Isolation and characterization of sphingolipid from Ochromonas danica

Mong Ching Wong Ganfield
Iowa State University

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ISOLATION AND CHARACTERIZATION OF SPHINGOLIPID
FROM OCHROMONAS DANICA

by

Mong Ching Wong Ganfield

A Dissertation Submitted to the
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Dean of Graduate College

Iowa State University
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1970
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Until the last decade little was known about the distribution of sphingolipids except in higher animals. The discovery of phytoglycolipid in seeds (1, 2, 3) and of cerebrosides in leaves (4, 5) as well as the isolation of a new class of sphingolipids, ceramide aminoethylphosphonates, from a sea anemone (6) showed that sphingolipids were of wider distribution than had previously been demonstrated and that some novel structures were to be found.

At the time this research was begun sphingolipids had been isolated from several metazoan phyla, but not from protozoa; from higher plants and fungi, but not from algae or bacteria. Since then reports have appeared of the isolation of sphingolipids from the zooflagellate protozoan Crithidia fasciculata (7) and the ciliate protozoan Tetrahymena pyriformis (8). More recently, the presence of a phytoglycolipid has been reported in a green alga - Scenedesmus obliquus (9). There is also a report of a ceramide phosphorylethanolamine and ceramide phosphorylglycerol in the lipids of Bacteroides melaninogenicus, an anaerobic bacterium (10).

Nevertheless our knowledge of the distribution and structures of sphingolipids is still very limited. It is clear that sphingolipids are found in most if not all the major groups of organisms and this wide distribution indicates a role of fundamental importance. Little is known about this role beyond the vague generalization that is is probably "structural".
In this work we have examined the lipids of *Ochromonas danica*, *Anacystis nidulans* and *Rhodospirillum rubrum* for the presence of sphingolipids, for comparative purposes. *Ochromonas danica* is a phytoflagellate protozoan in the order Chrysomonadidae. This order is considered one of the most primitive, phylogenetically, of eucaryotic organisms and is thought to be similar to the evolutionary progenitors of both higher plants and animals (11, 12). The cells of these organisms may be among the simplest having the typically animal-like property of ingesting particulate food. *Ochromonas danica* also contains a chloroplast, but it is only weakly photosynthetic. When only given carbon dioxide the population of *Ochromonas danica* will stay constant in light but diminish rapidly in the dark. This inadequate photosynthesis is attributed to its low chlorophyll content - since only chlorophyll a was found in these organisms and also the only carotene identified was β-carotene (13, 14, 15). *Ochromonas danica*, thus can be considered really as an obligate heterotroph which has retained only enough of its photosynthetic apparatus to survive in time of distress. It can be maintained easily on a chemically defined medium and obtained in large quantities.

The blue-green algae are the simplest organisms in which the photosynthetic apparatus has the structure similar to that of the chloroplast. Nuclear and mitochondrial membranes are not present so most of the complex lipid in the cell must be part of the structure of the photosynthetic apparatus. *Anacystis nidulans* was chosen as a representative of this group of organisms because it could be obtained in large enough quantities to
allow the isolation of a lipid present in very small quantities. These organisms, being intermediate in complexity between the bacteria and higher plants and animals, may be valuable in further studies of the metabolism and function of sphingolipids.

The photosynthetic bacteria are considered by some to be the most primitive of the currently living forms. The outstanding common physiological property is their ability to grow under strictly anaerobic conditions, using light as the ultimate energy source. These bacteria comprise three groups or families: Thiorhodaceae (purple sulfur bacteria), Athiorhodaceae and Chlorobacteriaceae (green bacteria). Rhodospirillum rubrum belongs to the Athiorhodaceae. The latter is composed of purple and brown no-sulfur-containing photosynthetic bacteria. These microorganisms closely resemble the purple sulfur bacteria, except they do not require H₂S and utilize organic matter as the preferred source of hydrogen. The Athiorhodaceae, therefore, tend to be heterotrophic in their metabolism. In addition, some species are capable of growing both anaerobically in the light and aerobically in darkness. Such is the case of Rhodospirillum rubrum. The nonsulfur purple bacteria are encountered mainly in waters containing organic matter, especially those with intensive disintegration processes; they are occasionally found in some soils.

A representative species (Rhodospirillum rubrum) of these photosynthetic bacteria was included in our investigations because of their unique position among other bacteria; also because there have been no reports of investigations of sphingolipids in these microorganisms.
Sphingolipids

The term sphingolipid is used as a convenient designation for those lipids containing the organic base sphingosine, related bases and their homologs.

\[
\text{C}_{18}\text{-sphingosine}
\]

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_{12}\text{CH}=\text{CH}-\text{CH}-\text{CH}-\text{CH}_2 \\
\text{OH} \quad \text{NH}_2\text{OH}
\end{align*}
\]

\[
\text{C}_{18}\text{-dihydrosphingosine}
\]

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_{14}\text{CH}-\text{CH}-\text{CH}_2 \\
\text{OH} \quad \text{NH}_2\text{OH}
\end{align*}
\]

\[
\text{C}_{18}\text{-phytosphingosine}
\]

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_{13}\text{CH}-\text{CH}-\text{CH}-\text{CH}_2 \\
\text{OH} \quad \text{OH} \quad \text{NH}_2\text{OH}
\end{align*}
\]

\[
\text{C}_{18}\text{-dehydrophytosphingosine}
\]

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_8\text{CH}=\text{CH}(\text{CH}_2)_3\text{CH}-\text{CH}-\text{CH}-\text{CH}_2 \\
\text{OH} \quad \text{OH} \quad \text{NH}_2\text{OH}
\end{align*}
\]

The need for a standardized nomenclature for the lipids has been recognized by both organic chemists and biochemists for a long time. A new nomenclature for lipids was proposed in 1967 by the IUPAC-IUB (International Union of Pure and Applied Chemistry-International Union of Biochemistry) Commission on Biochemical nomenclature (16). According to the new rules, the compound previously known as dihydrosphingosine
is called sphinganine. This name may be modified by prefixes to indicate additional substituents or higher or lower homologs. Configuration of substituents are indicated by "D" or "L" prefixes. Thus C_{18}-dihydrosphingosine is octadecasphinganine and phytosphingosine is 4D-hydroxysphinganine. However, in this thesis the old nomenclature will be used.

The bases are not found free in nature. The simplest sphingolipid is a ceramide, which is an N-acyl derivative of the long-chain base. The two best known classes of derivatives of long-chain bases are cerebrosides and sphingomyelins. Cerebrosides are glycosides in which the ceramide is attached to a carbohydrate moiety, usually galactose, at the 1-hydroxyl position of the base. Some molecules have more than one sugar residue, for examples gangliosides, strandin and lipids of red cell stroma. Sphingomyelins are ceramides joined to a phosphorylcholine group.

\[
\text{Cerebroside} \\
\begin{array}{c}
\text{CH}_3(\text{CH}_2)_n\text{CH} = \text{CH} - \text{CH} - \text{CH} - \text{CH}_2 - \text{O-galactose} \\
\text{OH} \quad \text{NH} \\
\text{CO} \\
\text{R} \\
\end{array}
\]

\[
\text{Sphingomyelin} \\
\begin{array}{c}
\text{CH}_3(\text{CH}_2)_n\text{CH} = \text{CH} - \text{CH} - \text{CH}_2 - \text{O-P-OCH}_2\text{CH}_2\text{N(CH}_3)_3 \\
\text{OH} \quad \text{NH} \\
\text{CO} \\
\text{R} \\
\end{array}
\]
Recently long-chain bases have been discovered in many unusual molecules such as phytoglycolipids and ceramide aminoethylphosphonates.

Sphingosine was first isolated from brain lipids by Thudichum in 1882 (17). For a long time animal tissue were thought to be the only source of this material. In 1911, Zellner (18) isolated from the mushroom *Amanita muscaria* a nitrogen-containing substance which later was identified by Carter et al. as phytosphingosine (19). Since that time the same base or a closely similar substance has been obtained from other mushrooms (20), yeast (21) and molds (22, 23, 24). The rather widespread distribution of phytosphingosine in fungi raised the question whether there was a general plant long-chain base. For a more detailed discussion of the earlier investigations, the reader is referred to the reviews by Celmer, Law and Carter (25, 26, 27).

In 1954, Carter et al. (19) reported the isolation of a long-chain base from corn and soybean phosphatides. In subsequent studies the long-chain base was found to be part of a rather complex molecule, which was given the name phytoglycolipid. The latter is unique among the sphingolipids, because it possesses the structural features of a glycolipid as well as those of a phosphatide. It contains fatty acid, phosphorus, inositol, glucuronic acid, glucosamine, mannose, galactose, arabinose and the long-chain base, phytosphingosine. Upon alkaline degradation, corn or soybean phytoglycolipid gave among other products cerebronyl-phytosphingosine phosphate and an oligosaccharide, which contains inositol, glucosamine, hexuronic acid, galactose, arabinose and mannose. Mild acid hydrolysis of the oligosaccharide fraction
yielded a trisaccharide containing inositol, glucuronic acid and glucosamine. The order of these sugars were then established by nitrite degradation (1, 2, 3). The data thus obtained supported the following formula:

$$\text{CH}_3(\text{CH}_2)_3\text{CH}-\text{CH}-\text{CH}_2-\text{O-}\text{P-}\text{O-} \text{inositol}$$

$$\text{OH} \quad \text{OH} \quad \text{NH} \quad \text{OH}$$

$$\text{mannose}$$

$$\text{glucuronic acid}$$

$$\text{galactose}$$

$$\text{glucosamine}$$

$$\text{arabinose}$$

$$\text{fucose}$$

Phytoglycolipids with similar compositions were also isolated from the phosphatides derived from peanuts, wheat germ, flax seed, cottonseed and sunflower seed (28). Little is known about the metabolism of these lipids, although preliminary studies indicated that phytosphingosine was not stored as reserve material and has its highest concentration in seeds (29).

In 1961, Carter et al. (30, 31) reported the isolation of a glucocerebroside containing dehydrophytosphingosine in wheat flour. Later Sastry and Kates (4, 5) reported the presence of a glucocerebroside containing both phyto- and dehydrophytosphingosine in runner bean leaves.

In recent years, the discovery of a new sphingolipid having a covalent carbon-phosphorus bond has led to a new interest in the study of the distribution of sphingolipids. α-Aminoethylphosphonic acid was first found in rumen protozoa and *Tetrahymena pyriformis* (32). The same compound was isolated later from sea anemones (*Anthopleura elegantissima*) where it occurs free and esterified with glycerol and...
ceramide (6). The structure of the ceramide aminoethylphosphonate was finally elucidated by Simon and Rouser (33) as:

\[
\text{CH}_3(\text{CH}_2)_{12}\text{CH}=\text{CH}-\text{CH}-\text{CH}_2-\text{O}-\text{P}-\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3
\]

They suggested that the ceramide aminoethylphosphonate is perhaps a substitute for the sphingomyelin present in other animal cells. Since then, the ceramide aminoethylphosphonate has been found in a limited number of snails (34), the gametes of some aquatic animals (35) and also in other invertebrates (36, 37).

It is interesting to note that the sphingolipids, discovered originally as components of the central nervous system of higher animals, now appear to be present in such a wide variety of living organisms.

**Ochromonas danica** Lipids

Of all Protozoa, the flagellates, or Mastigophora, are perhaps the most varied in their characteristics. This class is divided into two large subdivisions: the Phytomastigina (plantlike forms) and the Zoomastigina (animal-like flagellates), depending on their mode of nutrition.

The phytomastigina are regarded as probably the most primitive of Protozoa, because they are both phagotrophic and photosynthetic;
also the life cycle of many of them resembles that of the green algae.

Of the plantlike flagellates the order Chrysomonadida is undoubt-
edly closely related to the brown algae. They are relatively small
and lack a cell wall. Some of them are saprophytic but most of them
also possess chromatophores, and are therefore autotrophic too. They
are ubiquitous in fresh water and soil, and to this order belongs the
genus *Ochromonas* (38).

The chemical composition of the phytoflagellates is of interest
phylogenetically as these organisms are algal animals and the lipids
of *Ochromonas* species have proved to be particularly interesting in
this regard.

The animal fats differ in composition from typical plant fats in
containing a larger variety of fatty acids. Palmitic and oleic acids
are the predominant acids in the vegetable and animal lipids. However,
stearic acid which plays such a prominent role in the animal kingdom,
is absent or present in very small amounts in most of the vegetable
fats. On the other hand, vegetable fats frequently contain large pro-
portions of linoleic acid, while this diethenoid acid is seldom found
in appreciable quantity in animal fats. Also, both saturated and
unsaturated fatty acids of the C₂₀, C₂₂ and even C₂₄ series are found
in many animal fats, but rarely in lipids of plant origin. The fatty
acid composition in plants is related to the taxonomic groups and many
of these groups have rather unusual lipids such as acids with acetylenic
unsaturation, hydroxyl groups, carbocyclic rings and branched chains.
In case of animal lipids, their fatty acid compositions sometimes reflect the diet used (39).

The lipids of *Ochromonas danica* contain approximately 57% unsaturated fatty acids. The most abundant fatty acid is linoleic (16%) followed by myristic (15%), arachidonic (11%), palmitic and \(\gamma\)-linolenic, both 10%. The fatty acid composition is, therefore, rather similar to the plant lipid composition, except for the presence of arachidonic acid. This acid is usually found in animal lipids only; the occurrence of this acid in *Ochromonas danica* is, therefore rather interesting (40).

The sulfolipid, 1,2-di-O-acyl-sn-glyceryl-3-O-6-sulfo-D-quinovopyranoside (SQDG) was first recognized in photosynthetic microorganisms; it since has been found in all higher plants and algae investigated (41). Shibuya et al. (42) have observed that the sulfolipid in *Lemna* is specifically localized in the quantosomes and suggested that perhaps it plays a special role in the photosynthetic apparatus. This sulfolipid has also been found in *Ochromonas danica* (43). In the same paper, the authors observed that the sulfolipid accumulates five to six times more in photoautotrophic cells than in cells grown under heterotrophic conditions, which agrees with the observations of Shibuya.

In 1965 Haines reported the isolation of a novel sulfolipid from a variety of microbes, ranging from *Pseudomonas* species (sea water bacteria) to *Tetrahymena*, including *Ochromonas danica* and *Ochromonas malhennensis* (44). As a rule, polar lipids isolated from biological tissue consist of a long aliphatic chain of at least
fourteen methylene carbons with a polar functional group at one end. This novel compound, however, had polar groups on both ends of the molecule. The structure has been assigned as 1,14-docosyl-disulfate (45).

Elovson (46) reported last year the purification of a class of compounds, from *Ochromonas danica*, which is related to the 1,14-diol-disulfate. It differed from Haine's sulfolipid in that there was a varying number of chlorine atoms on the hydrocarbon chain; also the aliphatic chain lengths varied. One member of the class was identified as 13-chloro-docosane-1,14-diol-disulfate. The presence of two, three, four, five and even six atoms of chlorine in similar molecules has also been established. It seems that in cells in the stationary phase of growth the majority of the sulfolipids are chlorinated, with the hexachloro compound being the most abundant.

With the exception of *Tetrahymena pyriformis*, only recently have sphingolipids been reported in Protozoa (47). In 1966, Carter et al. (7) first reported the isolation of a sphingolipid from the trypanosomid flagellate *Crithidia fasciculata*. The major long-chain base portion was identified as a branched 19-methyl-C_{20}-phytosphingosine, while a small amount of C_{20}-phytosphingosine was also present. Both bases occurred as part of ceramides. In the subsequent year, the same group isolated two new sphingolipids from *Tetrahymena pyriformis* (8); the only sphingolipid previously reported in *Tetrahymena* was sphingomyelin (47). These new long-chain bases were also branched; they contained a total of seventeen and nineteen carbons, and have a double bond between carbons four and five. They were present as constituents of ceramides
and ceramide aminoethylphosphonates. Also in both light and dark grown *Euglena gracilis*, the major long-chain base was found to be C$_{20}$-sphingosine (8). It appears thus that sphingolipids might be ubiquitous components of protozoan lipids.

**Algal and Bacterial Lipids**

The possibility of a close relationship between blue-green algae and bacteria first was suggested as early as 1853 by Cohn (48). Recent studies of bacteria and blue-green algae in the electron microscope have shown striking similarities in the basic cellular architecture of the two groups (49). However, establishment of any phylogenetic relationship will undoubtedly also depend on biochemical and chemical properties of the organisms.

A comprehensive discussion of such relationships will not be attempted in the present review, but only a comparison of some of the lipid components of photosynthetic bacteria, blue-green algae and the higher plants.

The subject of bacterial lipids has been reviewed extensively by Asselineau (50) and Kates (51). In general, bacteria appear to contain no sterols, sphingolipids or polyunsaturated fatty acids. They have less neutral glycerides and more branched-chain, cyclopropane, hydroxy and free fatty acids and possibly more glycolipids than other organisms.

Reports of the presence of sphingolipids in bacteria are extremely rare (50). Until recently the only bacterial sphingolipid reported
were sphingomyelin, which was isolated from the pleuropneumonia-like organism \textit{Mycoplasma gallisepticum} (52) and an ethanolamine containing sphingolipid in the rumen anaerobe \textit{Bacteroides ruminicola} (53). In a recent publication LaBach and White (10) reported the finding of a ceramide phosphorylethanolamine and ceramide phosphorylglycerol in another anaerobic bacterium - \textit{Bacteroides melaninogenicus}. The long-chain base portion was found to be \(\text{C}_{17}, \text{C}_{18}\) and \(\text{C}_{19}\)-dihydrosphingosine; with the 17 and 19-carbon bases being branched (54). However, no sphingolipid has been reported in photosynthetic bacteria yet.

Wood \textit{et al.} (55) studied the major lipids of five species of photosynthetic bacteria: \textit{Rhodopseudomonas spheroides}, \textit{Rhodopseudomonas capsulata}, \textit{Rhodopseudomonas palustris}, \textit{Rhodopseudomonas gelatinosa} and \textit{Rhodospirillum rubrum}. They found that phosphatidyl ethanolamine, phosphatidyl glycerol, the sulfolipid SQDG, cardiolipin and \(\alpha\)-ornithyl phosphatidyl glycerol were the major components. The only lipids common to all five species were phosphatidyl ethanolamine and phosphatidyl glycerol.

Although the lipids from bacteria have been extensively investigated, there is only a small number of publications on the lipids of blue-green algae.

Hirayama (56) while studying lipids from the blue-green alga \textit{Anacystis nidulans} observed that the main components were mono- and digalactosyl diglyceride, phosphatidyl glycerol, the sulfolipid SQDG and an unknown lipid. Mono- and digalactosyl diglycerides, phosphatidyl
choline, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl glycerol and sulfolipid SQDG were reported to be present in photosynthetic tissues of spinach, *Chlorella*, *Scenedesmus* and other green plants and algae (57). Thus it seems that *Anacystis nidulans* lacks many of the complex lipids found in other photosynthetic organisms. The only complex lipid common to all five species of photosynthetic bacteria studied by Wood, green alga (*Chlorella vulgaris*), blue-green alga (*Anacystis nidulans*) and spinach is phosphatidyl glycerol.

The presence of a sphingolipid has not yet been reported in blue-green algae; its isolation from the green alga, *Scenedesmus obliquus*, was published only recently (9). The lipid seemed to be a phytoglycolipid. The bases were determined to be C\textsubscript{20}-phytosphingosine, C\textsubscript{18}-sphingosine, as well as some C\textsubscript{16}-dihydrosphingosine. The carbohydrate moiety comprised of galactose, mannose, rhamnose, glucosamine and one unidentified sugar.

In the investigation of the fatty acid composition, it was found that the bulk of constituents in blue-green alga cells have between ten and eighteen carbon atoms. Unlike the green alga *Chlorella* and higher plants, the fatty acids are saturated or have only one double bond. This is very similar to photosynthetic bacterial fatty acids. The major fatty acids present in *Anacystis nidulans* are palmitic and palmitoleic, but some polyenoic fatty acids have been found in *Anabaena variabilis*. The distribution pattern of the saturated and unsaturated acids in the individual lipid classes, however, resembles that of spinach (58).
EXPERIMENTAL MATERIALS AND METHODS

Materials

Biological materials

The Astasia longa culture was obtained from Algal Type Culture Collection, of the Department of Botany, University of Indiana, (Bloomington, Indiana). The Ochromonas danica cultures were from the Haskins Laboratory (New York, N.Y.). Starting cultures of Anacystis nidulans were gifts of Dr. Ruth Wildman, Department of Botany, Iowa State University (Ames, Iowa). Rhodospirillum rubrum culture was purchased from the Midwest Culture Service (Terre Haute, Indiana). Cat brain samples were gifts of Dr. D. Thake, National Animal Disease Laboratory (Ames, Iowa). Frozen spinach used was a product of Libby's.

Astasia cells were first cultured in a crude medium with the following composition:

Yeast extract (Difco) 1g/liter
Bacto-tryptone (Difco) 1g/liter
Sodium acetate 2g/liter

Later the cells were grown in a chemically defined medium (59). The cells were maintained in large Erlenmeyer flasks, about three fourths full and kept in the dark.

Ochromonas danica cells were also grown in a chemically defined medium (60) purchased from the General Biochemicals (Chagrin Falls,
Ohio). When cultivated under normal laboratory conditions or in the dark the cells were kept in four liter Erlenmyer flasks containing three liters of medium. When the cells were grown under continuous illumination, they were maintained in a twelve gallon carboy containing thirty liters of medium. The cells were harvested at the beginning of the stationary phase.

Anacystis nidulans cultures were grown in a chemically defined medium (61) in a twelve gallon carboy. The aeration was provided by 4% carbon dioxide in air (Matheson Company Inc., Joliet, Illinois) while illumination was provided by fluorescent lights which were kept on for sixteen hours and then off for eight hours.

Rhodospirillum rubrum was cultured anaerobically in a chemically defined medium (62). It was grown in one liter flasks under continuous illumination.

Chemical materials

All solvents used were reagent grade and redistilled. About 50 mg of the antioxidant: 4-methyl-2,6-di-tert-butylphenol (BHT) was added to each liter of the solvent (63).

Silica gel G, used for thin layer chromatography, was obtained from Brinkman Instruments, Inc. (Westbury, N.Y.). For gel-filtration chromatography, polystyrene beads with 2% divinylbenzene cross linkage, sold under the commercial name of Bio-beads X-2, 200-400 mesh, were purchased from Bio-rad Laboratories (Richmond, California). Unisil, an acid washed, activated silicic acid, 100-200 mesh, was purchased from Clarkson Chemical Company, Inc. (Williamsport, Pennsylvania). "Woelm"
silica gel (Eschwege, Germany) was purchased from Alupharm Chemicals (New Orleans, Louisiana).

Washed and pretested DEAE-cellulose for column chromatography, hexamethyldisilazane, trimethylchlorosilane and the gas chromatography column packing: 3% OV-1 (by wt.) on Gas Chrom Q, 100-200 mesh, were all products of Applied Science Laboratories (State College, Pennsylvania).

Dihydrosphingosine was purchased from Miles Laboratories, Inc. (Elkhart, Indiana). Sphingosine was isolated from beef lung according to the procedure of Tipton (64). Tetraacetyl phytosphingosine was a gift of Dr. Frank Stodola, Northern Regional Research Laboratories (Peoria, Illinois). The free base was isolated from it by an alkaline hydrolysis (65). Fatty acid methyl ester standards were purchased from Applied Science Laboratories (State College, Pennsylvania).

Method

Methods of chemical analysis

To isolate the carbohydrate from lipid, samples were first hydrolysed in 2N HCl for three hours at 70°C. The hydrolysate was extracted with either chloroform or hexane to remove lipids and the carbohydrate was analyzed by gas chromatography following trimethylsilation (68). The colorimetric determination of carbohydrate was accomplished using the anthrone method of Radin et al. (66) and the orcinol method of Svennerholm (67).

Phosphorus was determined by the molybdenum blue method as described by Harris and Popat (69).
Sulphate was determined by precipitating the sulphate in the form of amine sulphate using cetyltrimethyl ammonium bromide and 4-amino-4'-chlorodiphenyl hydrochloride. The unreacted reagent was then measured spectrophotometrically (70).

Nitrogen was analyzed by the micro-Kjeldahl method (71). Quantitative determination of long-chain bases was carried out utilizing the methyl-orange complex method (72).

To determine the fatty acid content of the dry cell residue, the sample was saponified with 6N KOH and then neutralized with HCl. The fatty acids were extracted with petroleum ether and chromatographed on thin layer plates. CHCl₃-MeOH-H₂O (65:25:4) solvent system was used to develop the plates and the spots were visualized by spraying with H₂SO₄ and charring.

Methods of structure determination

**Thin-layer chromatography**

Microscope slides (2.5 cm x 7.6 cm) were coated with silica gel G and dried at room temperature. The plates were developed in CHCl₃-MeOH-2N NH₃, 40:10:1 (v/v/v). The spots were visualized by spraying with either of the following reagents: a. 75% H₂SO₄, then charring at 120°C; b. 0.2% ninhydrin dissolved in 95 ml butanol and 5 ml pyridine, followed by heating in a hot oven for several minutes until pink spots appear (73); c. Clorox-benzidine (74). Reagent c is specific for amines and amide linkages.

**Methylation of fatty acids**

The methyl esters of fatty acids were made by the methanol-H₂SO₄ procedure (75).
Gas chromatography

A Varian Aerograph model 1520B equipped with a hydrogen flame detector was used. Nitrogen was used as carrier gas. The flow rates of both hydrogen and nitrogen were set at 30 ml/min, while the air flow rate was 380-400 ml/minute.

A six ft., 1/8 in. i.d. glass column was packed with 3% OV-1 on Gas Chrom Q, 100/200 mesh. The column was conditioned by baking for 30 minutes without gas flow and then with gas flow at 350°C overnight.

The long-chain base and carbohydrate samples were chromatographed as the trimethylsilyl ether derivatives (76, 68). The chromatography was carried out isothermally at 210°C for long-chain bases and 190°C for methyl esters of fatty acids and for sugars. The injector temperature was 265°C and the detector was maintained at 280°C for all runs.

Mass spectrometry

The instrument used was a Perkin-Elmer model 270 combined gas chromatograph-mass spectrometer. Long-chain bases were introduced into the instrument by means of the solid-samples probe, and the temperature was set between 45-50°C. The ionization voltage was set at 70 ev.

Isolation procedures

Isolation of long-chain base from Astasia longa

The cells of Astasia longa were hydrolyzed by refluxing in a mixture of MeOH-concentrated HCl (80:16) at 70°C for 16 hours (29). The residue was then removed by centrifugation. Enough water was added to the supernatant to make a 1:1 ratio of MeOH to H₂O, which was then made alkaline.
with concentrated KOH. The mixture was subsequently extracted three times with two volumes of diethyl ether. The diethyl ether extracts were combined, washed with water to remove any trace of KOH, and dried over anhydrous Na$_2$SO$_4$. The solvent was then concentrated to dryness under reduced pressure. The residue was analyzed for long-chain base content by the colorimetric method of Lauter and Trams (72).

**Extraction of complex lipids** 1. The following extraction procedure was used to isolate complex lipids from *Ochromonas danica*, *Anacystis nidulans*, *Rhodospirillum rubrum* and spinach.

The microorganisms were grown in chemically defined media, harvested by centrifugation and washed with distilled water. If not used immediately they were kept frozen.

The packed cells or frozen spinach leaves were first extracted by stirring with a mixture of CHCl$_3$-MeOH (2:1) and the insoluble residue removed by filtration. The extract was then evaporated to dryness under reduced pressure. The pigmented total lipid extract was further extracted with benzene and filtered to remove any benzene-insoluble material. The benzene was again removed under reduced pressure.

To separate the complex lipids from the neutral lipids and pigments, gel-filtration chromatography was used (77). Approximately 200 grams of polystyrene beads (200-400 mesh) were allowed to swell in benzene or tetrahydrofuran overnight. The slurry was packed into a 4 cm x 80 cm glass column with a teflon stopcock. The amount of lipid applied depended on the amount of pigments in the sample. It varied from 100-150 mg.
for *Rhodospirillum rubrum*, to 200-250 mg for *Anacystis nidulans* and 350-400 mg for *Ochromonas danica*. The entire elution was carried out with benzene or with tetrahydrofuran. The complex lipid was eluted in the first band, which was brown.

2. Cat brain lipids were extracted by the procedure of Folch et al. (78).

**Isolation of sphingolipids from the complex lipid** The method used to separate the alkali-stable sphingolipid from the rest of the complex lipids was a modification of the procedure described by Carter and Gaver (8).

Mild alkaline hydrolysis was carried out by the addition of approximately 0.5 ml of freshly prepared 1N methanolic NaOH to an equal volume of CHCl₃-MeOH (2:1) containing about 20 mg of lipid. The mixture was left at room temperature for 30 minutes with occasional mixing. The alkali was then neutralized by the addition of 1N HCl and the appropriate volumes of CHCl₃, MeOH and water were added to give the Folch's partition of CHCl₃-MeOH-H₂O (8:4:3). This mixture was shaken vigorously and centrifuged to separate the phases. The glass stoppered centrifuge tube was then left at room temperature for an additional 30 minutes. If any cloudiness developed in either layer, the centrifuge tube was left undisturbed until both phases became clear. After removing the upper phase, the sides of the vessel were washed down carefully with a mixture of MeOH-H₂O (1:1). Care was taken not to disturb the lower phase.

The lower phase was evaporated to dryness under a stream of nitrogen and the residue partitioned into acetone-soluble and acetone-
insoluble fractions. The acetone-insoluble fraction was further purified by washing with water. The water-insoluble residue was then redissolved in CHCl₃-MeOH (2:1). Any insoluble material was removed by centrifugation and the CHCl₃-MeOH (2:1) solvent evaporated under a stream of nitrogen. When this fraction was dissolved in CHCl₃ alone, it left a dark oily film on the sides of the vessel, which was in turn dissolved in MeOH. Thus two final fractions were obtained from the acetone-insoluble fraction: CHCl₃-soluble and MeOH-soluble.

In the original procedure of Carter and Gaver the Folch's lower phase was evaporated to dryness under a stream of nitrogen. The residue was applied to an Unisil (silicic acid) column and eluted first with CHCl₃, to remove neutral lipids, followed by MeOH, to elute the polar lipids. The sphingolipid-containing MeOH fraction was then used in the acid methanolysis.

**Isolation of long-chain base from sphingolipid**

To isolate the long-chain base from the alkali-stable fraction the acid methanolysis according to Carter and Gaver was used (8).  

**Solvent contamination**

During the early part of our investigation, a gas chromatographic peak with the same elution time as C₁₈-dihydrosphingosine was observed, regardless of the starting material used. It became obvious that the material was not a long-chain base when it appeared in samples which had not been converted to trimethylsilyl derivatives. This same peak, however, was not found in the long-chain bases isolated from cat brain.
Following is a diagram of the isolation of long-chain base from the cells.

```
Cells
  | CHCl₃-MeOH(2:1) extraction
  | total lipid
  | dry cell residue
  | benzene extraction
  | benzene-insoluble
  | benzene extract
  | Gel chromatography
  | pigments and neutral lipids
  | complex lipids
  | 1. mild alkaline hydrolysis
  | 2. Folch's partition
  | upper phase (discard)
  | lower phase
  | A. acetone Unisil fractionation column
  | B. MeOH (neutral lipid) used in acid methanolysis
  | acetone-insoluble
  | acetone-soluble
  | CHCl₃-MeOH (neutral lipid)
  | CHCl₃-soluble
  | MeOH-soluble
  | O. danica
  | A. nidulans
  | A. Modified procedure of Carter and Gaver
  | B. Original procedure of Carter and Gaver
  | 1. Acid methanolysis
  | 2. Hexane extraction
  | methylester fatty acids
  | aqueous layer
  | 1. concentrated KOH
  | 2. ether extraction
  | ether extract
  | aq. layer (LCB* fraction)

*LCB = long-chain base.
```
samples. The major difference in the procedures used was the amount of organic solvents, because larger volumes of solvent were needed for the extraction of microorganisms. By repeated evaporation of these solvents to dryness, what was present initially as small contaminants was concentrated enough in the final product to make it detectable by gas chromatography. A mock run of the whole isolation procedure gave the same "long-chain base" peak on the gas chromatogram. In subsequent work all the organic solvents were redistilled before use, and the spurious peak was eliminated.
RESULTS AND DISCUSSION

Results

Astasia longa

Astasia cells were first cultivated in complex media. The long-chain base was isolated by the procedure described in the experimental section and the amount determined spectrophotometrically (72). The yeast extract and bacto-tryptone used in media were treated similarly and used as a control. The amount of long-chain base detected in Astasia cells was so small it easily could have been contamination by the media used, since both yeast extract and bacto-tryptone contained long-chain bases.

The Astasia sample contained 0.001% long-chain base while yeast extract had 0.11% and bacto-tryptone, 0.12%. The results were computed on weight by weight basis.

To eliminate the possibility of contamination by the medium used, the cells were transferred to a chemically defined medium (59).

Approximately 728.2 mg (dry weight) of Astasia cells were used in the acid hydrolysis. The diethyl ether extract of the hydrolysate weighed 81.3 mg. Methyl-orange determination, however, only indicated presence of 74 µg of long-chain base. It was about 0.01% of the total dry weight. Sphingosine was used as standard.

Thin layer chromatography of the same product developed in CHCl₃-MeOH-2N NH₃ (40:10:1) and sprayed with "Clorox-benzidine" reagent (74) showed the presence of two spots. They corresponded to the two major
bases of a sample isolated from beef lung, sphingosine and dihydrosphin-
gosine.

We did not attempt to identify these bases further or to isolate
the sphingolipids because the cells were relatively difficult to grow
since they do not reach a very high cell density and also they were
easily contaminated by other microorganisms.

_**Ochromonas danica**_

**Isolation of complex lipids** The isolation of total lipids by
CHCl₃-MeOH (2:1) extraction was preferred over Folch's partition (78)
because it was easier and no detectable difference was found between
the two procedures. Analysis of the dried cell residue gave negligible
amounts of fatty acid; therefore, the extraction was essentially com­
plete. The total lipid was approximately 30% of the total dry weight
(Table 1).

The amount of benzene-insoluble material varied widely from one
batch of cells to another; the reason is unknown.

**Gel-filtration column chromatography** Gel-filtration of the
total lipids gave six colorful bands, however, they were not always
clearly discernible. The first band contained the complex lipids and
was light brown. The second band was dark green, which was followed
by a grayish green band. The next band was yellow, followed by a grayish
yellow band and finally by a bright orange band. Analysis indicated
that only the first band contained long-chain base.
The complex lipid thus obtained comprises approximately 7.6% of the total dry weight and 41.3% of the benzene extract (Table 1).

Fractionation of the complex lipids into phospholipids and glycolipids was accomplished by gel-filtration column chromatography using tetrahydrofuran as eluent (79). The phospholipids were eluted first, followed by the glycolipids. Chemical analysis indicated that there was a rather good separation of these classes of lipids (Table 2).

This method, however, was later abandoned because of the difficulty in obtaining peroxide-free tetrahydrofuran. Peroxides could be removed by shaking the solvent with a concentrated solution of FeSO$_4$: 60 g of FeSO$_4$ in 6 ml of concentrated H$_2$SO$_4$ and 110 ml of water (80). The tetrahydrofuran was then partially dried over Na$_2$SO$_4$ and distilled.
Table 1. Yields of lipids and lipids fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ochromonas danica</th>
<th>percent of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dry weight</td>
<td>Benzene extract</td>
<td>Complex lipid</td>
<td>CHCl\textsubscript{3}-sol.</td>
<td>MeOH-sol.</td>
</tr>
<tr>
<td>Total lipid</td>
<td>29.5</td>
<td>19.5</td>
<td>7.6</td>
<td>41.3</td>
<td>4.4x10^{-4}</td>
</tr>
<tr>
<td>Benzene extr.</td>
<td>19.5</td>
<td>15.1</td>
<td>7.4</td>
<td>49.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Complex lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>CHCl\textsubscript{3}-sol.</td>
<td>0.01</td>
<td>0.21</td>
<td>0.09</td>
<td>0.91</td>
<td>4.7x10^{-3}</td>
</tr>
<tr>
<td>MeOH-sol.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-chain base</td>
<td>4.4x10^{-4}</td>
<td></td>
<td>4.7x10^{-3}</td>
<td>1.10</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Anacystis nidulans

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ochromonas danica</th>
<th>percent of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dry weight</td>
<td>Benzene extract</td>
<td>Complex lipid</td>
<td>CHCl\textsubscript{3}-sol.</td>
<td>MeOH-sol.</td>
</tr>
<tr>
<td>Total lipid</td>
<td>19.1</td>
<td>15.1</td>
<td>7.4</td>
<td>49.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Benzene extr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Complex lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCl\textsubscript{3}-sol.</td>
<td>0.09</td>
<td>1.80</td>
<td>0.04</td>
<td>0.51</td>
<td>1.27</td>
</tr>
<tr>
<td>MeOH-sol.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-chain base</td>
<td>4.4x10^{-4}</td>
<td></td>
<td>4.7x10^{-3}</td>
<td>1.10</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Rhodospirillum rubrum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ochromonas danica</th>
<th>percent of</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dry weight</td>
<td>Benzene extract</td>
<td>Complex lipid</td>
<td>CHCl\textsubscript{3}-sol.</td>
<td>MeOH-sol.</td>
</tr>
<tr>
<td>Total lipid</td>
<td>20.6</td>
<td>13.4</td>
<td>10.4</td>
<td>70.8</td>
<td>0.14</td>
</tr>
<tr>
<td>Benzene extr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td>Complex lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCl\textsubscript{3}-sol.</td>
<td>0.14</td>
<td>1.32</td>
<td>0.28</td>
<td>2.69</td>
<td>0.06</td>
</tr>
<tr>
<td>MeOH-sol.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-chain base</td>
<td>4.4x10^{-4}</td>
<td></td>
<td>4.7x10^{-3}</td>
<td>1.10</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Table 2. Chemical analysis of the lipids fractions from *O. danica*

<table>
<thead>
<tr>
<th>Sample</th>
<th>% carbohydrate</th>
<th>%P</th>
<th>%N</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃-MeOH (2:1)</td>
<td>1.35</td>
<td>0.10</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td>Complex lipid</td>
<td>11.65</td>
<td>0.22</td>
<td>1.92</td>
<td>1.17</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>3.67</td>
<td>0.40</td>
<td>2.27</td>
<td>0.99</td>
</tr>
<tr>
<td>Glycolipid</td>
<td>12.32</td>
<td>0.00</td>
<td>0.62</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*The carbohydrate content was analyzed by the orcinol method (61).*

This method, however, did not completely remove the peroxides from the solvent, and in addition it left some water which remained in the solvent even after distillation. This small amount of water drastically changed the behavior of the complex lipids during gel chromatography with the result that phospholipids and glycolipids were no longer separated.

Fatty acid analysis of the phospholipids and glycolipids from the column showed that the major component found in both fractions were oleic and linoleic acids and there was a rather high percentage of polyenoic fatty acids in both classes of lipids. The fatty acid composition of the phospholipids and glycolipids differed quantitatively rather than qualitatively (Table 3).

**Isolation of sphingolipids** In the preliminary studies, long-chain bases were isolated by direct acid methanolysis. However, as our investigations proceeded, it was found that when the samples were subjected to the mild alkaline hydrolysis, followed by acid methanolysis,
Table 3. Fatty acid composition of phospho- and glycolipids from *O. danica*

<table>
<thead>
<tr>
<th>Fatty acids&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phospholipids %</th>
<th>Glycolipids %</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>8.87</td>
<td>11.67</td>
</tr>
<tr>
<td>15:0</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>12.95</td>
<td>6.32</td>
</tr>
<tr>
<td>16:1</td>
<td>7.51</td>
<td>0.86</td>
</tr>
<tr>
<td>17:0</td>
<td>2.24</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>3.92</td>
<td>0.66</td>
</tr>
<tr>
<td>18:1</td>
<td>14.39</td>
<td>21.93</td>
</tr>
<tr>
<td>18:2</td>
<td>10.71</td>
<td>19.60</td>
</tr>
<tr>
<td>18:3 α</td>
<td>4.64</td>
<td>5.75</td>
</tr>
<tr>
<td>18:3 γ</td>
<td>4.40</td>
<td>8.66</td>
</tr>
<tr>
<td>18:4</td>
<td>5.76</td>
<td>6.78</td>
</tr>
<tr>
<td>20:3</td>
<td>7.91</td>
<td>4.69</td>
</tr>
<tr>
<td>20:4</td>
<td>8.63</td>
<td>5.50</td>
</tr>
<tr>
<td>23:02 ECL&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>23:20 ECL</td>
<td>7.19</td>
<td></td>
</tr>
<tr>
<td>23:22 ECL</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>23:60 ECL</td>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>22:4</td>
<td></td>
<td>2.24</td>
</tr>
<tr>
<td>22.5</td>
<td></td>
<td>3.23</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>99.99</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fatty acid analysis was carried out by the Northern Regional Laboratory (Peoria, Illinois).

<sup>b</sup>ECL = equivalent chain length.
then the bases could not be detected either by methyl-orange or gas chromatographic methods. Although it was possible to identify the bases through acid methanolysis alone, the mild alkaline hydrolysis was essential in order to isolate the original sphingolipid. It seems that the latter was lost during some step of the mild alkaline hydrolysis.

To test our assumptions and at the same time look for a better method of obtaining complex lipids with less pigments, spinach was used. The latter was known to contain a sphingolipid (81), although it has not been identified and also the lipid composition of spinach was similar to that of Ochromonas danica.

Studies on spinach Libby's frozen spinach was extracted with CHCl₃-MeOH (2:1) and benzene as described in the Experimental section. The benzene soluble material was then divided into two parts. One part was used in polystyrene column chromatography and the second part used in acetone fractionation.
All four fractions: benzene extract, acetone-soluble, acetone-insoluble and band I from the polystyrene column, were subjected to the mild alkaline hydrolysis. After the Folch's partition, the lower phases were again divided into two parts. One part was used in the silicic acid column purification step as described by Carter and Gaver (8). The other part together with the upper phase of the Folch's partition were dried under a stream of nitrogen and used directly in the acid methanolysis, by-passing the silicic acid column.

Long-chain base analysis (72) was carried out on all these samples, together with a sample of complex lipid from *Ochromonas danica*, obtained by polystyrene column chromatography. No long-chain base was found in those samples which had been purified by silicic acid column chromatography. The long-chain base content of the other samples is given in Table 4.

\*LCB = long-chain base.
Table 4. Long-chain base analysis of the Folch's phases obtained after mild alkaline hydrolysis of lipid fractions of spinach and O. danica

<table>
<thead>
<tr>
<th>Lipid fractions</th>
<th>% long-chain base</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folch's upper</td>
<td>Folch's lower</td>
<td></td>
</tr>
<tr>
<td>phase</td>
<td>phase</td>
<td>phase</td>
<td></td>
</tr>
<tr>
<td>Benzene extract</td>
<td>0.06</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Acetone-soluble fraction</td>
<td>0.04</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Acetone-insoluble fraction</td>
<td>0.05</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Band I</td>
<td>0.03</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>O. danica (Band I)</td>
<td>0.05</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

*See text, pp. 30 and 31.

Long-chain bases were detected only in the Folch lower phases of those samples that by-passed the silicic acid column, although insignificant amounts were also detected in the upper phase. It was evident that the sphingolipid was being retained on the column during the purification step. However, the silicic acid column has been used on cat brain samples with satisfactory results.

With respect to the merit of acetone fractionation versus polystyrene column chromatography, the results were not so obvious. Although complex lipids obtained by acetone fractionation had lower pigment content, some of the sphingolipids were lost in the acetone-soluble fraction, thus we decided to continue the use of column separation.

Gas chromatography of the trimethylsilyl ether derivatives of the spinach long-chain bases obtained by acid methanolysis gave the following retention times, relative to C\textsubscript{18}-dihydrosphingesine: 0.59, 1.00 and 1.26. Additional data are necessary to identify these bases.
Purification of the sphingolipids Since the Folch's lower phase was a highly pigmented mixture, additional methods of purification were necessary.

At first different methods of column chromatography were used. The Unisil column was replaced by "Woelm" silica gel. No sphingolipid was recovered from the column. Then it was thought perhaps the molecule was too polar to be eluted with MeOH alone, thus a mixture of CHCl$_3$-MeOH-H$_2$O (65:25:4); MeOH-H$_2$O (1:1) and H$_2$O alone were used as eluents, without success.

DEAE-cellulose column chromatography was tried with the following scheme of elution (82):

- CHCl$_3$-MeOH (9:1) - to elute all non-acidic lipids, except phosphatidyl ethanolamine
- CHCl$_3$-MeOH (7:3) - to elute phosphatidyl ethanolamine and lipids with same ionic group
- MeOH - to elute water soluble non-lipids
- CHCl$_3$-MeOH-ammonia-ammonia acetate (4:1+20 ml concentrated (29%) aqueous ammonia per liter + 0.05 M salt) - to elute all acidic lipids.

Again no sphingolipid was recovered. Attempts at using preparative thin layer chromatography also failed. At last it was decided to try acetone fractionation again, although knowing in advance that the separation would not be complete.

The acetone-insoluble fraction was a lightly colored powder. Most of the pigments remained in the acetone-soluble part. The latter became
an oily residue when dried under a stream of nitrogen.

To remove any water soluble contaminants such as sugars, amino acids, inorganic phosphorus or sulfates, that would interfere with the subsequent chemical analysis, the acetone-insoluble fraction was washed with water and dried.

In order to obtain an IR spectrum of the water-insoluble material, the sample was dissolved in CHCl₃ alone. It was noticed that the sample only dissolved partially, and the oily film left behind could be dissolved in MeOH. Analysis of both CHCl₃-soluble and MeOH-soluble fractions for long-chain bases indicated that there was an enrichment of the long-chain base in the CHCl₃-soluble portion, although the separation was not complete. In subsequent experiments the CHCl₃-soluble fraction was used almost exclusively. It was about 0.19% of the complex lipid and 0.01% of the total dry weight (Table 1).

Characterization of the sphingolipid Due to the small amount of material, the methods used to determine the structures of the sphingolipid and the long-chain base were restricted to those that only required minute amounts of material for performance.

Identification of long-chain bases For characterization purposes the CHCl₃-soluble fraction was used.

a. Methyl-orange determination - the amount of base present was determined by the methyl-orange method (72). C₁₈-dihydrosphingosine was used as standard. The CHCl₃-soluble sample contained 1.11% long chain
while the MeOH-soluble fraction had 0.27%. The percentages were computed on the weight by weight basis (Table 1).

b. Thin layer chromatography - thin layer chromatography of the isolated long-chain bases gave a single ninhydrin-positive spot. It had a relative Rf of 1.10 as compared to the 0.85 relative Rf value of phytosphingosine in the same solvent system. The standard was $C_{18}$-dihydrosphingosine. This was consistent with the gas chromatographic results, since the solvent system used for developing the thin layer plates could not resolve chain-length homologs of the same base.

c. Gas chromatography - gas chromatograms of the trimethylsilyl ether derivatives of the long-chain bases showed these major peaks with the following relative retention times in relation to $C_{18}$-dihydrosphingosine: 0.42, 1.00, 1.29 and 1.90. They corresponded to the equivalent chain lengths of 16.6, 18.0, 18.3 and 20.0 respectively. Usually a branched chain is indicated by a fractional equivalent chain length. Thus the 16.6 base is probably a branched $C_{17}$ base while the 18.3, a branched $C_{19}$-dihydrosphingosine. By far the major base is $C_{20}$-dihydrosphingosine (Fig. 1).

d. Mass spectrometry - analysis of the long-chain base sample by mass spectrometry substantiated the above conclusions.

The mass spectrum of standard $C_{18}$-dihydrosphingosine showed that the ion with highest mass had a m/e of 300, which corresponded to the molecular ion minus one. The major peak was at m/e 270, which can be obtained by the loss of $-\text{CH}_2\text{OH}$ from the molecular ion. By the further
Fig. 1. Gas chromatogram of the trimethylsilyl ether derivatives of the long-chain bases of *Ochromonas danica*
C20 DIHYDROSPHINGOSINE

C18 DIHYDROSPHINGOSINE

RETENTION TIME (MINUTES)
loss of m/e 18, probably a water molecule, m/e 252 resulted (Fig. 2).

The spectrum of the bases from Ochromonas danica has peaks with m/e 286, 300, 314 and 328 which are consistent with the molecular weights of \( C_{17}^-, C_{18}^-, C_{19}^-, C_{20}^- \)-dihydrosphingosine minus one. Major peaks in the spectrum at m/e 256, 270, 284 and 298 can be attributed to loss of \(-\text{CH}_2\text{OH}\) from the molecular ions, in agreement with the breakdown pattern of the standard dihydrosphingosine. Peaks corresponding to the further loss of water from each of these ions can also be seen. The position of the methyl group of \( C_{17}^- \) and \( C_{19}^- \)-dihydrosphingosine has not been determined (Fig. 3).

**Analysis of the fatty acids**

Methyl esters of the fatty acids of the sphingolipid were extracted during the acid methanolysis. Complete methylation was carried out by incubating in the MeOH-H\(_2\)SO\(_4\) mixture (75). The methyl esters analyzed by gas chromatography showed that the major component was palmitic \((C_{16}^-\text{ saturated})\) followed by stearic \((C_{18}^-\text{ saturated})\) (Fig. 4).

**Chemical analysis**

Analysis of the carbohydrate content by the anthrone method and gas chromatography of trimethylsilyl ethers gave negligible amounts of sugar. Phosphorus content was also insignificant. However, the sulfate analysis was 1.46% on weight by weight basis. There are 6 moles of sulfate for each mole of long-chain base.

**Anacystis nidulans**

Complex lipid from Anacystis nidulans The complex lipids from Anacystis nidulans were isolated as outlined in the Experimental section.
Fig. 2. Mass spectrum of the C_{18}-dihydrosphingosine standard
CH₃(CH₂)₁₄CH⁻CH₂CH₃OH NH₂  

MW = 301

Relative Abundance

252 (M-a-H₂O)
269 (M-b-a)
270 (M-a)

m/z
230 240 250 260 270 280 290 300 310 320

300 (M-b) 301 (M)
Fig. 3. Mass spectrum of the long-chain bases of *Ochromonas danica*
Fig. 4. Gas chromatogram of the methyl esters of fatty acids of the sphingolipid from *Ochromonas danica*
The blue-green algae contained approximately 19.1% total lipid on a dry weight basis. Complex lipids made up about 7.4% of the total dry weight and 49.7% of the benzene extract. The sphingolipid, i.e., the MeOH-soluble fraction was 0.03% of the total dry weight and 0.51% of the total complex lipid (Table 1).

Partial determination of the structure of the sphingolipid

Chemical analysis The chemical analysis of MeOH-soluble fraction gave 0.1% phosphorus. This value was too low to be of any significance, even if the impurities in the sample were considered. Carbohydrate analyzed by gas chromatography were also very low, more likely to be contaminants. Sulphate analysis gave 1.3% on weight by weight basis. Although its presence was clearly indicated, it could not be ascertained whether all the sulphate groups are part of the sphingolipid structure.

Analysis of the fatty acids The fatty acid composition of the sphingolipid analyzed by gas chromatography showed that the major components are lauric acid (C_{12}-saturated) followed by palmitic ccid (C_{16}-saturated)

Identification of the long-chain bases a. methyl-orang determination - the long-chain base content as determined by the methyl orange method was 1.27% of the MeOH-soluble fraction, $6 \times 10^{-3}$% of the complex lipid and $5 \times 10^{-4}$% of the total dry weight (Table 1).
b. Thin layer chromatography - thin layer plates visualized by spraying with ninhydrin indicated the presence of a single spot. It had the same $R_f$ value as $C_{18}$-dihydrosphingosine used as standard. The relative $R_f$ value of phytosphingosine in the same solvent was 0.86.

c. Gas chromatography - when analyzed by gas chromatography as trimethylsilyl ether derivatives, the sample of long-chain bases gave seven major peaks (Fig. 5). They had the following relative retention time in relation to $C_{18}$-dihydrosphingosine: 0.50, 0.71, 1.00, 1.36, 1.90, 2.67 and 3.62. When plotted against equivalent chain lengths of dihydrosphingosine homologs, the following chain lengths were obtained: 17.0, 17.4, 18.0, 18.9, 20.0, 21.7 and 24.0

**Rhodospirillum rubrum**

The complex lipid of *Rhodospirillum rubrum* was isolated as described in the Experimental section. Total lipid comprised 20.6% of the total dry weight. Complex lipid made up 10.4% of the total dry weight and 70.8% of the benzene extract (Table 1).

Approximately 10.2 g of *Rhodospirillum rubrum* cells (dry weight) were used for isolation. Of the 14.1 mg of CHCl$_3$-soluble and 28.7 mg of MeOH-soluble fractions obtained only part of them were used in acid methanolysis.

Methyl-orange determination of the diethyl ether extract of the acid hydroslysates gave a 0.014% of long-chain base in MeOH-soluble fraction and 0.064% in CHCl$_3$-soluble fraction (Table 1). Obviously these amounts are at the limit of sensitivity of the method employed.
Fig. 5. Gas chromatogram of the trimethylsilyl ether derivatives of the long-chain bases from *Anacystis nidulans*
Discussion

The results of our investigation show that we have isolated long-chain base-containing compounds from *Ochromonas danica* and *Anacystis nidulans*. However, the exact structures of the molecules were not determined.

Based on the gas chromatographic and mass spectrometric data, it was concluded that there were four long-chain bases in *Ochromonas danica*. The major base was C₂₀-dihydrosphingosine, followed by a branched C₁₇-dihydrosphingosine. Besides C₁₈-dihydrosphingosine, which was present in smaller amounts, minute quantities of a branched C₁₉-dihydrosphingosine were also found. The points of branching have not been determined. The finding of branched long-chain bases in *Ochromonas danica* and *Anacystis nidulans* is not unique, since branched bases have been reported both in protozoan (7, 8) and bacterial lipids (54).

The bases of *Anacystis nidulans* were analyzed by gas chromatography only. The gas chromatogram indicated the presence of a series of chain length homologs of dihydrosphingosine. These results are not conclusive, since the equivalent chain length graph was obtained with only two points; but the good agreement between the various relative retention times and equivalent chain lengths led us to conclude that this might be the case. The bases present were probably C₁₇, C₁₈, C₁₉, C₂₀, C₂₄ and two branched long-chain bases as indicated by the fractional equivalent chain lengths. Unsuccessful attempts were made to obtain
separate mass spectra of each of the bases by combined gas-chromatography-mass spectrometry, since the mass spectrum of such a mixture of bases would have been meaningless. Although sphingolipid has been reported in an increasing number of organisms, such as protozoa (7, 8), green alga (9), bacteria (10) and invertebrates (32, 16, 33, 34), it is believed that this is the first report of the occurrence of sphingolipid in a blue-green alga.

When comparing the bases of *Ochromonas danica* with the bases isolated from the cat brain which has a composition typical of mammalian central nervous tissue, it was noticed that although *Ochromonas danica* contained a number of components, they were all homologs of the same base. This was also true with the bases from *Anacystis nidulans*. On the other hand, the cat brain contained a variety of bases such as sphingosine, dihydrosphingosine, phytosphingosine plus a few other minor bases (Fig. 6). Also the sphingolipids of the animal tissue belonged to many different classes such as cerebrosides, sphingomyelins, gangliosides, etc., while the sphingolipid found in protozoa is more likely to belong to one or two classes only. In this regard the composition of the protozoan and algal long-chain base and sphingolipid is considerably simpler than the animal lipids.

During the earlier part of the investigation it was thought that the long-chain base present in *Ochromonas danica* was probably part of a cerebroside, since this is the most widespread form of sphingolipid in nature. However, the long-chain base-containing material found in
Fig. 6. Gas chromatogram of the trimethylsilyl ether derivatives of the long-chain bases isolated from cat brain
the complex lipid fraction eluted from gel-filtration columns could not be eluted from a silica acid column. This behavior suggested the sphingolipid must be very polar and perhaps of relatively high molecular weight. Chromatography on DEAE-cellulose, from which such large, polar lipids as gangliosides may be eluted, was then attempted. Again, no long-chain base could be detected in the eluates from the column. A possible explanation of this behavior is that the sphingolipid may be a polyanion, with many strongly acidic groups on each molecule.

Chemical analysis of the sphingolipid preparations from both *Ochromonas danica* and *Anacystis nidulans* indicated that there was no phosphorus or carbohydrate present. This eliminated the possibilities that these sphingolipids might resemble phytoglycolipid, gangliosides or a ceramide aminoethylphosphonate.

The presence of sulfate residues, on the other hand, suggested the possibility of a ceramide sulfate ester, although such a molecule has not been reported before. The high polarity exhibited by this sphingolipid can thus be accounted in part by the presence of these sulfate groups; however, it could also be due to the presence of some additional polar groups attached to the ceramide sulfate ester. Yet the possibility that the unusual behavior of the sphingolipid on the different columns was caused by non-covalent association of the sphingolipid with some other material cannot be overlooked.

The difficulty of isolating larger amounts of samples with higher purity has prevented us from further analysis of the lipids from both
Ochromonas danica and Anacystis nidulans. During our purification procedures, much of the sample was lost in an effort to obtain pure products. Despite this fact, the products were still relatively crude and our quantitative analyses should, therefore, be regarded as estimates.

Although we did not pursue the identification of the long-chain bases from Astasia longa or spinach, we feel that in the case of Astasia, it will probably be of value to reinvestigate this organism in the future. The lack of pigments and also the larger quantity of sphingolipid present can probably compensate for the difficulty found in culturing these cells. In the case of Rhodospirillum rubrum, although we could not rule out the presence of long-chain base with certainty, it is more likely that these bases are absent. This conclusion is in agreement with the published results. Despite the recent report of sphingolipid in an anaerobic bacterium (10), their findings remain extremely rare.

Unlike such materials as secondary plant metabolites, the distributions of which are commonly used to determine phylogenetic relationships, sphingolipids of one sort or another have now been found in representatives of all the major groups of organisms; thus the presence of sphingolipid in itself has little phylogenetic significance. This widespread distribution does, however, point to the possibility that sphingolipids may have a role in membrane structure and function which cannot, except in the bacteria, be filled by another type of lipid. More information on the intracellular distribution of these lipids and the way in which they fit into membrane structures is needed.
SUMMARY

The total lipids from *Ochromonas danica*, *Anacystis nidulans*, *Rhodospirillum rubrum* and spinach were extracted by CHCl₃-MeOH (2:1). After reextraction of these lipids with benzene, the complex lipids were obtained through gel-filtration column chromatography.

The sphingolipids obtained after mild alkaline hydrolysis and acetone fractionation, were very crude preparations. These were purified by water extraction and then partitioning into CHCl₃-soluble and MeOH-soluble fractions. These fractions still contained some impurities.

Acid methanolysis of the CHCl₃-soluble fraction of *Ochromonas danica* and MeOH-soluble fraction of *Anacystis nidulans* enabled us to isolate some relatively pure samples of long-chain bases. From the combined data of thin layer chromatography, gas chromatography and mass spectrometry, we identified the major bases in *Ochromonas danica* as C₂₀, branched-chain C₁₇, C₁₈ and branched-chain C₁₉-dihydrosphingosines. C₂₀-dihydrosphingosine was the major component. The positions of the branching in the C₁₇ and C₁₉ bases have not been determined. These long-chain bases are believed to be part of a ceramide sulfate ester.

The bases of *Anacystis nidulans* were apparently a series of chain-length homologs of dihydrosphingosine. Their chain lengths obtained from an equivalent chain length graph were: C₁₇, C₁₈, C₁₉, C₂₀, C₂₄ and two branched bases as indicated by their fractional equivalent chain
lengths of 17.4 and 21.7. These results, however, are not conclusive. The lack of sufficient material, also the difficulty in separating them into individual bases did not allow us to identify them in detail.

The characterization of the sphingolipids was only partially accomplished. Chemical analysis of the sphingolipids from both Ochromonas danica and Anacystis nidulans showed no phosphorus or carbohydrate present. Sulfate groups, however, were found in both organisms; this fact suggested the possibility of a ceramide sulphate ester.

The major fatty acid components of the ceramides were palmitic acid for Ochromonas danica and lauric acid for Anacystis nidulans.

Long-chain bases were also isolated from Astasia longa cells and spinach, but no attempts were made to identify them. On the other hand, Rhodospirillum rubrum did not contain any long-chain base as analyzed by our methods.

It appears that our research has not been fruitless, since the presence of sphingolipids in a blue-green alga (Anacystis nidulans) is being reported for the first time. Also the possibility of a sulphate ester ceramide, heretofore unknown, would make future investigations of the sphingolipids of blue-green algae and Ochromonas danica interesting.
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