1953). In this respect, monoprotic phosphate shows a greater similarity to sulfate than to diprotic phosphate (in phosphatidic acid) which had the selectivity Li > Na > K.

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The Sterols of *Ochromonas danica* and *Ochromonas malhamensis**

M. C. Gershengorn,† A. R. H. Smith, G. Goulston, L. J. Goad, T. W. Goodwin,‡ and T. H. Haines‡

ABSTRACT: The sterols of *Ochromonas danica* have been identified as ergosterol, brassicasterol, 22-dihydrobrassicasterol, clionasterol, poriferasterol, and probably 7-dehydroporiferasterol. By contrast *Ochromonas malhamensis* contains only poriferasterol as the major sterol component. In addition, evidence has been obtained for the occurrence of cycloartenol and 24-methylenecycloartanol in *O. danica* and *O. Malhamensis*.

The chemical composition of the phytoflagellates is of importance phylogenetically as these organisms are algal animals and the lipids of *Ochromonas* species have proved particularly interesting in this regard. Thus only chlorophyll a was found in *Ochromonas danica* (Allen *et al.*, 1960a) and the only carotene identified was β-carotene (Allen *et al.*, 1960b). The fatty acid composition yielded large amounts of α-linolenic as well as γ-linolenic and arachidonic acids (Haines *et al.*, 1962). A unique sulfatide has been isolated from its lipids (Mayers and Haines, 1967), while the chloroplast sulfonolipid has also been identified (Miyachi *et al.*, 1966).

Although the sterols of *O. danica* and *Ochromonas malhamensis* have been investigated several times, a positive identification of all the sterols has not been achieved to date. Stern *et al.* (1960) reported ergosterol (III) in *O. danica* on the basis of its ultraviolet spectrum, and this was later verified (Aaronson and Baker, 1961). On the basis of gas–liquid partition chromatography and silver nitrate thin-layer chromatography, Halevy *et al.* (1966) confirmed the presence of ergosterol (III) and reported that the major sterol of *O. danica* was stigmasterol (VII). A number of additional unidentified sterols were also observed by gas–liquid partition chromatography.

* From the Department of Chemistry, The City College of The City University of New York, New York, New York 10031 (M. C. G. and T. H. H.), and the Department of Biochemistry, University of Liverpool, Liverpool 3, England (A. R. H. S., G. G., L. J. G., and T. W. G.). *Received December 11, 1967.* This research was supported by the National Science Foundation, Undergraduate Research Participation Program (M. C. G.), and by Grant WP-00675 from the U. S. Public Health Service (T. H. H.). The work in Liverpool was financed in part by the Science Research Council. A. R. H. S. was supported by a grant from Unilever Research, Colworth House.

† Present address: Department of Biochemistry, New York University School of Medicine, New York, N. Y. 10016.

‡ Authors to whom correspondence should be addressed.

TABLE I: Steryl Acetates of *O. danica*.[a]

Steryl Acetate	Double-Bond Position	C_{24} Substituent	Elution Order from Glpc	Elution Order from Magnesium Silicate	Rel Amt of Each Sterol (%)
Brassicasterol (II)	5,22	β-Me	1	3	12.8
Ergosterol (III)	5,7,22	β-Me	2	5	2.7
22-Dihydrobrassicasterol (I)	5	β-Me	3	1	5.7
Poriferasterol (V)	5,22	β-Et	4	4	58.0
7-Dehydroporiferasterol (VI)	5,7,22	β-Et	5	6	12.1
Clionasterol (IV)	5	β-Et	6	2	8.7

[a] Elution order from gas–liquid partition chromatography (glpc) can be seen in Figure 1. Although it was not possible to separate the acetates on the magnesium silicate column, the elution can be determined from Figure 2. The relative quantities of the sterols were determined with a planimeter from the gas chromatogram.

In *O. malhamensis* the major sterol present was identified as stigmasterol (VII) by Bazzano (1965) and Avivi *et al.* (1967). However the data obtained by Williams *et al.* (1966) indicated that this sterol is poriferasterol (V), the C_{24} epimer of stigmasterol (VII).

The present paper describes work carried out simultaneously in our two laboratories. The results were derived independently, were clearly complementary, and led to the same conclusions.

Results

The Sterols of O. danica. The cells were grown on a synthetic medium described by Aaronson and Scher (1960) under continuous illumination for a period of 5 days. They were harvested and stored frozen. Extraction of the nonsaponifiable lipid as described in the Experimental Section yielded a dark green gum. Chromatography on a column of magnesium silicate, using increasing amounts of diethyl ether in cyclohexane as the eluting solvent, yielded a crude preparation of mixed sterols.

The sterols were analyzed as their acetates by gas–liquid partition chromatography in a column of 3% JXR (Vandenheuvel, 1965) at 230° and the presence of at least six sterols was demonstrated (Figure 1). The mixture was acetylated (Heftmann *et al.*, 1960) and chromatographed on a column of magnesium silicate using cyclohexane–diethyl ether (99:1, v/v) as the eluting solvent. The column was monitored by gas–liquid partition chromatography and the results are illustrated in Figure 2. Table I compares the elution

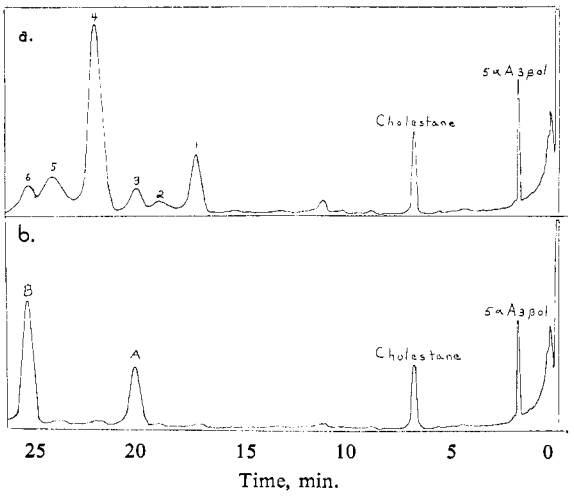


FIGURE 1: Gas–liquid partition chromatographic studies. (a) On 3% JXR of the original mixture of *O. danica* steryl acetates. Peaks correspond to: 1, brassicasterol; 2, ergosterol; 3, 22-dihydrobrassicasterol; 4, poriferasterol; 5, 7-dehydroporiferasterol (or 7-dehydrostigmasterol); and 6, clionasterol. (b) Of the hydrogenated steryl acetates; (A) campestanyl acetate; (B) stigmastanyl acetate.

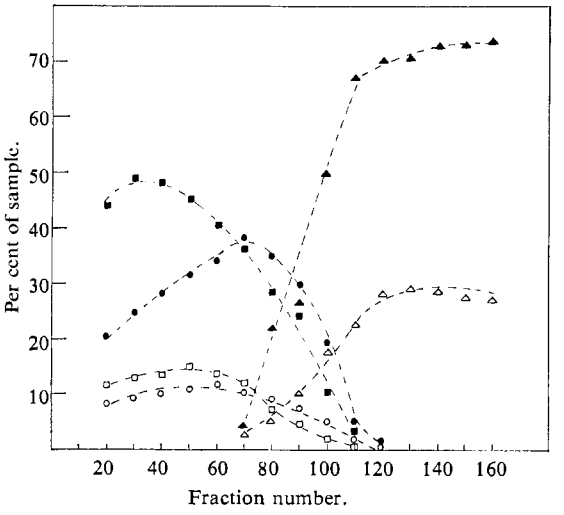
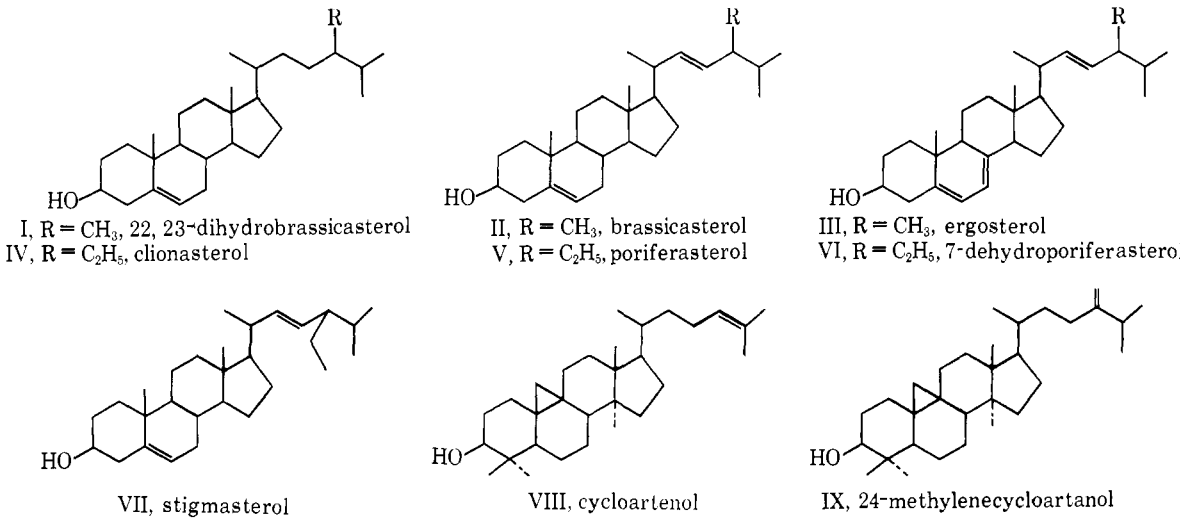


FIGURE 2: Elution pattern obtained by chromatographing the steryl acetates of *O. danica* on magnesium silicate using cyclohexane–diethyl ether (99:1, v/v) as the eluting solvent. Gas–liquid partition chromatograms were taken of every tenth fraction from the column. The relative amount of each acetate in each fraction was determined with a planimeter (brassicasteryl acetate, o; ergosteryl acetate, △; 22-hihydrobrassicasteryl acetate, □; poriferasteryl acetate, ●; 7-dehydroporiferasteryl acetate, ▲; and clionasteryl acetate, ■).

CHART I



order of the steryl acetates on magnesium silicate with their order on gas–liquid partition chromatography. Although the double bonds dominate the order of elution from the magnesium silicate column, the alkyl side chain dominates the elution pattern on gas–liquid partition chromatography. The gas–liquid partition chromatography retention times of the six *O. danica* steryl acetates were compared with those of available standards. Four had retention times identical with those of known sterols: (2) erogsteryl acetate, (3) campesteryl acetate, (4) stigmasteryl acetate, and (6) β-sitosteryl acetate. However the gas–liquid partition chromatography systems employed will not separate the sterol C_{24} isomers such as stigmasteryl acetate and poriferasteryl acetate.

This preliminary evidence suggested that the structures of the steroids were those of I–VI of Chart I. Confirmation was sought by reduction of the entire mixture of steryl acetates by hydrogen in the presence of palladium. As expected the gas–liquid partition chromatography (Figure 1) showed two peaks which had retention times identical with those of campestanyl acetate and stigmastanyl acetate, respectively. It must be noted that the gas–liquid partition chromatography system cannot separate a stanol from its isomer, nor a 24α from a 24β compound regardless of double bonds present in the molecule.

Preparative thin-layer chromatography on a silver nitrate–silica gel support (Vroman and Cohen, 1967) separated both the free sterols and the steryl acetates into two groups. The bands were eluted and gas–liquid partition chromatography showed that steryl acetates 2 and 5 had been separated from the others and were in the more polar material (band A) which cochromatographed with authentic ergosteryl acetate. The ultraviolet spectrum of the steryl acetates of band A showed λ_{max} at 262, 272, 282, and 294 mμ which are typical of sterol 5,7-dienes.

Steryl acetate 2 had a retention time on gas–liquid partition chromatography identical with that of ergosteryl acetate while steryl acetate 5 had the retention time expected for a sterol with a C_{24} ethyl group (*i.e.*, with the stigmastane skeleton). The free sterols from band A were further characterized by mass spectrometry (Budzikiewicz *et al.*, 1964; Friedland *et al.*, 1959) (Figure 3). This showed molecular ions at *m/e* 410 (M_1^+) and 396 (M_2^+) with further fragmentation ions at *m/e* 377 [$M_1^+ - (CH_3 + H_2O)$], 363 [M_2^+

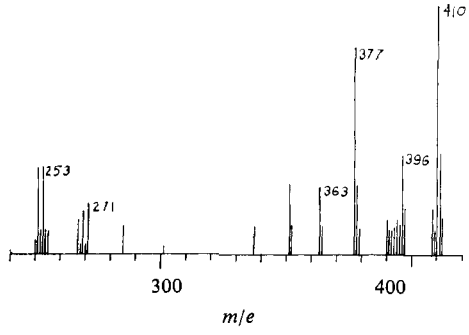


FIGURE 3: Mass spectrum of the band A sterols obtained by $AgNO_3$–silica gel thin-layer chromatography of the *O. danica* sterols mixture.

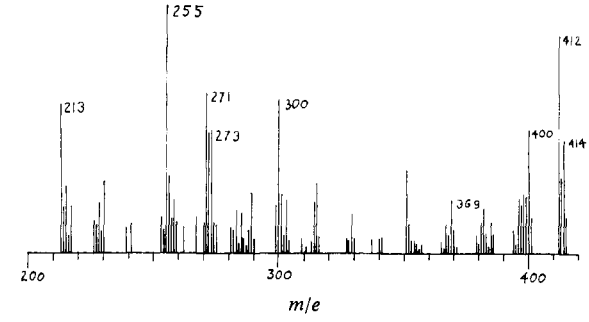


FIGURE 4: Mass spectrum of the band B sterols obtained by $AgNO_3$–silica gel thin-layer chromatography of the *O. danica* sterol mixture.

TABLE II: Summary of the Mass Spectrum (see Figure 4) of Band B Sterols of *O. danica*.

	Peaks Observed (*m/e*)			
	Clionasterol	Poriferasterol	22-Dihydro-brassicasterol	Brassicasterol
Molecular ion (M^+)	414	412	400	398
$M^+ - CH_3$	399	397	385	383
$M^+ - H_2O$	396	394	382	380
$M^+ - (CH_3 + H_2O)$	381	379	367	365
M^+ — terminal isopropyl		369		
M^+ — (terminal isopropyl + H_2O)		351		
M^+ — part of side chain[a]		300		(300?)
M^+ — side chain[b]	273	271	(273?)	(271?)
M^+ — (side chain + H_2O)[b]			255	
M^+ — (side chain and part of ring D + H_2O)[c]			213	

[a] Cleavage of the C_{20}–C_{22} bond (Lenfant *et al.*, 1967). [b] Cleavage of the C_{17}–C_{20} bond (Budzikiewicz *et al.*, 1964; Friedland *et al.*, 1959; Benveniste *et al.*, 1966a). In the case of poriferasterol this fragmentation apparently involves the loss of two additional hydrogen atoms from the nuclear fragment (*cf.* with Bergmann *et al.*, 1965). [c] See Budzikiewicz *et al.* (1964).

– (CH_3 + H_2O)], 271 [M_1^+ – side chain ($C_{10}H_{19}$)] and [M_2^+ – side chain (C_9H_{17})], and 352 [M_1^+ and M_2^+ – (side chain + H_2O)]. These data are in accord with structures 7-dehydroporiferasterol (VI) and ergosterol (III) for M_1 and M_2, respectively.

The less polar sterol or steryl acetate fraction (band B) obtained by the silver nitrate–silica gel thin-layer chromatography gave the slow-reacting response typical of Δ^5 sterols (Moore and Baumann, 1952). Gas–liquid partition chromatography revealed four components with retention times corresponding to those of (1) brassicasterol (II), 3() 22-dihydrobrassicasterol (I), (4) poriferasterol (V), and (6) clionasterol (IV), with poriferasterol predominating. Additional evidence for all these compounds was obtained by mass spectrometry of the free sterol mixture (Figure 4). Analysis of the mass spectrum of this mixture was facilitated by comparison with the mass spectra of known sterols. Many of the peaks observed were assigned as indicated in Table II.

The data so far presented allow identification of the six sterols with the exception of the orientation of the C_{24} alkyl group. Melting point and optical rotation have previously been used to distinguish between 24α and 24β isomers. (Bergmann *et al.*, 1945, 1960).[1] In order to obtain pure samples for melting point determinations the steryl acetates were separated into the two groups on silver nitrate thin-layer chromatography as indicated above and each group then separated into its components by preparative gas–liquid partition chromatography. The individual steryl acetates were collected from the end of the column into glass capillary tubes. The sample was carefully rinsed onto a microscope slide with a drop of diethyl ether; as the solvent evaporated a ring of steryl acetate formed on the slide. The melting point was then determined on a Kofler micro hot stage. The melting points of the *O. danica* steryl acetates were compared with those of authentic samples or with the literature values (Bergmann, 1960) (Table III). The melting points of the *O. danica* compounds were such as to strongly favor the identity of the following five acetates as: (1) brassicasteryl acetate, (2) ergosteryl acetate, (3) 22-dihydrobrassicasteryl acetate, (4) poriferasteryl acetate, and (6) clionasteryl acetate. Steryl acetate 5 could not be identified with certainty. Although 7-dehydrostigmasterol is present in marine organisms (Matsumoto *et al.*, 1955; Takagi *et al.*, 1956), the 24β compound (VI) has apparently not been reported in the literature. Furthermore, from the wide melting point range (145–149°) it was not possible to exclude the possibility that the compound was 7-dehydrostigmasteryl acetate, although this does not appear likely on the basis of the C_{24} configuration of the other *O. danica* sterols and the large variance with the melting point of 7-dehydrostigmasterol (mp 172).

The biosynthesis of plant sterols is being actively investigated at the present time (Benveniste *et al.*, 1966b; Ehrhardt *et al.*, 1967; Goad and Goodwin, 1966, 1967; Williams *et al.*, 1967; Aexel *et al.*, 1967; Reid, 1966; Castle *et al.*, 1963; Goad, 1967) and cycloartenol (VIII) may play an important intermediary role by replacing lanosterol the known cholesterol precursor in animals and fungi. (Clayton, 1965). It was therefore of interest to examine the 4,4-dimethyl sterol fraction of *O. danica*. By use of methods de-

[1] 24*R* and 24*S* in the specific notation of absolute configuration as proposed by Cahn *et al.* (1956), correspond to 24α and 24β, respectively.

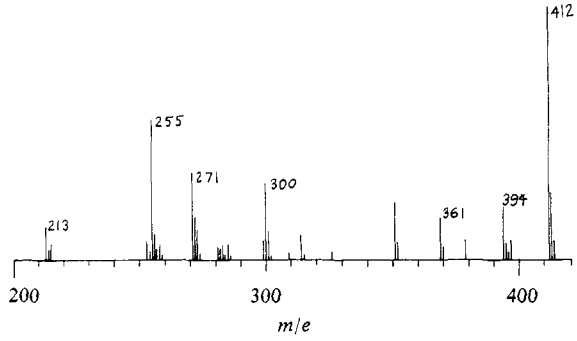


FIGURE 5: Mass spectrum of the sterol isolated from *O. malhamensis*.

scribed previously (Goad and Goodwin, 1966, 1967; Williams *et al.*, 1967) a very small sample (~1 mg) of the 4,4-dimethyl sterols was obtained. Gas–liquid partition chromatography analysis on 0.7% Hi-EFF8B showed three major components, the first with a short retention time did not correspond to any available reference sterol. The other two components had retention times identical with cycloartenol (VIII) and 24-methylene cycloartanol (IX), respectively. There was no evidence of lanosterol. Further characterization of this minor sterol fraction was not possible because of the small amount of material obtained.

The Sterols of O. malhamensis. Gas–liquid partition chromatography analysis of the main sterol fraction of *O. malhamensis*, showed one major compound (98%) which had retention data on all gas–liquid partition chromatography columns identical with that of poriferasterol (V) or its C_{24} epimer, stigmasterol (VII). A minor component (1%) was also observed (*cf.* Avivi *et al.*, 1967) with a retention time corresponding to brassicasterol. With the Liebermann–Burchard reagent the major sterol gave the slow-reacting green color of a Δ^5 sterol. The mass spectrum of the sterol (Figure 5) had a molecular ion at *m/e* 412 and other ions at *m/e* 397 [$M^+ - CH_3$], 379 [$M^+ - (CH_3 + H_2O)$], 369 [M^+ – terminal isopropyl group], and 351 [M^+ – (terminal isopropyl + H_2O)]. A peak at *m/e* 300, corresponding to loss of part of the side chain (Lenfant *et al.*, 1967), was present while the peak at *m/e* 271 can be ascribed to loss of the side chain by cleavage of the C_{17}–C_{20} bond accompanied by loss of two hydrogen atoms from the ring portions. This mass spectrum is in accord with the structure of poriferasterol (or stigmasterol). The infrared spectrum was identical with that of stigmasterol and showed a peak at 975 cm^{-1} characteristic of a Δ^{22}-*trans* double bond (Johnston *et al.*, 1957). The nuclear magnetic resonance spectrum at 100 Mc of the *O. malhamensis* sterol was essentially identical with the nuclear magnetic resonance spectrum of stigmasterol and showed peaks at τ 4.7 (multiplet) for the C_6 proton, τ 4.8–5.2 (unresolved multiplet) for the C_{22} and C_{23} protons, and τ 6.4–6.7 (multiplet) for the C_3 proton. The above data established that the *O. malhamensis* sterol was either poriferasterol (V) or stigmasterol (VII). However, the melting point of the sterol (154°) and its acetate (146°) after crystallization to constant melting point provided very good evidence for its identity with poriferasterol (lit. (Bergmann, 1960) poriferasterol, mp 156°; poriferasteryl acetate, mp 147°; stigmasterol, mp 170°; and stigmasteryl acetate, mp 144°).

TABLE III: Comparison of Melting Points of Steryl Acetates of *O. danica* and Standards.[a]

Steryl Acetate	C_{24} Substituent	Mp (°C)
Brassicasteryl acetate	β-Me	157
24α-Methyl-22-dehydrocholesteryl acetate	α-Me	
O. danica steryl acetate 1		156–158
Ergosteryl acetate	β-Me	181
24α-Methyl-7,22-bisdehydrocholesteryl acetate	α-Me	
O. danica steryl acetate 2		179–181
22-Dihydrobrassicasteryl acetate	β-Me	145[b]
Campesteryl acetate	α-Me	138
O. danica steryl acetate 3		144–145
Poriferasteryl acetate	β-Et	147
Stigmasteryl acetate	α-Et	144
O. danica steryl acetate 4		146–147
7-Dehydroporiferasteryl acetate	β-Et	
7-Dehydrostigmasteryl acetate	α-Et	172
O. danica steryl acetate 5		145–149
Clionasteryl acetate	β-Et	144
β-Sitosteryl acetate	α-Et	127
O. danica steryl acetate 6		143–144

[a] Bergmann (1960). [b] The melting point of synthetic 22-dihydrobrassicasteryl acetate was recently reported as 153–155° (Martinez *et al.*, 1967).

Lanosterol has rarely been found in plants but commonly in animals. Because it has been reported in *O. malhamensis* (Bazzano, 1965), the 4,4-dimethyl sterols were therefore examined in the present work. Gas–liquid partition chromatography showed a number of components, two of which had retention times corresponding to cycloartenol (VIII) (peak 1) and 24-methylenecycloartanol (IX) (peak 2), respectively. There was no trace of a peak with a retention time corresponding to lanosterol. Acetylation followed by gas–liquid partition chromatography (Figure 6) showed two main peaks corresponding to cycloartenyl acetate and 24-methylenecycloartanyl acetate, respectively. The mass spectrum of the mixture is shown in Figure 7. While the mass spectrum showed peaks for the non-sterol impurities in the sample, a peak at *m/e* 482 corresponding to the molecular ion of 24-methylenecycloartanyl acetate was clearly distinguished. Other

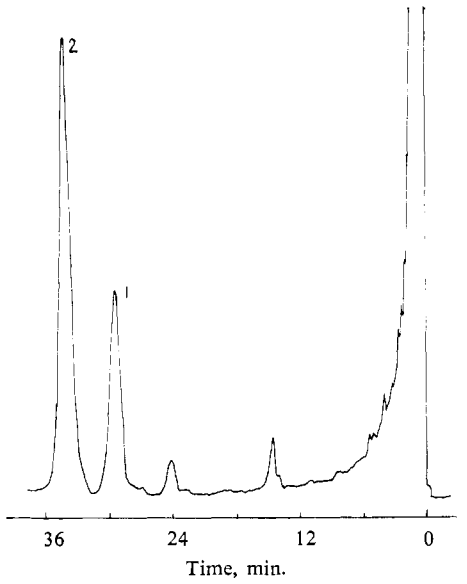


FIGURE 6: Gas–liquid partition chromatographic studies on 1% QF-1 of the 4,4-dimethylsteryl acetates isolated from *O. malhamensis*. Peak 1 has a retention time equal to cycloartenyl acetate. Peak 2 corresponds to 24-methylenecycloartanyl acetate.

peaks at *m/e* 467 [$M^+ - CH_3$], 422 [M^+ − acetate], 407 [$M^+ - (CH_3 + \text{acetate})$], 379, 353, 300 [$M^+$ − ring A], and 297 [M^+ − (side chain + acetate)] confirmed the identification of 24-methylenecycloartanyl acetate (Benveniste *et al.*, 1966a; Audier *et al.*, 1966; Aplin and Hornby, 1966) in the mixture. Peaks indicating cycloartenyl acetate in the mixture were difficult to distinguish in the mass spectrum possibly because of the small amount present. However on the basis of the identification of 24-methylenecycloartanol it is reasonable on biogenetic grounds to conclude that peak 1 in the gas–liquid partition chromatography (Figure 6) was due to cycloartenol. It is hoped later to confirm the presence of cycloartenol in *O. malhamensis* by radiochemical means.

Discussion

The absolute configuration at C_{24} of stigmasterol is 24*R*[1] and opposite to that at C_{24} of ergosterol which is 24*S* (Tsuda *et al.*, 1959, 1960; Kishida, 1960a,b). Consideration of the literature shows that various known sterols have the configurations indicated in Table IV (Shoppee, 1964; Rowe, 1965). The present results reveal that the sterols of *O. danica* and *O. malhamensis* probably all have the 24*S* configuration. Moreover it is notable that the sterols so far characterized in other algae (for example, in *Chlorella* species (Patterson and Krauss, 1965), *Scenedesmus obliquius* (Bergmann and Feeney, 1950), and *Navicula pellicalosa* (Low, 1955)) are also the 24*S* isomers. This contrasts with the vast majority of higher plants which contain 24*R* sterols, usually β-sitosterol, campesterol, and stigmasterol. Although an insufficient number of green algae have so far been examined it is tempting to speculate that sterols with a (24*S*)-alkyl substituent may be

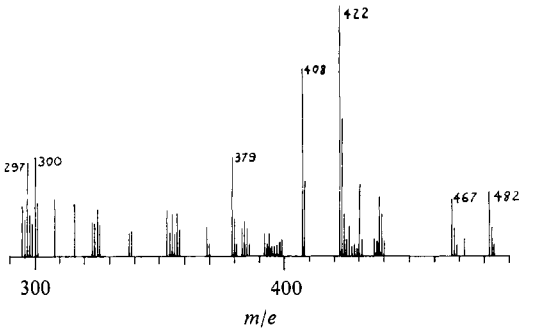


FIGURE 7: Mass spectrum of the 4,4-dimethylsteryl acetate fraction isolated from *O. malhamensis*.

characteristic of these organisms. The C_{24} alkyl group of phytosterols is derived from methionine (Lederer, 1964) and a biosynthetic transmethylation mechanism has been proposed (Castle *et al.*, 1963; Lederer, 1964; Goad *et al.*, 1966). This involves production of C_{24} methylene and ethylidene steroids which are subsequently reduced to give the C_{24} methyl and ethyl sterols, respectively. Evidence for the involvement of C_{24} methylene and ethylidene intermediates has been obtained for ergosterol production by fungi (Jaureguiberry *et al.*, 1965; Akhtar *et al.*, 1966; Barton *et al.*, 1966), β-sitosterol formation in higher plants (Goad *et al.*, 1966), and poriferasterol biosynthesis in *O. malhamensis* (Smith *et al.*, 1967). (However, an alternative transmethylation mechanism not involving a 24-ethylidene intermediate is reported in the slime mold *Dictyostelium discoideum;* Lenfant *et al.*, 1967.) One explanation for the presence of both (24*R*)- and (24*S*)-alkyl sterols in nature is a stereospecific reduction of 24-methylene and ethylidene intermediates (Rowe, 1965). This may presumably reflect a fundamental difference in the relevant reductase enzymes found in green algae and fungi on the one hand and in most higher plants on the other. This possibility is now being actively investigated.

During cholesterol biosynthesis in animals the Δ^5 bond is introduced *via* the sequence Δ^7 sterol → $\Delta^{5,7}$ sterol → Δ^5 sterol (Frantz *et al.*, 1964; Dempsey, 1965; Akhtar and Marsh, 1967). A similar biosynthetic path-

TABLE IV: Configuration at C_{24} of Various Phytosterols.[a]

24*R* (24α)	24*S* (24β)
Campesterol	22,23-Dihidrobrassicasterol
22,23-Dehydrocampesterol	Brassicasterol
	Ergosterol
β-Sitosterol	Clionasterol
Stigmasterol	Poriferasterol
α-Spinasterol	Chondrillasterol
7-Dehydrostigmasterol	7-Dehydroporiferasterol

[a] Shoppee (1964).

way in phytosterol formation will satisfactorily explain the presence of ergosterol and 7-dehydroporiferasterol in *O. danica* since they might be anticipated as precursors of brassicasterol and poriferasterol, respectively. However the accumulation of these $\Delta^{5,7}$-dienes in *O. danica* is in contrast to *O. malhamensis* where no evidence of such sterols was found. A further difference between the two species is the observation that the alkylation of the sterol side chain is more complete (*i.e.*, more C_{24} ethyl is formed than C_{24} methyl) in *O. malhamensis* where the C_{29} sterol, poriferasterol, predominates while in *O. danica* considerable amounts of C_{28} sterols are present. These observations are an interesting reflection on the biochemical capabilities of two closely related algal species.

The present evidence for the presence of cycloartenol and 24-methylenecycloartanol in *O. danica* and *O. malhamensis* along with its occurrence in *Enteromorpha linza* (G. F. Gibbons, L. J. Goad, and T. W. Goodwin, unpublished results), *Ulva lactuca* (G. F. Gibbons, L. J. Goad, and T. W. Goodwin, unpublished results), and *Fucus spiralis* (L. J. Goad and T. W. Goodwin, unpublished results) reveals that in algae sterol biosynthesis may follow a similar route to that at present postulated in higher plants.

Experimental Section

Growth of the Cells. O. danica. The cells were grown in 2-l. Fernbach flasks on a chemically defined medium (Aaronson and Scher, 1960) at 29° with continuous illumination for 5 days. Each flask was inoculated with 10 ml of a 5-day-old culture. At the end of the incubation period, the cells were collected by centrifugation, washed with deionized water, and stored frozen.

O. malhamensis (933/1A) was obtained from the Cambridge Culture Collection and grown in 10-l. batches on the medium of Aaronson and Baker (Aaronson and Baker, 1959). Flasks were inoculated from a fully grown culture and then grown for 6 days at 25–38° with continuous aeration and under illumination provided by fluorescent lights. In all about 500 l. was cultured. The cells were harvested by centrifugation and freeze dried (80 g) before storage in a deep freeze.

Extraction of the Nonsaponifiable Lipids and Isolation of the Sterols. O. danica cells (300 g wet weight) were extracted three times with 5 l. of chloroform–methanol (2:1, v/v). The solvent was removed *in vacuo* and the extract was saponified in 1 l. of 0.2 N KOH in methanol at 37° for 45 min. The suspension was diluted with 1 l. of water and extracted with 2 l. of chloroform. The chloroform was removed *in vacuo* and the nonsaponifiable lipid dissolved in 1 l. of petroleum ether (bp 30–60°)–methanol (3:2, v/v). The layers were separated by the addition of 10 ml of water to the mixture. The petroleum ether fraction was evaporated to dryness yielding 6.05 g of a green gum. This was chromatographed on a 175-g column (5 × 40 cm) of Adsorbosil M-2 and fractions were collected at 90-min intervals (approximately 10 ml/fraction). The sample was first eluted with cyclohexane–diethyl ether (90:10, v/v). The solvent was changed to cyclohexane–diethyl ether (70:30, v/v) at fraction 80 (after approximately 800 ml) and then to cyclohexane–diethyl ether (50:50, v/v) at fraction 130. The fractions were monitored by thin-layer chromatography and those containing sterol were combined, yielding 1.4 g of an orange solid. The sample was rechromatographed on a 375-g column (6.5 × 60 cm) of Adsorbosil M-2 with cyclohexane–diethyl ether (90:10, v/v) as the solvent. The sterol-containing fractions were combined yielding 0.978 g of an off-white solid. A sample of the 4,4-dimethyl sterols from *O. danica* was obtained by the methods outlined below for the isolation of *O. malhamensis* sterols.

O. malhamensis. Dry cells (80 g) were refluxed first with several liters of petroleum ether and then with petroleum ether–ethanol (1:1, v/v) until no further pigment was extracted. Removal of solvent left a greenish gum (21.5 g) which was saponified by refluxing for 90 min with 500 ml of ethanol and 50 ml of 60% aqueous KOH. The nonsaponifiable lipid (4.0 g) was subjected to preliminary chromatography on 200 g of Brockmann grade III alumina eluting with 2 l. of 2% diethyl ether in petroleum ether (E/P) (91 mg), 2 l. of 40% E/P (1.907 g), 1 l. of diethyl ether (518 mg), and 1 l. of ethanol (980 mg). The sterols were obtained from the 40% E/P by precipitation with 10 g of digitonin (Goad and Goodwin, 1966) and cleavage of the digitonide with dimethyl sulfoxide (Issidorides *et al.*, 1962). The white sterol fraction (1.0 g) was separated into the three structural groups by chromatography on 100 g of Brockmann grade III alumina and elution with 1 of2 % E/P (1.5 mg), 1 l. of 6% E/P (4,4-dimethyl sterols, 5.0 mg), 800 ml of 9% E/P (4α-methyl sterols, 43.6 mg), and 1 l. of 40% E/P (major 4-desmethyl sterols, 950 mg). The 6 and 9% E/P fractions were further purified by silica gel thin-layer chromatography to give the 4,4-dimethyl sterol fraction (5.0 mg). The 4,4-dimethyl sterols could not be isolated by any method other than digitonin precipitation because of the presence of considerable amounts of oily material which always cochromatographed with them on column or thin-layer chromatography and which made further analysis impossible.

Thin-Layer Chromatography. (a) For the *O. danica* work, plates (250 μ) of magnesium silicate, Adsorbosil M-1 (Applied Science Laboratories, State College, Pa.) activated for 15 min at 110°, were used. Petroleum ether–diethyl ether (80:20, v/v) was used to chromatograph free sterols and petroleum ether–diethyl ether (99:1, v/v) was used for steryl acetates. The spots were made visible under ultraviolet light and by spraying with 0.2% (w/v) 2,7-dichlorofluoroscein in methanol. (b) For the *O. malhamensis* work plates of silica gel incorporating Rhodamine 6G and developed with chloroform were employed (Goad and Goodwin, 1966). (c) Preparative thin-layer chromatography was performed with either 25% (w/w) silver nitrate in silica gel ADM-1 (Applied Science Laboratories, State College, Pa.) and developing with benzene–hexane (3:5, v/v) for the steryl acetates or on 10% (w/w) silver nitrate in Kieselgel H (E. Merck, A. G.

Darmstadt) and developing with chloroform for the free sterols.

Acetylation of the O. danica Sterol Mixture. The sterol mixture was dissolved in 40 ml of freshly distilled anhydrous pyridine to which was added 0.4 g of acetic anhydride. The solution was kept at room temperature, in the dark, for 2 days. The solvent was then removed *vacuo* and the product was stored in a vacuum desiccator with KOH for several days. The sample was chromatographed on a 400-g column (6.5 × 65 cm) of Adsorbosil M-2 with cyclohexane–diethyl ether (99:1, v/v) as the solvent.

Gas–Liquid Chromatography. (a) A Perkin-Elmer 881 Gas Chromatograph with a 6 ft × 1/8 in. stainless-steel column packed with 3% JXR (a nonpolar silicone oil) on 100–120 mesh Chromosorb Q (Applied Science Laboratories, Pa.) column 230°, helium flow rate 30 cc/min, was used in New York. The sample (1 μl) (1 mg of steryl acetate in 2 ml of carbon disulfide containing 40 μg/ml of cholestane as internal standard) was injected. (b) A Varian Aerograph 1522B instrument fitted with 6 ft × 1/8 in. columns of 1% SE-30, 1% QF-1 (nonpolar silicone oils), or 0.7% HiEFF8B (cyclohexanedimethanol succinate polyester) on 80–100 mesh HMDS (silanized) Chromosorb W (Applied Science Laboratories, Pa.) was used in Liverpool. All operating parameters were as described previously (Gibbons *et al.*, 1967).

Hydrogenation of the Steryl Acetates. The steryl acetates (20 mg) were dissolved in 3 ml of cyclohexane to which was added 20 mg of 30% (w/w) palladium on carbon. The mixture was bubbled with nitrogen for 5 min followed by hydrogen for 25 min. The suspension was filtered and the solvent was removed *in vacuo*.

Determination of Melting Points. The two groups of steryl acetates were separated into their component sterols by preparative gas–liquid partition chromatography (3% JXR). The individual steryl acetates were collected from the end of the gas–liquid partition chromatography column directly into disposable pipets (Aloe Scientific, St. Louis, Mo.) which fit snugly into the gas chromatograph splitter. Each steryl acetate was carefully rinsed on to a microscope slide with a drop of diethyl ether. The solvent was allowed to evaporate leaving a ring of crystalline solid behind. The sample was dried at 50° for 15 min in a vacuum oven. The melting point was determined on a Kofler micro hot stage (Arthur H. Thomas Co., Philadelphia, Pa.) which was mounted on a low-power (35×) microscope.

Mass and Nuclear Magnetic Resonance Spectra. The nuclear magnetic resonance spectrum of poriferasterol as a CDI_3 solution was kindly determined on a Varian MA 100 instrument by Miss C. Jordan of the Chemistry Department, The University, Liverpool. The mass spectra were kindly determined by Miss J. A. Shaw and Mr. J. Berkley on an AEI MS 9 instrument in the Chemistry Department, The University, Liverpool. Mass spectra obtained by the New York group were obtained through the kindness of Perkin-Elmer Corp., Norwalk, Conn., on the Hitachi MU6.

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