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Lipid composition of the plasma-membrane of the halotolerant alga, *Dunaliella salina*

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The major lipids of the isolated plasma-membrane of the halotolerant alga *Dunaliella salina* are diacylglyceroltrimethylhomoserine (DGTS, 23.5%), sterol peroxides (7-dehydroporiferasterol peroxide and ergosterol peroxide, 22%), phosphatidylcholine (13%) and phosphatidylethanolamine (11%). Free sterols comprised 5% of the lipids and contained predominantly 7-dehydroporiferasterol and ergosterol. The major fatty acids of the plasma-membrane were palmitic (31%), oleic (13%), linoleic (20%) and γ-linolenic (17%) acids. In constrast to the whole cells, the plasma-membrane contained less (11%) α-linolenic acid and no 16-carbon unsaturated fatty acids. Sterol peroxides were identified by ^{1}H-NMR and ^{13}C-NMR spectroscopy, mass spectrometry, and by comparison on thin-layer chromatography to the product of ergosterol photooxygenation. We believe that this is the first report on the occurrence of sterol peroxides as major constituents of a biological membrane. It is suggested that they may play a role in the unusual membrane-permeability properties of the plasma-membrane of *Dunaliella*.

Introduction

Dunaliella, a unicellular green alga which lacks a rigid cell wall, occurs in medium or extreme saline habitats. Salt tolerance in *Dunaliella* involves the accumulation of high concentrations of glycerol. Glycerol balances about 80% of the external osmotic pressure and serves as a 'compatible solute' [1–4]. The cells therefore maintain very high concentration gradients across the plasma-membrane, both of glycerol and of NaCl. Evidently, the plasma-membrane is involved in this essential requirement to maintain such high gradients. Indeed, it was shown [5,6] that the cells are extremely impermeable to glycerol in a temperature-dependent manner.

Further study of the properties of the plasma membrane necessitates a preparation of a relatively pure isolated plasma-membrane. The isolation of the plasma-membrane of *Dunaliella*, described in the accompanying article [7], made it possible to study its lipid composition as described in this article.

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Abbreviations: DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine; MGDG, monogalactosyl diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

Most studies of the lipid composition of *Dunaliella* have been confined to whole cells [8–15]. Fried et al. [16] determined, in addition, the composition of the carotene globules of *Dunaliella bardawil*. The lipid composition of an undefined subcellular fraction, called the 'cell envelope', was determined by Jokela [17]. Lynch and Thompson [18–20] analysed the lipids in the chloroplastic and the microsomal fractions of *Dunaliella salina*.

In most of the above studies, the neutral lipid fraction was not studied. As will be shown here, DGTS and sterol peroxides, which have not been previously suggested as constituents of plasma-membranes, are the two major lipids of the plasma-membrane of *Dunaliella*.

Materials and Methods

Cells and growth conditions. Dunaliella salina was obtained from the culture collection of Dr. Thomas, La Jolla, CA. The cells were grown in batch culture as described in the accompanying article [7]. For quantitative analysis of total lipids, $NaH^{14}CO_3$ was added (10 $mCi \cdot mol^{-1}$) and the $NaHCO_3$ initial concentration was 10 mM; for ^{13}C-NMR studies, $Na_2^{13}CO_3$ was added to obtain about 15% ^{13}C, and the initial concentration of $NaHCO_3$ was 5 mM. Growth rate was normal under these conditions.

Preparation of the plasma-membrane. The plasma-membrane was prepared as described in the accompanying article [7]. Where indicated, membranes were purified by Ficoll layering without sucrose density gradient centrifugation.

Lipid extraction and fractionation. Lipids were extracted twice in chloroform/methanol (1 : 2, v/v) at 50°C for 30 min [21]. Phase separation of the extract was obtained at about 1 : 1 : 0.9 (v/v) chloroform/methanol/water. The lower phase, which contained the Total lipids, was dried under reduced pressure and dissolved in a small volume of chloroform. The Total lipids were fractionated on 2 g silicic acid (Unisil, Clarkson; or Bio-Sil A, Bio-Rad) column (8 mm i.d.) as follows. (A) The Neutral lipids fraction was eluted by 40 ml chloroform; (B) most glycolipids were eluted by 25 ml of 4 : 1 (v/v) acetone/chloroform; (C) DGTS and most phospholipids were eluted by 25 ml of 1 : 1 (v/v) methanol-chloroform; and (D) remaining PC was eluted by 25 ml of 4 : 1 (v/v) methanol/chloroform. Elution by a further 25 ml methanol yielded nonsignificant amounts of ^{14}C labelled lipid. All fractions were dried under reduced pressure and dissolved in a small volume of chloroform.

Thin-layer chromatography. TLC was carried out on Silica gel 60 glass plates (Merck; 0.25 mm thick). The solvent mixtures for development were: (A) 50 : 40 : 2 : 0.2 (v/v) benzene/diethyl ether/ethanol/acetic acid for Neutral lipids [22] and (B) 100 : 20 : 12 : 2.5 (v/v) chloroform/methanol/acetic acid/water for all other fractions. At the end of development, the plates were air-dried and exposed to iodine vapor to locate spots. The isolated lipid spots were identified by staining: ninhydrin, periodate-Schiff, Dragendorff and ferric chloride [23]. For quantitative analysis the fractions were scraped off and mixed directly with scintillation fluid. From the amount of ^{14}C incorporated into the different lipid fractions, the relative composition of the lipids was calculated as the percentage of the total carbon in the extract.

Fractionation of the neutral lipids. The Neutral lipids fraction in hexane or in 1 : 19 (v/v) diethyl ether/hexane was fractionated on 1.5 g silicic acid column (8 mm i.d.) as follows: (A) 20 ml of 1 : 19 (v/v) diethyl ether/hexane; (B) 20 ml of 3 : 17 (v/v) diethyl ether/hexane, to eluted Free sterols; and (C) 20 ml diethyl ether, to elute the Sterol peroxides. Alternatively, the Neutral lipids fraction in chloroform was fractionated by TLC with solvent mixture A, the spots were located by iodine vapor and scraped off. The lipids were then extracted twice by 5 : 5 : 1 (v/v) chloroform/methanol/water, and partitioned into the chloroform phase at 1 : 1 : 0.9 (v/v) chloroform/methanol/water.

Fatty acid analysis. Lipids were subjected to alkaline hydrolysis at 0.5 M KOH in methanol at 60°C for 30 min, and extracted into hexane after acidification. The fatty acids were methylated for GLC by diazomethane [24]. GLC was performed by a Hewlett-Packard HP 5790A gas chromatograph equipped with a HP 3390A integrator and a flame ionization detector. The column was 15% SP2340 on Chromosorb W (Supelco) (2 m; 1/8 inch i.d.). Temperature programming was 165°C

for 6 min and then increased to 230°C at 5 Cdeg · min^{-1}. The carrier gas was nitrogen at 30 ml · min^{-1}.

Analysis of free sterols. The Free sterols were silylated by adding 50 μl *N,O*-bis(trimethylsilyl)acetamide to a dry sample. GLC was performed on a capillary column (25 m; 0.31 mm i.d.; 0.52 μm film thickness) of cross-linked methylsilicone (SE-30). The temperature was 280°C and carrier gas was nitrogen at 1 ml · min^{-1}.

Nuclear magnetic resonance. The ^{1}H-NMR spectra were obtained on a Bruker WH-270 spectrometer at 270 MHz. ^{13}C-NMR spectra were obtained on a Bruker AM-300 spectrometer at 75.4 MHz. In addition to a broad-band proton-decoupled spectrum, two single-frequency off-resonance decoupled spectra were obtained, with irradiation at different frequencies, in order to determine signal multiplicities.

Mass spectrometry. Low resolution electron impact (70 eV) mass spectra were obtained on a Varian Mat-731 mass spectrometer, using a direct-inlet system.

Results

The total lipid extract of the isolated plasma membrane of *Dunaliella* was initially fractionated into four fractions by silicic acid chromatography as described under Materials and Methods.

The Neutral lipid fraction contained 35% of the total lipids of the plasma-membrane. Three distinct spots were revealed by iodine vapor. Spot I ($R_F = 0.35$) contained 58% of the radioactivity in this fraction, and was identified as sterol-peroxides as described below. Spot II ($R_F = 0.50$) was identified as free sterols by sterol standards, and was further analysed by GLC. Spot III had an R_F value of 0.65, which is close to the diacylglycerol standard ($R_F = 0.68$). This fraction had a ^{1}H-NMR spectrum typical of diacylglycerols which contain also polyunsaturated fatty acids (not shown). Two minor lipids comigrated with standards of monoacylglycerol ($R_F = 0.21$) and triacylglycerol ($R_F = 0.83$), respectively.

The 4:1 (v/v) acetone/chloroform fraction contained 5% of the total lipid and was comprised primarily of DGDG ($R_F = 0.32$) and MGDG ($R_F = 0.84$).

The 1:1 (v/v) methanol/chloroform fraction contained 51% of the total lipid. Seven spots could be distinguished by iodine vapor. Spot I ($R_F = 0.16$) was not identified. Spot II ($R_F = 0.24$) was identified as SQDG by a standard. Spot III ($R_F = 0.29$) was Dragendorff-positive and identified as PC by a standard. Spot IV ($R_F = 0.43$) was periodate-Schiff-positive and identified as PG against standard. Spots V and VI ($R_F = 0.49$ and 0.56, respectively) were ninhydrin-positive and identified as PE by a standard and comparison to intact cells [16]. Spot VII ($R_F = 0.80$) was Dragendorff-positive and identified as DGTS by a standard. DGTS was clearly the major lipid in this fraction.

The 4:1 (v/v) methanol/chloroform fraction contained about 8% of the total lipid. It consisted essentially of PC ($R_F = 0.29$) only, which was Dragendorff-positive and comigrated with PC standard.

The lipid composition of the plasma-membrane of *Dunaliella* is given in Table I. The results of a parallel sample of whole cells are given for comparison. The latter are in general agreement with

TABLE I

LIPID COMPOSITION OF THE PLASMA-MEMBRANE AND OF WHOLE CELLS OF *DUNALIELLA SALINA*

For details see Materials and Methods. The neutral lipid fraction of the whole cells contained mainly pigments and was not analysed. Values are percentages of total lipids.

Lipid	Plasma-membrane	Whole cells
Phospholipids + DGTS	52.7	12.3
phosphatidylcholine (PC)	13.2	1.7
phosphatidylglycerol (PG)	5.3	4.6
phosphatidylethanolamine (PE)	10.7	0.8
diacylglyceroltrimethylhomoserine (DGTS)	23.5	5.2
Glycolipids	7.7	49.7
monogalactosyldiacylglycerol	3.1	37.0
digalactosyldiacylglycerol	2.9	10.8
sulfoquinovosyldiacylglycerol	1.7	1.9
Neutral lipids	35.4	37.3
monoacylglycerol	1.6	–
diacylglycerol	6.5	–
triacylglycerol	0.9	–
sterol peroxides	21.8	–
free sterols	4.6	–
Unidentified	4.0	0.7

other recent reports on whole cells of *Dunaliella* [11,12,16,18]. The two major lipids in the plasma-membrane are DGTS (23.5%) and sterol peroxides (21.8%). A marked enrichment can also be seen for PC and PE. PG level in the plasma-membrane is similar to the whole cells, but the glycolipid content is much lower.

The fatty-acid composition of the plasma-membrane and of whole cells is given in Table II. The plasma-membrane lacks the C-16 unsaturated fatty acids, but contains C-18 unsaturated fatty acids. These results are in agreement with Lynch and Thompson [18], who analysed the chloroplast and 'microscomal' fractions of *Dunaliella*.

The similarity of the ultraviolet absorption spectrum of the Free sterols fraction to an authentic ergosterol (Fig. 1) indicates that this fraction contains a substantial amount of sterols which have conjugated double bonds at C-5 and C-7. Two major sterols and five minor sterols were found in the plasma-membrane, as determined by GLC (Table III). The sterol at $R_t = 20.25$ was identified as ergosterol by a standard. Based on the results of Wright [14] for sterol composition of whole cells of *Dunaliella*, the other major sterol ($R_t = 24.25$) is tentatively identified as 7-dehydroporiferasterol. The structural formulae of the two major sterols are given in Fig. 2. Cholesterol ($R_t = 17.35$), stigmasterol ($R_t = 22.50$), and β-sitosterol ($R_t = 25.20$) were not found in the plasma-membrane of *Dunaliella*.

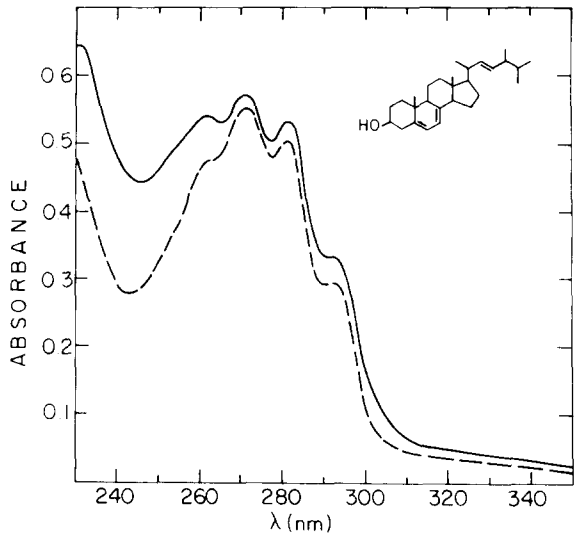


Fig. 1. Ultraviolet absorption spectrum of the Free sterol fraction in hexane. The absorption spectrum of ergosterol is given for comparison (dashed line). Absorption maxima are at 261, 271, 281 and 282 nm. The structural formula is of ergosterol.

TABLE II

FATTY-ACID COMPOSITION OF THE PLASMA-MEMBRANES AND OF WHOLE CELLS OF *DUNALIELLA SALINA*

Determined by GLC after alkaline hydrolysis. Plasma-membranes were prepared without sucrose density contrifugation. For further details see Materials and Methods. Values are percentages of total fatty acids

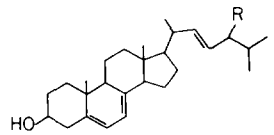
Fatty acid		Plasma-membrane		Cells
		neutral lipids	polar lipids	total lipids
Myristic	14:0	0	5.0	1.6
Palmitic	16:0	32.5	30.7	12.3
Palmitoleic	16:1	0	0	3.7
	16:2	0	0	1.3
	16:3	0	0	1.8
	16:4	0	0	23.5
Stearic	18:0	3.9	3.2	0.2
Oleic	18:1	34.9	9.5	8.0
Linoleic	18:2	12.0	21.5	10.2
α-Linolenic	18:3 (*cis* 9,12,15)	4.4	12.3	34.3
γ-Linolenic	18:3 (*cis* 6,9,12)	12.5	17.7	3.0

TABLE III

COMPOSITION OF THE FREE STEROLS IN THE PLASMA-MEMBRANE OF *DUNALIELLA SALINA*

Plasma-membranes were prepared without sucrose density centrifugation. The fraction was analysed by GLC. For further details see Materials and Methods.

Sterol		Retention time (R_t) (min)	Free sterols (% of total)
Ergosterol	28:5,7,22	20.25	30.8
		22.20	1.9
		22.80	4.9
		23.75	12.7
7-Dehydroporiferasterol	29:5,7,22	24.25	44.4
		27.05	2.9
		28.20	2.5



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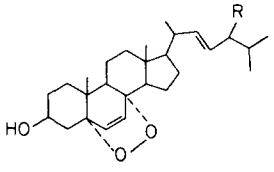

II

(1) R=CH_3
(2) R=CH_2CH_3

Fig. 2. Structural formulae of the major sterols in the plasma-membrane of *Dunaliella salina*. I. Free sterols: (1) erogsterol; (2) 7-dehydroporiferasterol. II. Sterol peroxides: (1) ergosterol peroxide; (2) 7-dehydroporiferasterol peroxide.

Conclusive identification of the sterol peroxides (epidioxysterols; Fig. 2) was achieved by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry and TLC. The results indicate that the fraction was composed of two significant components, with a molar ratio of about 0.7 : 1. Although not separated, they were identified as 7-dehydroporiferasterol peroxide (5,8-epidioxystigmasta-6,22-dien-3β-ol) – the major component, and ergosterol peroxide (5,8-epidioxyergosta-6,22-dien-3β-ol) – the minor component.

The ^{1}H-NMR assignments, which are similar to literature values [25], are given in Table IV. The high-field region has strong absorptions due to the methyl groups ($\delta = 0.78$–1.01). Interestingly, the protons on C-18 and C-19 appear at lower field as compared to free sterols, due to the deshielding effect of the dioxy group. Evidence for the molecular structure is provided by the lower field of the spectrum. The signals of H-6 and H-7 indicate an isolated *cis* double bond. They are strongly deshielded by the adjacent dioxy group ($\delta = 6.25$ and 6.50). The methine proton of C-3 also appears at lower field ($\delta = 3.97$). On the other hand, the side-chain double bond has chemical shifts typical of the respective sterols ($\delta = 5.06$, 5.10, 5.20 and 5.24).

Tentative assignments of ^{13}C-NMR, which are based on two single-frequency off-resonance decoupled spectra, as well as comparison with previous sterol literature (see below), are given in Table V. The two sterol peroxides differ in the chemical

TABLE IV

^{1}H-NMR ASSIGNMENTS OF THE STEROL PEROXIDE FRACTION

s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; e, ergosterol peroxide; p, 7-dehydroporiferasterol peroxide. The numbers in parentheses are the splittings in Hz.

H	δ (ppm)
7	6.50, d (8.5)
6	6.25, d (8.5)
22, p	5.24, dd (15;7)
22, e	5.20, dd (15;7)
23, p	5.10, dd (15;8)
23, e	5.06, dd (15;8)
3	3.97, m
21, p	1.01, d (6.5)
21, e	1.00, d (6.5)
28, e	0.91, d (7)
19	0.88, s
Other methyls	0.78–0.85

TABLE V

^{13}C-NMR ASSIGNMENTS OF THE STEROL PEROXIDE FRACTION

e, ergosterol peroxide; p, 7-dehydroporiferasterol peroxide; a, b, c assignments with the same letter may be interchanged.

δ (ppm)	Assignment	δ (ppm)	Assignment
12.5	C-29,p	39.4	C-12
12.9	C-18	39.7	C-20,e
17.6	C-28,e	40.0	C-20,p
18.2	C-19	42.8	C-24,e
18.9	C-26,p	44.6	C-13
19.7	C-27,e	51.1	C-9,b
20.0	C-26,e	51.2	C-24,p
20.7	C-11	51.7	C-14,b
20.9	C-21	56.2	C-17
21.1	C-27,p	66.5	C-3
23.2	C-15	79.4	C-5,c
25.4	C-28,p	82.2	C-8,c
28.7	C-16,e	130.0	C-23,p
28.9	C-16,p	130.8	C-7,a
30.2	C-2	132.3	C-23,e
31.8	C-25,p	135.2	C-22,e
33.1	C-25,e	135.4	C-6,a
34.7	C-1	137.6	C-22,p
37.0	C-4 and C-10		

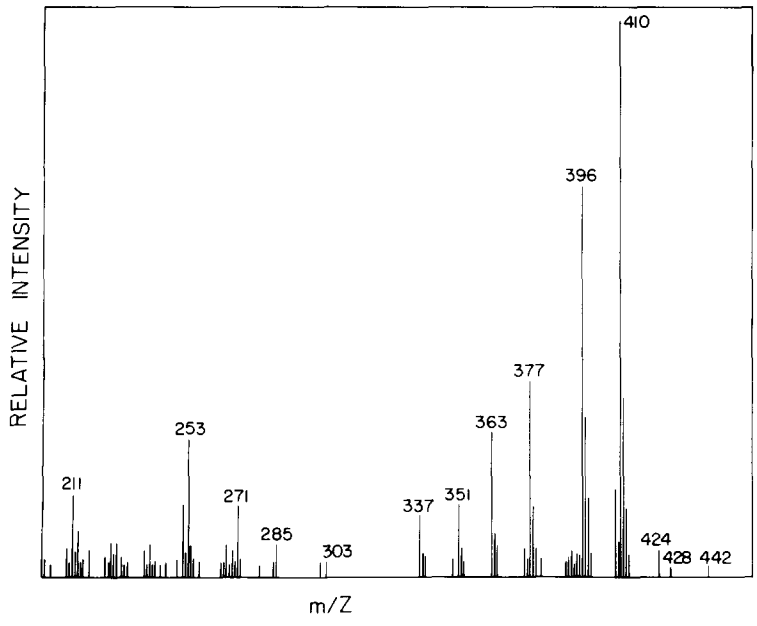

Fig. 3. Electron-impact mass spectrum of the Sterol peroxide fraction. The spectrum was taken at 150°C after 1–2 min at 100°C.

shifts of the side-chain carbon atoms but have identical shifts for most carbons of the nucleus. The chemical shifts for the side-chain carbon atoms were in agreement with the literature for sterols having side-chains similar to those of ergosterol and poriferasterol, respectively [26,14,27]. The molar ratio between ergosterol peroxide and 7-dehydroporiferasterol peroxide is about 0.7 : 1. C-3 ($\delta = 66.5$) is shielded by the dioxy group (a γ effect; C-3 of most 3β free sterols appear at $\delta = 71$–73). C-5 and C-8 appear at 79.4 and 82.2 ppm, due to the dioxy group which is bound to them.

The mass spectrum of the Sterol peroxide fraction (Fig. 3) is in good agreement with the previous literature [28,25], both for the m/z values and the relative abundance of the fragment ions, assuming a mixture at a ratio of about 0.7 : 1 for ergosterol peroxide and 7-dehydroporiferasterol peroxide, respectively. The molecular ions (m/z = 428 and 442) have low relative abundance due to loss of O_2 [28]; thus, the major fragments are identical to the molecular ions of the respective free sterols, i.e., ergosterol and 7-dehydroporiferasterol, respectively.

After the identification of this fraction as sterol peroxides the behaviour on TLC of ergosterol peroxide was examined. An authentic ergosterol in chloroform was illuminated for 3 days to synthesize ergosterol peroxide [29]. The product of this reaction had $R_F = 0.35$, identical to that of the fraction isolated from the plasma-membrane.

Discussion

The lipid composition of the plasma-membrane is strikingly different from that of whole cells.

As related to fatty acids it contains a high proportion of saturated fatty acids (39%). On the other hand, the degree of unsaturation is high: about 1.4 double bonds per fatty acid. Thus, 29% of the total fatty acids contain three double bonds and an additional 20% two double bonds. The majority of the saturated fatty acids contain 16 carbon atoms with no unsaturated 16-carbon components. All the unsaturated acids contain 18 carbon atoms. This fatty acid composition is similar to those of plasma-membranes of other plants [30].

The composition of the lipid classes is also very different from that of the whole cells. Galactolipids, which were suggested to be exclusive to the chloroplasts [31], were found in low amounts in the plasma-membrane preparation. Their presence may be due to contamination with membranes of the chloroplast envelope. The major phospholipids

in the plasma-membrane are phosphatidylcholine and phosphatidylethanolamine, as in plasma-membranes from other plants [30]. However, phospholipids comprise only 29% of the total lipids in the plasma-membrane. The major polar lipid in the plasma-membrane is DGTS (trimethylhomoserinediacylglycerol). It accounts for only 5% of the total lipids in the whole cells, but comprises 23.5% in the plasma-membrane lipids. Thus DGTS may not be enriched in the thylakoids, as suggested for *Chlamydomonas* [32], but is rather a major constitutent of the plasma-membrane.

A most striking result is the sterol composition of the plasma-membrane. Free sterols comprise only about 5% of the total lipids in the plasma-membrane. This fraction contain primarily 5,7,22-trien sterols: ergosterol and 7-dehydroporiferasterol. This result is in agreement with Wright [14], who analysed the free sterols in whole cells of *Dunaliella*. Ergosterol is the major sterol found in fungi, but is quite rare in plants [33,34]. The two substances were found as the major sterols in three other unicellular algae – *Ochromonas* [35], *Chlorella* [36], and *Chlamydomonas* [37]. The 24β-methyl and 24β-ethyl side-chains are characteristic of many algae, including Chlorophycophyta [33].

Most sterols in the plasma-membrane are sterol peroxides. They comprise 22% of the total lipids of the plasma-membrane. Thus, the total sterols (free sterols and sterol peroxides) comprise 26.5% of the total C-lipids, or 36% of the total lipids on a molar basis.

The fraction of the sterol peroxides was identified by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry and TLC. Sterol peroxides can be formed by photooxygenation of sterols having conjugated double bonds at carbons 5,6 and 7,8 [29,38]. Thus, ergosterol peroxide in extracts from fungi, was regarded as an artifact due to photooxygenaton of ergosterol [39]. To check for this possibility, authentic ergosterol was added to lipid extracts of *Escherichia coli*, liver, or brain and the extracts were fractionated as described in Materials and Methods. In no case was ergosterol peroxide found, excluding the possibility that the sterol peroxides were formed due to incorrect handling of the lipids. A possibility still exists that they are formed after cell breakage, due to some intrinsic oxidation mechanism. However, we consider this unlikely, since no degradation products of other lipids were observed. Sheikh and Djerassi [25] suggested that the sterol peroxides are formed by biological processes. Enzymic and photooxidative conversions of ergosterol into ergosterol peroxide were found to take place in vivo in *Penicullum* and *Gibberella* [40].

The ratio between ergosterol and 7-dehydroporiferasterol, and between their respective peroxides is about 0.7 : 1. Thus, it is likely that the same mechanism is involved in the production of the two sterol peroxides from their respective free sterols. The significance of sterols as constituents of biological membranes has been discussed [41], but we have not found any reference to the existence of sterol peroxides as membrane constituents. It is tempting to suggest that, as major components of the plasma-membrane of *Dunaliella*, they are involved in determining the membrane structure and its unusual permeability properties. The significance of the high content of DGTS in the plasma-membrane of *Dunaliella*, and possible interactions between DGTS and the sterol peroxides deserves further studies.

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