms autotrophically, they were used in much of the early work to determine the effects of different growth conditions on lipid production. Others on the list were chosen to establish the generality of the findings or because of reports of their ability to produce large amounts of fatty acid containing lipid in conventional aqueous media [16,17]. Adaptation efforts were carried out on a test-tube scale with initial transfers to 50% D_2O media followed by successive transfers to 90 and 99.8% D_2O. The other *Scenedesmus* and *Chlorella* organisms were induced to grow at least moderately well in 99.8% D_2O, but organisms 8,9, and 10 did not grow well even in 50% D_2O.

Organisms 2 and 3 were not bacteria free, and although the other organisms were pure cultures initially, they also became contaminated with bacteria with repeated subculturing. Bacterial contamination is usually ignored by algae mass culturists, and we also found that the biomass contribution of bacteria in a healthy algal culture was insignificant. However, where the culture was allowed to incubate

for a long period of time in an effort to maximize the lipid production, the proportion of bacteria increased and thus may contribute a small amount of lipid to the total.

Media

The following formulae were usually employed:

Chlorella

$NaNO_3$	1.25 g/l
KH_2PO_4	1.25
$MgSO_4 \cdot 7H_2O$	0.256
$FeSO_4 \cdot 7H_2O$	0.0054

Scenedesmus

$MgSO_4 \cdot 7H_2O$	0.49 g/l
NH_4NO_3	0.20
Na_2HPO_4	0.10
$KHCO_3$	0.10
$Ca(NO_3)_2 \cdot 4H_2O$	0.22
NaCl	0.010
$FeSO_4 \cdot 7H_2O$	0.014

Trace Elements (in both media)

B	0.5 ppm
Mn	0.25
Zn	0.05
Cu	0.02
Mo	0.05

A variety of other media were used in attempts to grow organisms 8, 9, and 10.

The presence of hydrogen in the water of crystallization of some of the salts and in ammonium nitrate introduces only a few tenths of an atom percent hydrogen in the D_2O media. The D_2O was obtained from Aldrich Chemical Co. (99.8 at. %), and it was redistilled before use. The D_2O solutions used as growth media were filtered through 0.2-μm Millipore filters into sterile culture vessels immediately before inoculation with the desired alga.

Culture Conditions

Initial culturing was carried out in large glass test tubes (5 by 350 cm) suspended in a glass-walled water bath at 29.5°C. Agitation was provided by a magnetic stirring bar, and illumination was by two 40-W "Cool White" fluorescent tubes placed about 20 cm from the culture tubes outside the water bath. To reduce the culture size and increase the efficiency of stirring and intensity of illumination, the culture vessels subsequently were changed to 0.5 and 1.0 liter Erlenmeyer flasks illuminated from below through a sheet of Lucite supporting the flasks and attached to a rotary shaker run at about 100 rpm. Light intensity was at about 6000 lux, but shielding by metal screens was sometimes necessary initially to prevent bleaching of low-density inocula. The apparatus was kept at about 26°C in an incubator with rapid air circulation.

Nitrogen gas with 5% CO_2 or air with 3.6% CO_2 was used to aerate the cultures. The gas was dried by passage through a Drierite column, rehumidified by bubbling through D_2O, and filtered through a 0.3-μm glass air filter before passing into the culture medium through a small Teflon tube at 10 to 20 ml/min. Exposure of the algal cultures to atmospheric humidity was minimized to prevent contamination by H_2O.

Determination of Growth

As a measure of the density of the suspension of algal cells in a culture, the light absorbance of 1.0 ml of culture diluted with water to 6.0 ml was determined in a Klett colorimeter filled with a red filter. For relative chlorophyll concentrations, 1.0 ml of the same diluted cell suspension was diluted further to 8.3 ml. A 2.0-ml aliquot was treated with 20 μl of a 1.0-μM methanol solution of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea, and the fluorescence was measured in a fluoromicrophotometer equipped with an R-136 photomultiplier tube and Corning glass filters No. 5543 and 2418 (see Ref. 18 for details).

Lipid Extraction

The algal cultures were harvested by centrifugation. After one wash in D_2O, a small aliquot was dried at 80°C and weighed to establish the biomass of the culture. The packed cells (50 to 100 mg dry weight) were resuspended in 1 ml of D_2O and ruptured by heating 1 to 2 min in a boiling water bath followed by cooling in crushed ice.

The algal lipids were extracted by the method of Bligh and Dyer as modified by Kates [19]; the solvents were redistilled. To the cell suspension (50 to 100 mg dry weight), 3.75-ml methanolchloroform (2:1, v/v) was added and shaken intermittently for several hours. After centrifugation the lower chloroform phase was removed, and the residue resuspended in 4.75 ml methanol-chloroform-D_2O (2:1:0.8, v/v), shaken several times over a half hour or more, and centrifuged. The supernates of the two extractions were combined, and 2.5 ml each of D_2O and chloroform were added and centrifuged. The lower chloroform phase was removed and evaporated in a stream of dry nitrogen at 35°C while swirling on a rotary shaker. A small amount of benzene was added occasionally to aid in the removal of water. The dry weight of the crude lipid extract was recorded after a constant weight was attained. The procedure was scaled up proportionally for larger quantities of cells. During the investigation, it was learned that the yield could be improved somewhat by acidifying the initial extracting solvents with 1% HCl (3 M), and this change was incorporated as a routine part of the procedure [20].

Chemical Derivatization

Fatty acids are present in algal lipids largely in the form of glycerol esters. To free the individual fatty acids and to make them volatile enough for gas chromatographic analysis, it was necessary to form the fatty acid methyl esters (FAMEs). This was done in one step by transesterification [21]. A methanol solution (1 ml) containing 14% BF_3 was added to 10 to 20 ml of the dry crude lipid extract and heated in a closed vial at 80°C for 0.5 h. Water (3 ml) was added, and the FAMEs were extracted with three or more 5-ml portions of pentane with intervening centrifugations to clarify the phases. The combined extracts were evaporated under a stream of dry nitrogen at 35°C on a rotary shaker, and the residue was weighed. The residue was dissolved in hexane and appropriately diluted for gas chromatography (GC). For larger quantities of lipid extract, the reagent volumes used were scaled up proportionally.

Fatty Acid Analysis

GC of the FAMEs was performed on a Hewlett-Packard 5830 gas chromatograph by using a 3-m (10-ft), 2 mm-i.d. glass column packed with 10% SP-2330 on 100/120 Chromosorb. Helium was used as the carrier gas with flame ionization detection (FID). The column was heated from 200° to 230°C at 2°C per min with a 1-μl injection volume from 0.01 to 1.0 mg/ml sample solutions. The GC/FID data given here represent averages of at least three runs.

GC of FAMEs is the traditional method of analysis of fatty acids from lipids. The time-consuming nature of the lipid extraction, purification, and esterification procedures, and the large number of algal samples generated in our algal growth and lipid optimization studies required that we investigate and find a more rapid method of fatty acid screening in algal samples for economy.

We have applied fast-atom bombardment mass spectrometry (FABMS) for the rapid and direct analysis of fatty acids from complex lipids in intact algal cells [22]. (See the appendix of this report for description.) Because the FABMS method required only small quantities of sample, avoided the lengthy preparation procedure required for GC samples, and yielded the same relative amounts of major fatty acids found by GC/FID, FABMS was used extensively for screening the fatty acid composition of the algal cultures.

The atom-percent deuteration of the fatty acids was determined from the electron ionization mass spectra obtained by gas chromatography/mass spectrometry (GC/MS) on the FAMEs. The atom-percent deuteration of any of the fatty acids was calculated from the relative abundance of selected ions in the molecular ion region as described by Wendt and McCloskey [23]. The average atom-percent deuteration for five samples was measured as 98.3 ± 0.5%. The $[RCOO]^-$ region of the negative ion FAB mass spectra of the algal samples also allowed the atom-percent deuteration to be determined, and FABMS yielded values similar to the GC/MS data.

RESULTS

Fatty Acid Composition of Deuterated Algae

Table 1 presents a comparison of the relative fatty acid composition of several *Chlorella* and *Scenedesmus* organisms grown in the same media made up in H_2O and D_2O and harvested near the end of the exponential growth phase. The 16:0 (carbon chain length:number of double bonds) fatty acid was higher for *Scenedesmus* cultures grown in D_2O than in H_2O, but the converse was true for *Chlorella*. The 16:1 and 18:3 fatty acids tended to be lower for all organisms grown in D_2O, and 18:1 was markedly higher for *Chlorella* grown in D_2O than in H_2O.

Table 1 also summarizes the relative fatty acid composition of all the algae that were grown successfully in D_2O. The two strains of *Chlorella* had essentially the same deuterated fatty acid composition with the major constituents being 16:0, 18:1, and 18:2. *Scenedesmus obliquus* strains 393 and Arg were also very similar in deuterated fatty acid composition with 16:0 constituting about half of the total fatty acid. *Scenedesmus obliquus* strains 746 and 1237 had lower 16:0 and higher 18:2 than the other two strains, and almost half of the total fatty acids of strain 1237 was 18:1.

Effect of Nitrogen-Deficient Media

Chlorella vulgaris (Arg) and *S. obliquus* (Arg) were grown in 100-ml volumes of D_2O culture medium for 23 days. Fifty milliliters were removed from each, and the cells were spun down and resuspended in 50 ml of fresh medium without a nitrogen source (nitrate or ammonium salts). To the remaining 50 ml of uncentrifuged cell culture was added the same amount of nitrogen-containing salts

Table 1 — Fatty Acid Composition of a Variety of Algae Grown in H_2O and D_2O Media

Organism	Fatty Acids[a]							
	Medium	16:0	16:1	16:2	18:0	18:1	18:2	18:3
Scenedesmus obliquus								
strain 393	H_2O	37	7.4	--	5.2	45	5.1	1.1
strain 393	D_2O	51	5.1	--	6.0	31	7.6	--
strain Arg	H_2O	39	11	--	2.3	21	7.9	19
strain Arg	D_2O	47	9.4	--	--	33	8.1	3.0
strain 746	D_2O	30	4.5	4.5	2.3	36	18	4.5
strain 1237	D_2O	31	2.9	--	--	46	14	5.7
Chlorella vulgaris								
strain 397	H_2O	53	9.3	--	1.6	5.6	19	12
strain 397	D_2O	28	2.3	--	6.4	30	23	9.5
strain Arg	H_2O	53	9.0	--	2.1	5.4	19	12
strain Arg	D_2O	29	1.4	--	10	28	23	7.3

[a] Number of carbon atoms in molecule: number of double bonds.

as was present in the original medium. Both nitrogen-enriched and nitrogen-deprived cells were allowed to grow for 17 to 18 days, and then they were harvested for fatty acid analyses. Table 2 shows the results. The fatty acid composition was similar for the nitrogen-enriched and nitrogen-free cultures, but the dry cell weight was lower for the nitrogen-free cultures, especially for *Scenedesmus*. However, the fraction of cells composed of lipid and fatty acid was higher for cells from nitrogen-free media.

Effect of Iron-Enriched Media

During this investigation, we learned that the addition of iron to the normal medium significantly increased the rate of chlorophyll production and turbidity in *Chlorella* cultures. Experiments were carried out to determine the effect of the added iron on lipid production. Table 3 shows typical results. The yields of cell weight and lipid for all of these cultures were higher than normal and tended to increase with increased iron concentrations. The relative amounts of the different fatty acids were about the same for all cultures, but the yield of fatty acids was improved markedly with increased levels of iron, particularly for the 30-day culture. Figure 1 shows the growth (Klett turbidity reading) and chlorophyll concentration with culture age for the culture with an iron concentration sixteenfold higher than normal. Excess iron did not have comparable growth effects on *Scenedesmus* organisms.

In another experiment, *C. vulgaris* (Arg) was grown in normal D_2O media for 47 days. Then iron was added at five times the normal level, and the culture was allowed to incubate for another 25 days. In this case, the proportions of 16:0 and 18:2 fatty acids decreased to 17 and 15%, and the 18:1 fatty acid increased to 55%. Thirty-one percent of the cell dry weight was extractable lipid, and 10% was fatty acid.

Effect of Drying

D_2O cultures of *C. vulgaris* (Arg) and *S. obliquus* (Arg) grown in normal media for 52 days were centrifuged, resuspended in a small amount of D_2O, and dried partially by passing dry air over the

Table 2 — Lipid and Fatty Acid Content of *Chlorella vulgaris* (Arg) and *Scenedesmus obliquus* (Arg) Grown With and Without Nitrogen Sources in D_2O Medium

Organism	Nitrogen	Dry Wt. (mg/ml)	Crude Lipid (% of dry wt.)	FAMEs (% of dry wt.)	FAMEs (mg/ml)	
Chlorella vulgaris	+	5.18	17.3	15.5	0.80	
	−	4.71	31.8	31.6	1.49	
Scenedesmus vulgaris	+	3.94	22.8	20.7	0.52	
	−	1.69	32.2	27.3	0.46	
	Fatty Acid Composition (percent of total)					
		16:0	18:0	18:1	18:2	18:3
Chlorella vulgaris	+	44	2	10	34	10
	−	38	6	14	30	12
Scenedesmus vulgaris	+	40	<2	47	9	2
	−	33	<3	44	14	6

Table 3 — Effect of Ferrous Iron Concentration in D_2O Media on Growth and Lipid Production of *Chlorella vulgaris* (Arg)

	Iron Concentration ($\times 10^{-5}$M)			
	1.9 (normal) (22 days)	7.6 (22 days)	31 (22 days)	31 (30 days)
Cell dry wt. (mg/ml)	10.2	9.7	12.1	13.4
Crude lipid (% cell wt.)	57	62	67	65
Fatty acid (% cell wt.)	31	>37[a]	52	61
Fatty acid (mg/ml)	3.2	>3.7[a]	6.3	8.5

[a] The values for fatty acids may be slightly greater because of an experimental loss.

Iron Concentration ($\times 10^{-5}$M)	Fatty Acid Composition (percent of total)						
	16:0	16:1	18:0	18:1	18:2	18:3	X[b]
1.9 (normal; 22 days)	28	1.3	13	24	25	7.0	1.7
7.6 (22 days)	29	1.4	10	28	23	7.3	1.5
31 (22 days)	30	1.2	11	26	23	7.4	1.2
31 (30 days)	28	1.3	13	27	23	5.5	1.5

[b] Unidentified

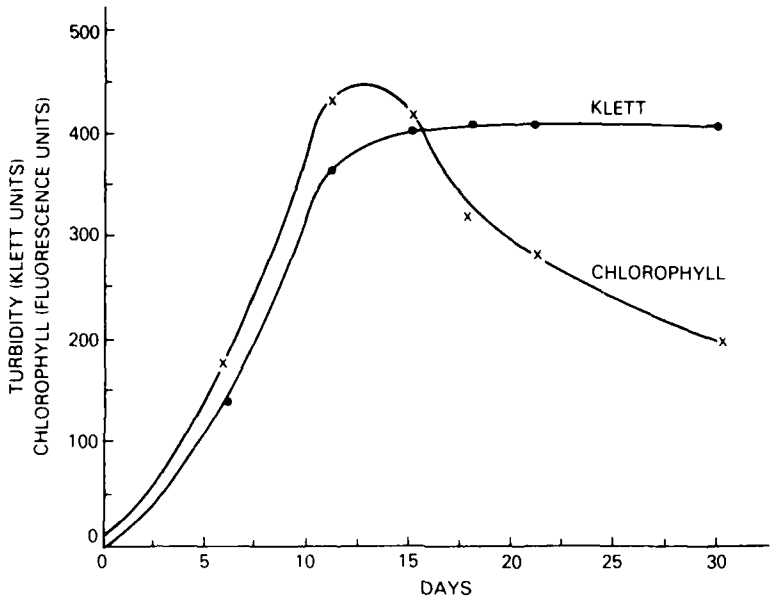


Fig. 1 — Turbidity and chlorophyll content of *Chlorella vulgaris* grown in D_2O medium containing high iron concentration

suspension for 1 to 2 days. This drying process produced no change in the lipid content of *S. obliquus* and lowered the proportion of lipid in *C. vulgaris.*

Chlorella vulgaris (Arg) was grown also on an agar surface for 50 days (50 ml of 2% agar equilibrated with D_2O and containing the normal salts); Table 4 shows the results. The proportion of fatty acid was increased compared to the results with normal liquid cultures (Table 1), but the production of lipid was not improved.

Table 4 — Lipid Production of *Chlorella vulgaris* (Arg) Grown on Surface of 50 ml D_2O-Agar

Cell Dry Wt.	Crude Lipid	Fatty Acid	Fatty Acid Composition (Percent of total)					
mg	mg	mg	16:0	16:1	18:0	18:1	18:2	18:3
178	51	35	21	2.6	5.9	42	23	4.5

DISCUSSION

The experimental results of this investigation show that selected species of the simpler green algae, adapted to growth in heavy water (D_2O) media, can be induced to produce substantial amounts of fatty acid-containing lipids. As might be expected from the results of earlier studies using aqueous media, the total lipid content and relative amounts of fatty acids present depended on the particular organism and the environmental conditions of growth. The C_{16} and C_{18} fatty acids were predominant, and the qualitative composition was similar to that of the same organisms grown in H_2O, but the relative amounts of the various acids differed. Compared to the analyses by Graff et al. [24] of fatty acids in *S. obliquus* grown in D_2O, we found qualitatively the same array of fatty acids but higher 16:0, especially with the Arg and 393 strains, lower 16:2, higher 18:0, and much lower 18:2.

Several approaches to promote the production of perdeuterated fatty acids by algae were tried based largely on the experience of previous investigators using conventional aqueous cultures. Limiting nitrogen availability by transferring an active culture to nitrogen-free medium significantly increased the lipid concentration in *Chlorella* but not in *Scenedesmus.* More striking increases in lipid production by *Chlorella* were observed in old cultures, especially where iron was in high concentration. Lipid and fatty acid yields reached 65 and 61% of the cell dry weight. These were the highest yields obtained in this investigation. The favorable effects of iron with *Chlorella* are not readily explainable, because iron is considered to be precipitated readily and unavailable in media without added chelating agents.

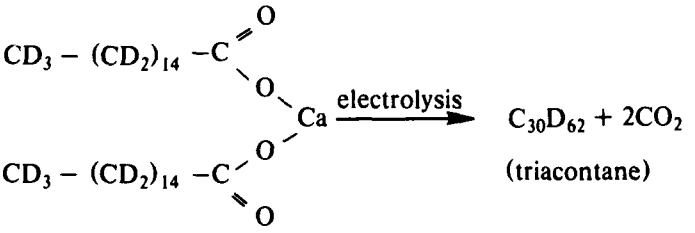
The extent of deuteration of the fatty acids produced by algae in this investigation usually exceeded 98%. A more rigorous exclusion of water by completely preventing contact of the D_2O media with air and by recrystallizing in D_2O certain of the mineral nutrients in the growth media would likely bring the extent of deuteration still closer to 100%. The use of undeuterated methanol for lipid extraction and esterification of the fatty acids did not appear to be a likely source of hydrogen substitution on the deuterocarbon chain, but no experimental verification of this was made.

Enhancement of lipid production by *Chlorella* subjected to drying conditions has not been as marked as Spoehr and Milner obtained in H_2O media [25]. The unusually high concentration of oleic acid (18:1) obtained with *Chlorella* growing on an agar surface (Tables 1 and 4) may be of interest for synthesis of particular deuterated materials.

Although much remains to be learned about the capabilities of various algae to grow and produce lipid in heavy water under different environmental conditions, sufficient information now appears to be available to formulate lubricant synthetic strategies using the deuterated compounds produced in greatest abundance by algae.

The production of a particular fatty acid by an alga can be promoted by choice of organism and culture conditions, but it appears likely that a mixture of fatty acids will always be present in the lipid extract. Where only one fatty acid is wanted for synthesis of a particular compound, it will be necessary to make a separation. Preparative chromatographic procedures are available, but for larger quantities it will be more economical to make at least initial fractionations by using film evaporation for acids with different carbon chain lengths and differential crystallization from selected solvents for acids with different degrees of unsaturation [26].

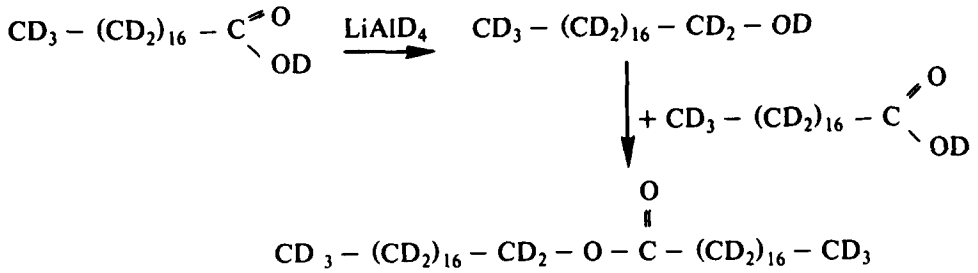
An approach to synthesizing a long-chain deuterocarbon lubricant from fatty acids is illustrated.



(hexadecanoic acid, calcium salt)

This is a classical Kolbe synthesis in which metallic salts of fatty acids are decarboxylated electrolytically. Recombination results in long-chain compounds with possible applications as lubricant greases. Maximum use of the fatty acids produced by algae could be realized, and fractionation of individual fatty acids avoided by reacting the mixture of fatty acids (almost exclusively C_{16} and C_{18}) and subsequently reducing the double bonds present.

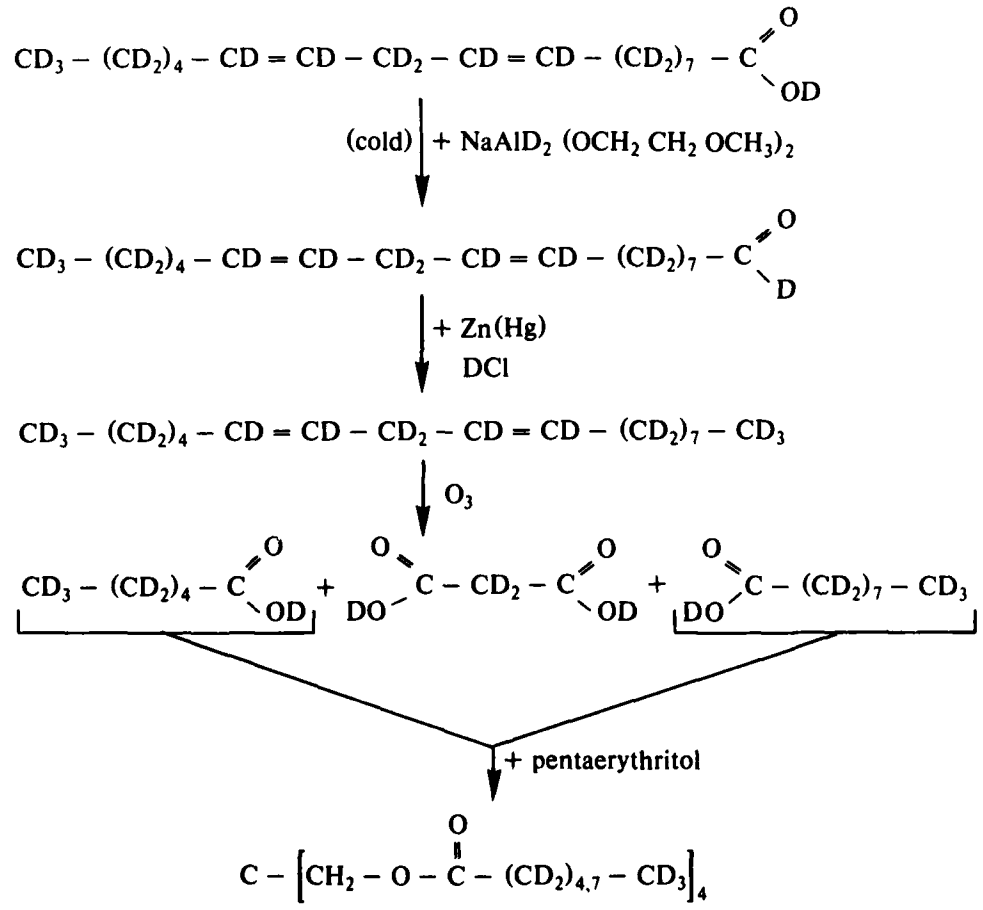
Long-chain esters analogous to sperm oil might be synthesized by reduction of the fatty acid carboxyl group and subsequent esterification with a fatty acid with the same or a different number of carbon atoms:



(octadecyl octadecanoate)

A variety of similar possibilities obviously exists with the deuterated fatty acids from algae. Liquidity of the product will depend on the double bonds present.

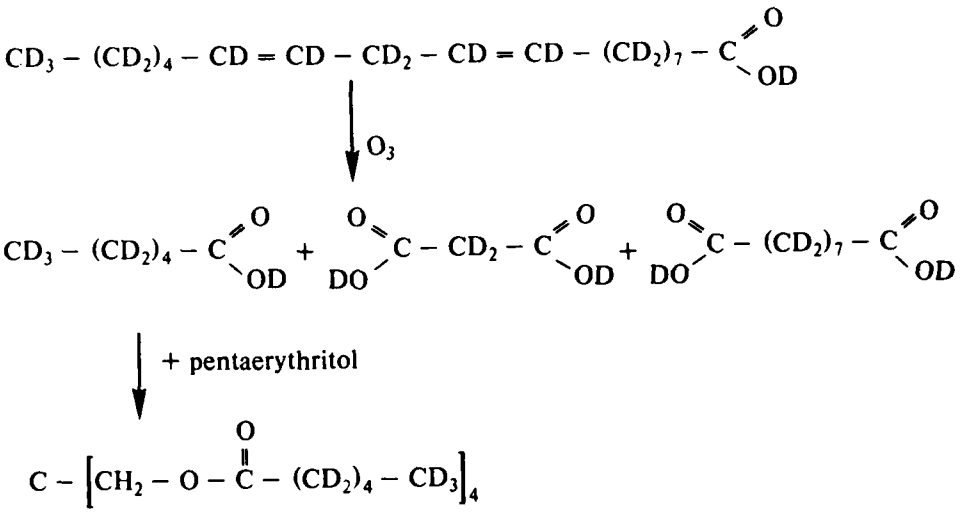
A possible approach to the preparation of relatively short-chain fatty acids suitable for synthesis of polyol ester lubricants is as follows:



pentaerythritol perdeutero mixed tetraalkanoate

The carboxyl group of linoleic acid is reduced to the aldehyde by using a selective reagent (Vitride, Eastman) [27] followed by a Clemmensen reduction to the unsaturated deuterocarbon. The double bonds are cleaved with ozone to yield a mixture of C_6 and C_9 monocarboxylic and malonic acids. The former are reacted with the pentaerythritol to give a mixed tetraalkanoate ester suitable as a lubricant. The pentaerythritol moiety need not be deuterated to obtain good oxidation stability [7,12,14].

A much simpler reaction sequence can lead to the pentaerythritol tetrahexanoate:



pentaerythritol perdeuterotetrahexanoate

Hexanoic acid should be readily separable from the dicarboxylic acids resulting from ozonolysis by differential solubility.

Other strategies exist for preparing deuterated lubricant materials from the fatty acids available from algae. Obviously, the choice of the final product, the selection of the algae and culture conditions, and the processing and chemical procedures used will all be important in determining whether the biosynthetic approach to preparing deuterated lubricants will be cost-effective compared to conventional methods.

The use of microorganisms to prepare deuterated materials would be most advantageous where the product is impossible or difficult to make by the usual chemical methods. Microorganisms carry out their syntheses under mild conditions so that compounds can be deuterated that would not withstand the high temperatures, pressures, and the catalysts necessary for conventional H/D exchange. Double bonds, for example, are always saturated by the customary methods of hydrocarbon deuteration [28,29]. Possible products include polyisoprene for making elastomers with resistance to high temperature or ozone [30,31]. Another possible compound is polyhydroxybutyric acid, a material accumulated by microorganisms as an energy reserve and recently proposed as a biologically produced thermoplastic [32]. The practical value of deuterating this compound is not established, but it might make an interesting demonstration compound for comparison with the hydrogen analog.

With the present rapid growth in knowledge of the genetic controls that operate in algae and other microorganisms capable of growing in D_2O, it is reasonable to expect that an organism can eventually be engineered to elaborate a particular deuterated compound a great deal more efficiently than is now possible.

Other possibilities exist, but a basic question must be addressed when considering deuteration of compounds more labile than hydrocarbons. Namely, whether deuteration will make enough of a difference in the stability of the final compound to be worth the cost of the deuterium and the effort required for preparation? This can probably be decided satisfactorily by conducting oxidation tests on a few model compounds isolated from algae.

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Appendix

FAST-ATOM BOMBARDMENT MASS SPECTROMETRY OF ALGAL SAMPLES

INTRODUCTION

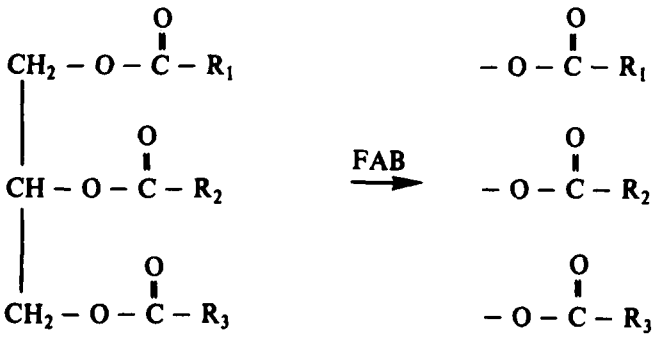
The method of fast-atom bombardment mass spectrometry (FABMS) has been used for the analysis of a wide variety of thermally labile biological compounds [A1]. This method is relevant to many general problems of a similar nature as described here in biotechnology, environmental science, and the medical sciences. Previously, FABMS has been used to profile fatty acids from lipids, the results of which were in excellent agreement with the results of gas chromatography (GC) on the fatty acid methyl esters (FAMEs) [A2-A4]. However, in all of these reports, extracted and/or purified lipid compounds were used. We have used FABMS to screen fatty acids from intact algal cells with no sample preparation.

EXPERIMENTAL

A reverse-geometry, double-focusing mass spectrometer was used for the FABMS experiments. Samples were prepared in a triethanolamine (TEA) matrix, and they were bombarded with an 8-keV xenon atom beam. All mass spectra were obtained at a resolving power of at least 1000. The mass spectral relative ion abundances of the carboxylate ions represent averages of at least 10 scans over a mass-to-charge ratio (m/z) 50 to 500 range. Approximately 1 to 10 mg of intact dried algal cells or 50 μl of a 1 to 3 mg/ml crude lipid extract solution were added to 100 μl of TEA so that 1 to 100 μg of total lipid were deposited on the probe tip for analysis.

GAS CHROMATOGRAPHY VS FAST-ATOM BOMBARDMENT MASS SPECTROMETRY

We have found that negative ion FABMS of intact algal cells suspended in a liquid matrix yielded abundant carboxylate negative ions, $[RCOO]^-$, corresponding to the fatty acids from the lipid compounds. In FABMS, these very stable carboxylate ions (also observed from the free acids [A5]) arise from the fragmentation of complex lipid compounds such as a triacylglycerol as shown in Scheme 1.



Scheme 1

The FABMS measurement of the relative abundances of the carboxylate ions yielded semiquantitative data that compared well with results obtained by the gas chromatography/flame-ionization detection (GC/FID) of the FAMEs (see Table A1). Because little sample preparation was necessary, the FABMS analyses were rapid and direct.

PROOF OF METHOD

To determine if FABMS could be used for the semiquantitative determination of fatty acid moieties on triacylglycerols, a proof-of-concept experiment was performed. The mixed triacylglycerol, glyceryl-1,2-palmitate-3-stearate (PPS), mixed in TEA, yielded the negative ion fast-atom bombardment (FAB) mass spectrum shown in Fig. A1. In addition to the ions from the TEA matrix (m/z 148, $[TEA-H]^-$, and m/z 297, $[2(TEA)-H]^-$), the carboxylate ions, $[RCOO]^-$, of the palmitate group (16:0, m/z 255) and the stearate group (18:0, m/z 283) of the triacylglycerol PPS were observed. The ratio of the average relative abundances of these two ions (16:0/18:0) was measured to be 2.0 ± 0.2 from four experiments. This study was repeated by using the triacylglycerol glyceryl-1,2-stearate-3-palmitate (SSP) with analogous results. The FABMS results were confirmed by GC analysis of the corresponding FAMEs, and the accuracy and precision were comparable to that of the FABMS result.

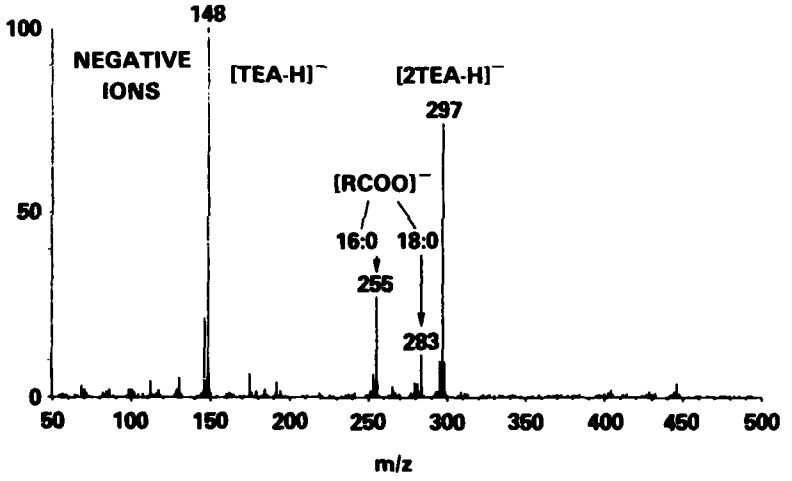


Fig. A1 — A negative ion fast-atom bombardment mass spectrum of the mixed *triacylglycerol, glyceryl-1,2-palmitate-3-stearate* (PPS),in triethanolamine

In addition to the $[RCOO]^-$ species observed for each acid moiety, an ion is observed at 2 amu below the $[RCOO]^-$ species. The abundances of these lower mass species are 5 to 15% of the $[RCOO]^-$ abundance and apparently result from dehydrogenation during the FAB ionization process. The relative abundances of the fatty acid moieties reported here are not corrected for these contributions because the semiquantitative data and correlation coefficients are not affected significantly by the correction.

The positive ion FABMS of the triacylglycerols yielded abundant diacylglycerol ions, $[(M+H-RCOOH)]^+$, and low abundances of the ions corresponding to either the protonated triacylglycerol molecule, $[(M+H)-H_2O]^+$. No abundant positive ions were observed from which the different fatty acid moieties could be determined directly and unambiguously. For this reason, negative ion FABMS was used for the fatty acid screening of algal lipids.

ALGAL SAMPLES

Figure A2 shows an example of FABMS of an algal sample. This algal sample, *Scenedesmus*, was grown in conventional water media, and the ions observed correspond to undeuterated fatty acid species. Figure A2(a) shows the mass spectrum obtained from the intact cells where the most abundant carboxylate negative ions correspond to the oleate group (18:1, m/z 281) and the palmitate group (16:0, m/z 255). The $[2(TEA)-H]^-$ species is observed at m/z 297. The FAB mass spectrum of the crude lipid extract, shown in Fig. A2(b), is almost identical to that of the intact cells, indicating that no major changes in the composition of the lipids occurred due to extraction (e.g., preferential extraction). The crude lipid extract provided a more concentrated sample; consequently, greater ion abundances were observed in the FAB mass spectra (although the signal-to-noise ratio did not improve). Finally, Fig. A2(c) shows the FAB mass spectrum of the algal residue remaining after extraction, and it demonstrates that little unextracted fatty acid-containing material was present.

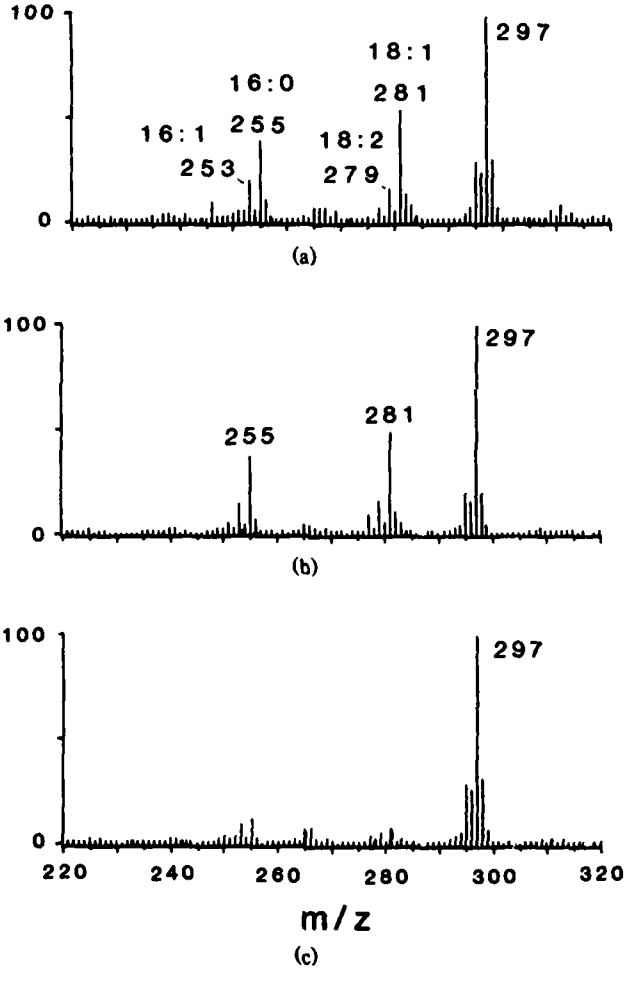


Fig. A2 — Negative ion fast-atom bombardment mass spectrum of (a) intact *Scenedesmus* cells in triethanolamine, (b) chloroform/methanol crude lipid extract of *Scenedesmus* cells in triethanolamine, and (c) *Scenedesmus* extract residue in triethanolamine

Algal samples were analyzed by FABMS in the above manner except that carboxylate ions were detected at mass-to-charge ratios corresponding to the deuterated analogs. Table A1 presents data obtained by the FABMS analysis of the algal cells compared with data obtained by the GC/FID of the corresponding FAMEs. These algal data represent different samples of *Scenedesmus* and *Chlorella* algal strains. Only the most abundant (>1%) fatty acid species are tabulated. Sample number 1 is the only deuterated algal sample shown. It is evident from these and other data that the same fatty acids predominate (i.e., palmitate (16:0), oleate (18:1), and linoleate (18:2) in deuterated and undeuterated samples). The correlation coefficients given in the last column indicate the excellent agreement between the data taken by the two methods.

Table A1 — Comparison of Analyses of Fatty Acids of Complex Lipids in Various Algal Samples

Sample	Method	16:0	16:1	18:0	18:1	18:2	18:3	Correl. Coeff.
1[a]	FABMS	20	5.4	1.9	48	18	6.7	0.99
	GC/FID	18	2	1.4	56	16	6.2	
2	FABMS	52	9.1	2.2	5.6	19	12	0.93
	GC/FID	39	7.1	2.6	7.3	25	19	
3	FABMS	36	7.4	5.2	45	5.1	1.3	0.99
	GC/FID	35	6.2	3.1	48	4.3	3.3	
4	FABMS	39	11	2.2	21	7.8	19	0.99
	GC/FID	42	7.1	1.6	20	8.3	20	
5	FABMS	37	7.1	3.4	28	16	8.5	0.95
	GC/FID	32	3.3	2.7	32	17	13	

[a] 98 at.% deuteration

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