STEROLS OF OOCYSTIS POLYMORPHA, A GREEN ALGA

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Received: June 29, 1970

ABSTRACT

Analysis by gas liquid chromatography and mass spectrometry indicates that the major sterols of Oocystis polymorpha are cholesterol, $\Delta^{7,22}$-ergostenol (24β-methylcholesta-7,22-dien-3α-ol) Δ^{7}-ergostenol (24β-methylcholest-7-en-3β-ol), chondrillasterol (24β-ethylcholesta-7,22 dien-3β-ol) and Δ^{7}-chondrillastenol (24ρ-ethylcholest-7-en-3β-ol). Cholesterol constitutes a greater proportion of the sterol ester fraction than of the total sterols. Fatty acid composition of the sterol ester fraction is markedly different from the total fatty acid composition. The percentage of total sterols present as cholesterol is greater in cells grown on glucose or acetate than in cells grown on CO_2, and in heterotrophic cells the cholesterol level is higher in light than in darkness.

INTRODUCTION

Plant sterols are currently of interest because of their potential significance in mammalian diets, their possible utility in biochemical taxonomy, and the possibility that they can be of importance in studies of evolution and phylogeny. In addition, the functions of sterols in both plants and animals is largely unknown, and it is anticipated that

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[2] The research reported in this paper was conducted by personnel of the Environmental Systems Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas. Further reproduction is authorized to satisfy the needs of the U.S. Government.

further study may reveal various roles for these materials in living organisms (1).

Oocystis polymorpha, Groover and Bold (2), is a recently described unicellular green alga which has been considered for use in photosynthetic life support systems for long-term aerospace missions (3, 4). The general composition of the organism has been determined (3), but no detailed study of its biochemical constituents has been made. The objective of the research reported here was to characterize the sterols of the species.

MATERIALS AND METHODS

Culture and Harvest Techniques. Tissue was obtained from a photosynthetic gas exchange system (4), an annular chamber culture system (5), and flask cultures. Tissue from the gas exchange system was grown in Knop's basal medium with urea as a nitrogen source and 5% CO_2-air as the carbon source. The system was operated as a turbidostat, with temperature maintained at 38° C and light provided by fluorescent lamps at 1200 ft.-c. The harvest tissue was centrifuged (Sharples model AS-16, Pennsalt Chem. Corp., Philadelphia, Pa.), washed twice with deionized water, recentrifuged, frozen, lyophilized (Vir Tis model 10-145 MR-BA, Research Equipment Div., Gardiner, N.Y.), and ground to pass a 40-mesh screen with a Wiley #3 mill.

The annular chamber system was operated as a chemostat at 38° C with Knop's potassium nitrate medium and 5% CO_2-air as the carbon source. Cells were harvested (Sharples T-1 centrifuge, Pennsalt Chem. Corp., Philadelphia, Pa.), and lyophilized as described above.

Flask-cultured cells were grown on Knop's potassium nitrate medium supplemented with 5% glucose or 5% sodium acetate under light and dark conditions. Cultures were maintained at 38° C in a shaker-incubator (model R-27, New Brunswick Scientific Co., New Brunswick, N.J.) and aerated with air. Fluorescent lighting at 150 ft.-c. was provided when required. The tissue was harvested and frozen until extraction.

Extraction. Initial sterol identification studies were done on tissue obtained from the gas exchange system. Twenty-five gms of dried algae was mixed with a volume of purified sand equal to one-half the volume of tissue. The tissue was suspended in 100 ml chloroform:methanol

(2:1, v/v) and sonified 10 minutes at 15 amps (Branson model 8125, Heat Systems Co., Great Neck, N.Y.). Extraction vessels were cooled by submergence in a chilled water bath. The extract was collected by filtration through the fritted glass portion of a Millipore filter apparatus. The sample was extracted three times, after which the extracts were combined and partitioned by the addition of one-third volume 0.1 M NaCl solution. The chloroform extract was then washed with 0.1 M sodium chloride-chloroform-methanol (50:3:47), evaporated to dryness (model 1007-41N rotary evaporator, Rinco Instrument Co., Greenville, Ill.), and redissolved in approximately 40 ml ethanol. The sample was saponified with 60% aqueous potassium hydroxide (1 ml/10 ml ethanol) at room temperature under nitrogen for 18 hours. Unsaponifiable material was separated by the addition of one volume of ethyl ether and three volumes of water. The ether layer, containing unsaponifiable components, was then dried with sodium sulfate, filtered, evaporated to dryness, and redissolved in nanograde n-hexane.

Extraction of lipid material for sterol ester analysis was done in the same manner as above, differing only in omission of the saponification procedure.

Tissue from acetate and glucose grown cells was extracted with a Sorvall model OM Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). The tissue in this instance was wet and in smaller quantity (1 gm). All other procedures were as outlined above.

Isolation of the Sterol and Sterol Ester Fractions. To a glass column (30 x 1.5 cm) was added a 5 mm layer of purified sand followed by a slurry of 100/200 mesh Bio-Sil BH silicic acid (Bio-Rad Laboratories, Richmond, Calif.) in hexane. Another 6 mm layer of sand was added to the top of the gel to prevent disturbance upon addition of the extract. The extract was added in the smallest volume possible and eluted with the following series:

(1) 50 ml n- hexane

(2) 120 ml 5% ether-hexane

(3) 100 ml 10% ether-hexane

(4) 150 ml 25% ether-hexane

(5) 150 ml 50% ether-hexane

(6) 50 ml 20% methanol-ether

(7) 100 ml methanol

The fractions were collected separately and sterols were detected by gas liquid chromatography (GLC). Sterols were usually eluted in the 25% ether fraction. Thin layer chromatography (TLC) was used for the isolation of sterols from acetate and glucose grown tissue. Silica gel G plates (200 x 200 x 0.6 mm) were activated at 110° C for 1 hour. Usually half the unsaponifiable extract was spotted across the plate, leaving 5 cm margins on both sides. A sterol standard (cholesterol) was then spotted at one margin and the plate developed in hexane-ether-acetic acid (85:15:1). The developed plate was air dried and visualized with 2',7'-dichlorofluorescein, 0.2% in isopropanol (Applied Science Labs, Inc., State College, Pa.). The sterol band was scraped from the plate and eluted three times with chloroform.

Sterol esters were isolated by column chromatography as described above for free sterols, with the esters being eluted in the 5% ether-hexane fraction. The ester fraction was taken to dryness and hydrolyzed with 15 ml 0.5 M methanolic-KOH for three minutes at 90° C. Fatty acid methyl esters were formed by adding 15 ml of 14% BF_3-methanol directly to the hydrolyzed solution and heating five minutes at 90° C. Partitioning in n-hexane three times separated the methyl esters and sterols into the n-hexane phase. The hexane layer was reduced to a small volume and subjected to TLC, as previously described except that the solvent used was hexane-ether, 99:1. The sterols and fatty acid methyl esters were eluted and analyzed by GLC.

Formation of Derivatives. Trimethylsilyl ethers were formed by reacting an aliquot of dried sterol with 1-2 ml pyridine-trimethylchlorosilane-hexamethyldisilazane (10:2:1)(Applied Science Labs, State College, Pa.) in a screwcap tube for 30 minutes at room temperature.

Acetylation was achieved by addition of acetic anhydride-pyridine (4:1) and heating for 15 minutes at 60° C. The solution was then evaporated and redissolved in n-hexane.

Hydrogenates were formed by dissolving the sterol fraction in n-hexane in the presence of 0.2 ml of glacial acetic acid and a few grains of 84.5% platinum oxide on charcoal. The container was flushed with N_2 to eliminate residual O_2 and then bubbled with H_2 until complete hydrogenation had occurred, as revealed by GLC.

Gas Liquid Chromatography. Two series 5000 Barber Colman gas liquid chromatographs (Barber Colman Co., Rockford, Ill.) were used in the analysis of fatty acids and sterols. Both instruments were equipped with dual hydrogen flame ionization detectors. Glass columns (5 mm O.D. x 15 m) packed with 3% QF-1 on 80/100 mesh Chromosorb W(HP) and 3% OV-1 on 100/200 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) were used for sterol analysis. Fatty acids were analyzed on glass columns (7 mm O.D. x 2 m) packed with 15% DEGS on 80/100 mesh Chromosorb W(AW). The QF-1 and

DEGS columns were operated at 200° and 178° C, respectively. The column temperature for OV-1 was varied from 220° to 255° C, depending on the resolution desired. Air (<2ppm hydrocarbon as CH_3) and hydrogen were optimized for maximal response with helium as the carrier gas. Injections were made by means of solvent flush technique using a 10 microliter Hamilton syringe (Hamilton Co., Whittier, Calif.).

Sterol composition was determined as percent of total GLC peak areas. Peak areas were calculated by the height times the width at half height technique. Cholestane was used as an internal standard and sterol quantities were expressed as percent dry weight of algae. Retention times were measured from the solvent front and expressed in relation to cholestane. Methyl erucate was used as an internal standard for the fatty acid analyses.

A mixture of cholesterol, ergosterol, campesterol, β-sitosterol, and stigmasterol (each component 2 mg/ml) in n-hexane was used as a standard to aid in sterol identification. The derivatives described above were also made for the standard sterol mixture.

Mass Spectrometry. Mass spectrometry was done at the Lipid Research Institute, Baylor Medical School, Houston, Texas. Trimethylsilyl derivatives were analyzed with an LKB model 9000 mass spectrometer (LKB Instruments, Inc., Rockville, Md.). A 3 m glass coiled column packed with 1% SE-30 was used for resolution of the sterol mixture at 240° C.

Melting Point Determination. To obtain individual sterols for melting point determinations an aliquot of total sterols was acetylated and chromatographed on a 42.5 x 1.8 cm column of Anasil B (Analabs, Inc., Hamden, Conn.), with 800 ml 2% ether in hexane as the eluent. Fractions of 20 ml were collected by means of a refrigerated fraction collection (model VL, Gilson Medical Electronics, Middleton, Mich.). Elution was monitored by GLC, and pure fractions were combined for further purification by TLC. Melting points were determined with a Fisher-Johns apparatus (Fisher Scientific, Houston, Tex.).

RESULTS

Identity of Sterols. GLC of the sterol fraction on OV-1 revealed the presence of five components (fig. 1). GLC of the silyl ethers and of the acetates on OV-1 and on QF-1 failed to reveal any additional peaks. GLC of the hydrogenated sterols on OV-1 (fig. 2) and comparison of retention times with those of hydrogenated standards (table 1) indicated the presence of C27, C28, and C29 compounds. Calculation of the

relative peak areas and monitoring of the course of hydrogenation indicated that peak H-2 (C28) was formed from sterol peaks 2 and 3, while H-3 (C29) was derived from peaks 4 and 5 of the untreated sterols.

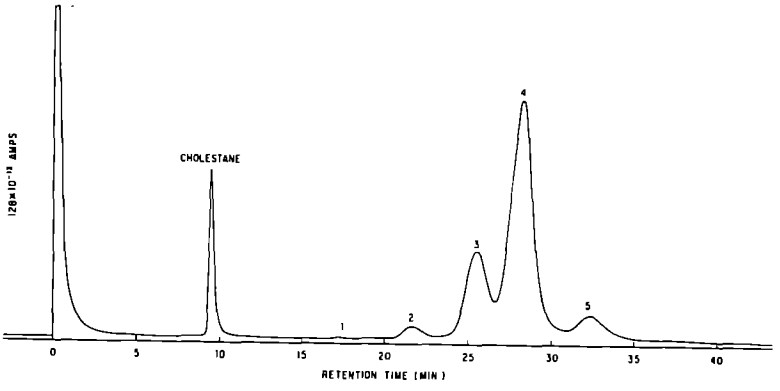

Figure 1. GLC retention pattern of O. polymorpha sterols on OV-1.

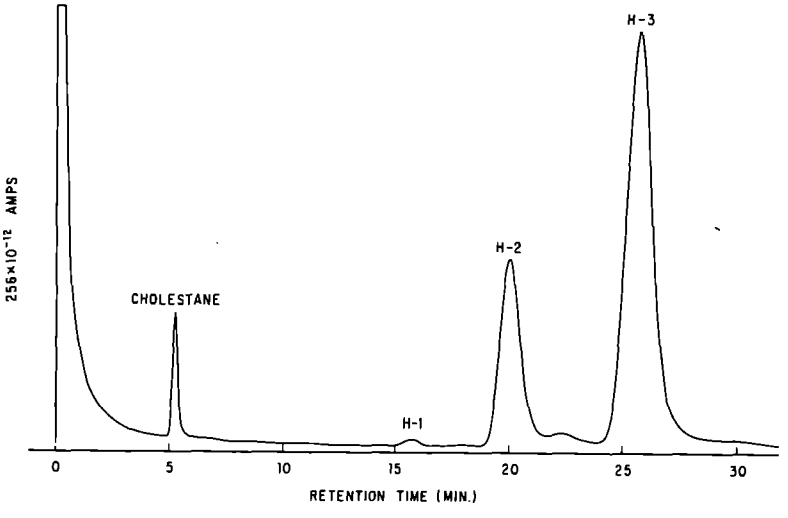

Figure 2. GLC retention pattern of hydrogenated acetates of O. polymorpha sterols on OV-1.

Table 1. Relative retention times of O. polymorpha sterols, known standard, and their derivatives.*

Sterol	OV-1**				QF-1***		
	Free sterol	TMS ether	Acetate	Hydrogenate	Free sterol	TMS ether	Acetate
1	1.99	2.34	2.74	2.9	3.57	2.43	6.53
2	2.35	2.99	3.44	3.7	4.53	3.17	7.37
3	2.76	3.50	4.03	3.7	5.59	3.92	10.56
4	3.03	3.84	4.43	4.7	6.00	4.21	10.56
5	3.49	4.42	5.09	4.7	7.03	4.88	11.97
Cholesterol	1.98	2.33	2.73	2.9	3.56	2.45	6.53
Ergosterol	2.32	2.88	3.36	3.7	4.45	3.29	8.35
Campesterol	2.47	3.06	3.59	3.7	4.93	3.55	9.37
Stigmasterol	2.70	3.34	3.92	4.7	4.93	3.55	9.37
β-sitosterol	3.13	3.87	4.54	4.7	5.97	4.25	11.27

*Relative to cholestane.
**Retention times of cholestane for free sterols, TMS ethers, and acetates were 9.45, 10.00, and 9.93 minutes, respectively.
***Retention times of cholestane for free sterols, TMS ethers, and acetates were 7.60, 7.50, and 7.30 minutes, respectively.

The relative retention data for the C27 sterol (peak 1) corresponded closely to that of cholesterol in all cases (table 1). Relative retention times (RRT's) for peaks 2-5 did not match those of any sterols on hand. The RRT for peak 2, however, approximated that of ergosterol, suggesting that it was a closely related analogue. Peaks 3-5 all had slightly longer retention times than the corresponding Δ^5-C28 and Δ^5-C29 standards, suggesting that they were Δ^7 analogues of those materials (table 1). In addition, it could be shown by calculation that the relationship between peaks 3-5 was the same that exists for campesterol, stigmasterol, and β-sitosterol (table 2). Thus, the GLC data suggested the presence of a C27 monoene (peak 1), a C28 di- or triene (peak 2), a C28 monoene (peak 3), a C29 diene (peak 4), and a C29 monoene (peak 5), the latter three sterols having a double bond at carbon 7.

Table 2. The relationship of unknowns 4 and 5 to unknown 3, as compared with the relationship between known C28 and C29 sterols.

	Free sterol	TMS ethers	Acetates
RRT of unknown 4, based on unknown 3.	1.098	1.097	1.100
RRT of stigmasterol, based on campesterol.	1.093	1.090	1.090
RRT of unknown 5, based on unknown 3.	1.260	1.260	1.260
RRT of β-sitosterol, based on campesterol.	1.270	1.260	1.260

The mass spectrum of the trimethylsilyl (TMS) ether of unknown 1 was essentially identical to published spectra for cholesterol (6, 7). The intense peaks at m/e 129 and 329 (M-129) are highly indicative of a

Δ^5-3β-trimethylsilyl ether group (6); and among biological C27 sterols, they are probably completely specific for the Δ^5 bond.

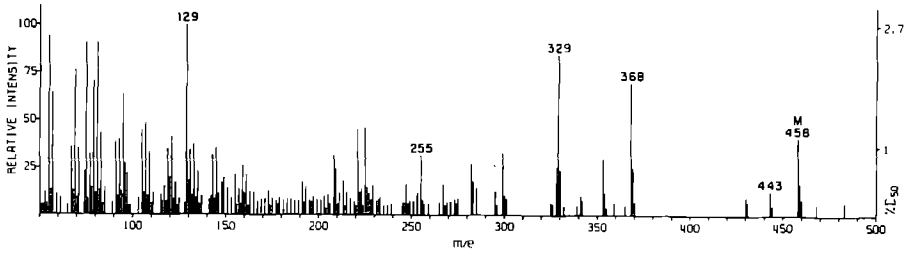

Figure 3. Mass spectrum of the TMS ether of unknown 1 (peak 1 in fig. 1).

All of the unknown sterols yielded a major peak at m/e 255, representing a monoene steroid nucleus after loss of the side chain and trimethylsilanol group. The spectra of unknowns 2-5 contained significant peaks at m/e 213 and 229 (fig. 4). These ions have been observed in spectra of Δ^7-cholestenol (6, 8) and are apparently characteristic of the degradation of Δ^7-monoene steroid nuclei with the basic structure of cholestenol.

Subtraction of the sum of 90 mass units (for trimethylsilanol) plus 255 mass units (for the monoene steroid nucleus) from the molecular ion yields the molecular weight of the sterol side chain. The results of this calculation, and the derived empirical formulae, are shown in table 3. As anticipated, the side chain of unknown 1 is the saturated hydrocarbon characteristic of cholesterol. Unknowns 2 and 3 each have an

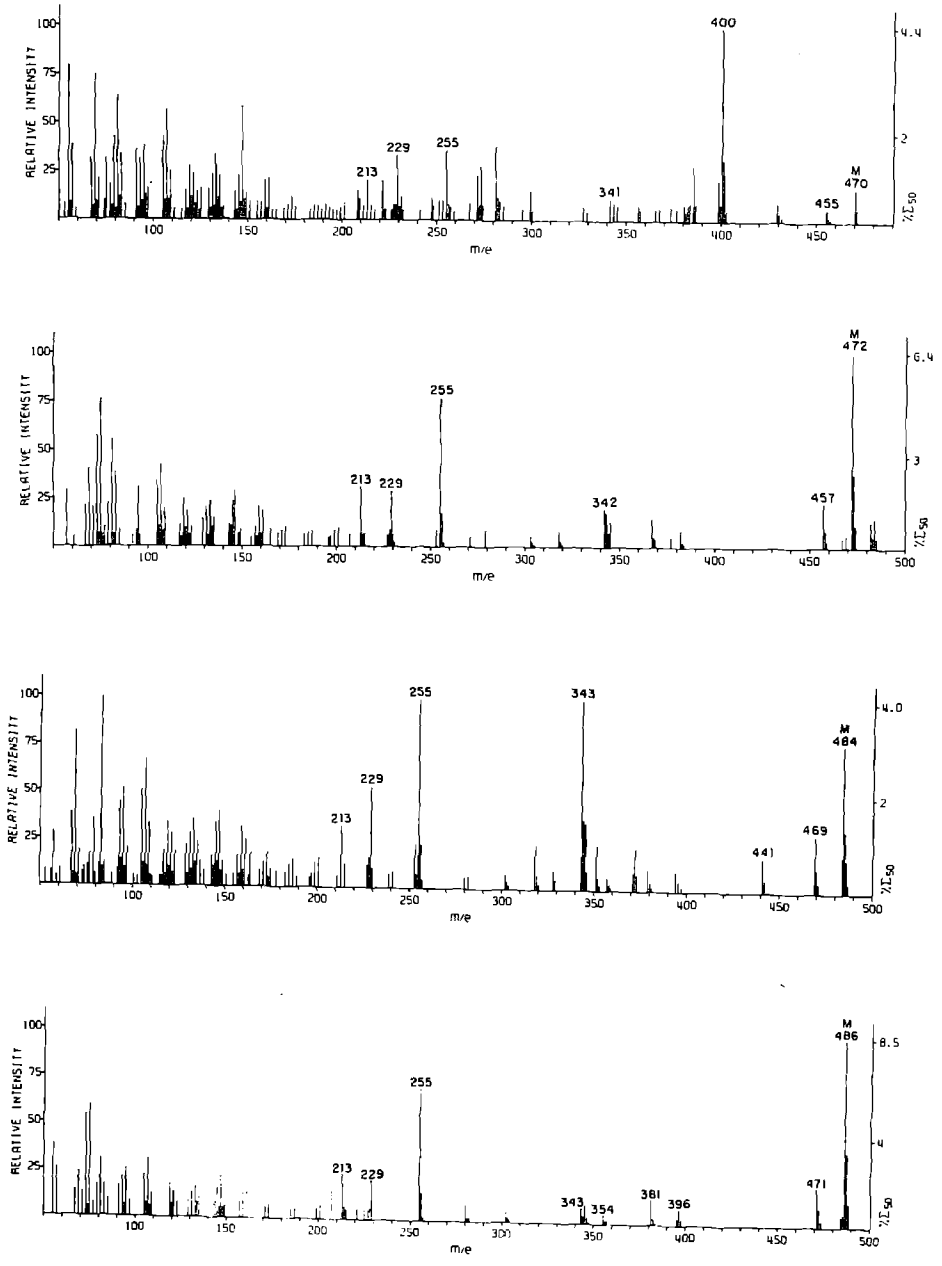

Figure 4. Mass spectra of the TMS ethers of unknowns 2 (top), 3 (upper center), 4 (lower center), and 5 (bottom). Unknown numbers correspond to peak numbers in fig. 1.

additional methyl group, while peaks 4 and 5 have an additional ethyl group. The side chains of unknowns 2 and 4 each contain one double bond. The peak at m/e 441 (M-43) in the spectrum of unknown 4 represents loss of the side chain terminal isopropyl group and is characteristic of Δ^{22} sterols (8). That mode of fragmentation is not pronounced for this compound, and the 343 ion, representing loss of the side plus two hydrogen atoms, is the base peak. The major peak in the spectrum of unknown 2, at m/e 400 (M-70), may represent loss of a C23-C29 fragment with fragmentation occurring at the Δ^{22} bond.

Table 3. Side chain molecular weights and empirical formulae derived from mass spectra of O. polymorpha sterols.

Sterol	M	Side chain molecular weight	Side chain empirical formula
1	458	113	C 8 H17
2	470	125	C 9 H17
3	472	127	C 9 H19
4	484	139	C10 H19
5	486	141	C10 H21

Column chromatography of the sterol acetates on Anasil B yielded the major component (unknown 4) in sufficient purity for melting point determination. The melting point obtained (174°-175° C) is in agreement with literature values for chondrillasterol (24β-ethylcholesta-7,22-dien-3β-ol)(9), which has the 24-ethyl group in the β orientation. Since mixtures of α and β alkylated sterols have never been found in biological material, it seems reasonable to conclude that the alkyl groups of

unknowns 2, 3, and 5 are also of the β configuration. The five sterols present are therefore cholesterol (unknown 1), $\Delta^{7,22}$-ergostenol (24β-methylcholesta-7,22-dien-3β-ol) (unknown 2), Δ^{7}-ergostenol (24β-methylcholest-7-en-3β-ol) (unknown 3), chondrillasterol (unknown 4), and Δ^{7}-chondrillastenol (24β-ethylcholest-7en-3β-ol) (unknown 5).

Composition of the Sterol Ester Fraction. The composition of the sterol ester fraction, as compared to the total sterols and fatty acids, is illustrated in figure 5. The most notable feature of the esterified

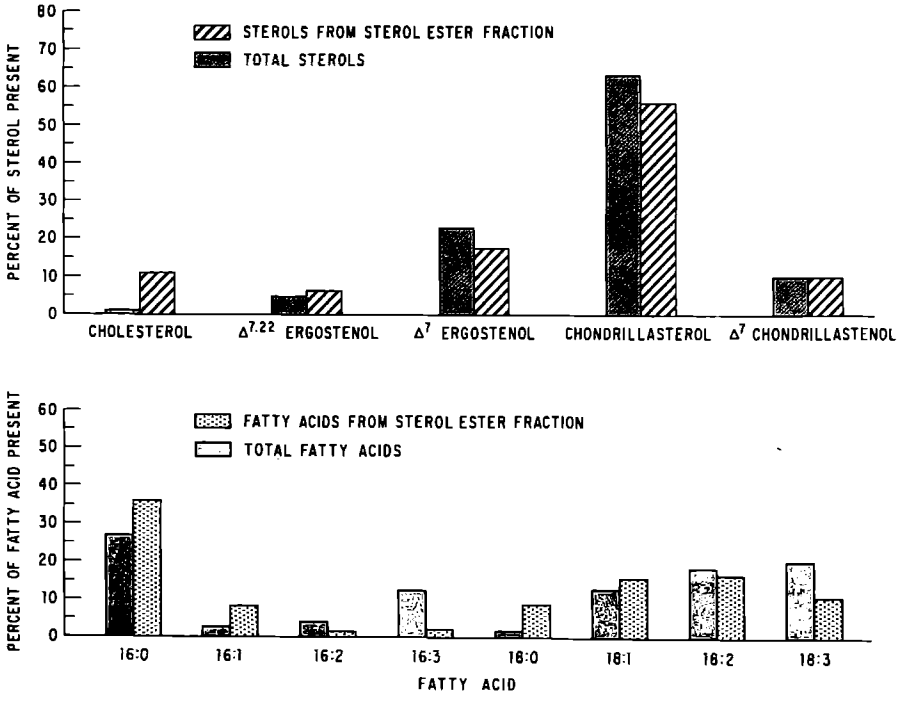

Figure 5. Composition of the sterols (%) and fatty acids (mole %) present in the sterol ester fraction of O. polymorpha, as compared to the total sterols and total fatty acids.

sterols is the tenfold increase in the proportion of cholesterol present. Lesser differences in the percentages of Δ^7-ergostenol and chondrillasterol are apparent, while the $\Delta^{7,22}$-ergostenol and Δ^7-chondrillastenol quantities are essentially the same. A minor peak with a retention time slightly greater than that of cholesterol was observed in the ester fraction but was never present in detectable amounts in total sterol samples. Compared to the total fatty acids, the esterified fatty acid fraction was markedly higher in 16:0, 16:1, and 18:0 but contained considerably less 16:2, 16:3, and 18:3. No quantitative determinations were made, but it appears that the esters constitute perhaps 5% or less of the total sterols present.

Effects of Environment on Sterol Composition. Cells grown photosynthetically with carbon dioxide and light in three different culture systems did not greatly differ in the composition of their sterol fractions (table 4). Nitrogen source, light intensity, and method of tissue handling seem to have no effect on the sterols obtained. Replicate extractions and calculations based on replicate gas chromatograms yielded only negligible differences. Cholesterol was always considerably less than 1% of the total sterols.

In heterotrophically cultured cells, however, carbon source and the presence or absence of light had a pronounced effect on the level of cholesterol (fig. 6). With glucose and darkness, cholesterol amounted to 0.4% of the total sterols, comparable to the autotrophically grown tissue. Light increased the level to 2%, while with acetate as the carbon source, cholesterol levels were 13.4% and 10.9% in light and

Table 4. Composition of O. polymorpha sterols from different photosynthetic culture systems.

Culture system	Composition of sterol fraction (% of total sterols)				
	cholesterol	$\Delta^{7,22}$-ergostenol	Δ^{7}-ergostenol	chondrillasterol	Δ^{7}-chondrillastenol
Annular chamber, KNO_3, lyophilized tissue	0.3	2.1	22.4	66.8	8.4
Gas exchanger, urea, lyophilized tissue	0.5	3.1	24.5	62.9	8.9
Flask culture KNO_3, wet tissue extraction	0.4	3.8	22.1	65.7	8.0

darkness, respectively. These increases in cholesterol seem to be accompanied by relative decreases in chondrillasterol, which constituted 65.2% of the sterols in glucose-dark cells but only 49.9% in acetate-light cells. The proportion of sterol present as Δ^{7}-chondrillastenol was somewhat greater in light than in darkness, while Δ^{7}-ergostenol and $\Delta^{7,22}$-ergostenol were essentially the same in all the heterotrophic cultures.

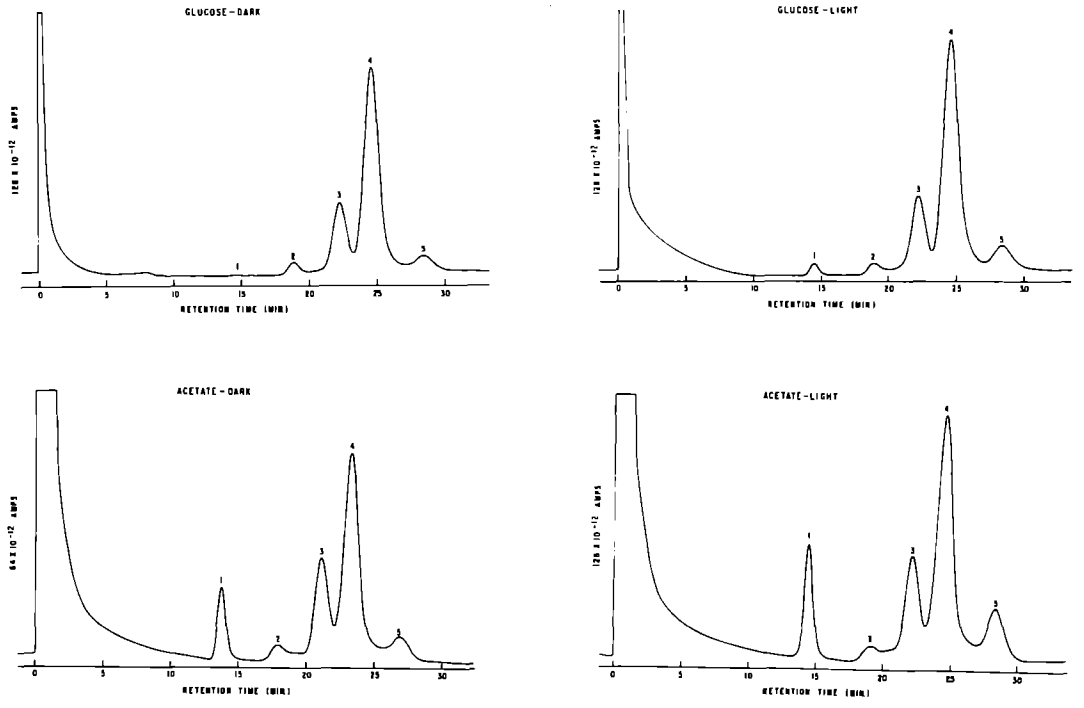

Figure 6. Effect of carbon source and light on the sterol composition of O. polymorpha. GLC retention patterns on OV-1.

DISCUSSION

The results reported here are the first evidence for the presence of cholesterol in the unicellular green algae. The multicellular green algae Ulva pertusa and Chaetomorpha crassa have been reported to contain cholesterol (10), and its widespread distribution in higher plants has been well documented (11). Its occurrence in Oocystis as the only Δ^5 sterol in a mixture of Δ^7 sterols prompts questions as to its mode of

synthesis and function. Whether one considers possible biosynthetic mechanisms, the composition and role of the sterol ester fraction, or the effects of environment, cholesterol seems to constitute a special situation, different from the other sterols.

The generally accepted scheme for the latter stages of cholesterol synthesis in animals, and for Δ^5 sterols in plants, is $\Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$. It is assumed that plants containing only Δ^7 or $\Delta^{5,7}$ sterols lack the enzyme systems for the further conversion to Δ^5 compounds. The occurrence in Oocystis of a C27 sterol (cholesterol) as the only Δ^5 compound, while the C28 and C29 sterols are Δ^7, suggests the possibility that in this organism alkylation of carbon 24 occurs relatively early in sterol biosynthesis and blocks the $\Delta^7 \rightarrow \Delta^5$ conversion. Alternately, cholesterol could arise by de-alkylation of the C28 or C29 sterols to a Δ^7C27 sterol which is then rapidly converted to cholesterol. The apparent inverse relationship between cholesterol and chondrillasterol in the carbon source studies reported above would support such a pathway; but although de-alkylation is well documented in animals (12), it has never been demonstrated in plants. Since no immediate precursors of cholesterol, or of the other sterols, were observed, it must be assumed that they are transformed soon after formation and do not accumulate.

The major portion of the Oocystis sterol fraction (Δ^7-ergostenol, chondrillasterol, and Δ^7-chondrillastenol) is similar in identity and proportions to that of several of the Chlorella species studied by Patterson and his co-workers (13, 14). Since the two species are relatively closely related, in the order Chlorococcales, this is not

surprising. The evidence that the 24-alkylated sterols of Oocystis are of the β configuration lends additional weight to the idea that β-alkylation is a general characteristic of the green algae (15).

The elevated level of cholesterol in the ester fraction generally parallels work done by Kemp and Mercer with maize organelles (16), but its significance is impossible to determine at the present time. Similarly, the composition of the fatty acids in the ester fraction indicates that esterification is not simply a random process; but as Goad has noted (15), the role of sterol esters awaits further study.

The great variation in cholesterol content with carbon source may have considerable practical importance. The inordinately high cholesterol level in acetate grown cells indicates that biosynthetic studies in which plant tissues are incubated with acetate or mevalonate can result in sterol compositions that are not typical of the species as it occurs in nature. Conversely, the fact that in CO_2 cultured cells cholesterol was barely detectable suggests that failure to find cholesterol in many species may be due to the quantity present being below the level of detectability with the environmental conditions employed. This quantitative variation with culture conditions should be validated with other species, but the sampling of tissue from various environments may be an advisable step in characterizing plant sterols for chemical taxonomy and other purposes.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Dr. James A. McCloskey for conducting the mass spectrometry analyses and Dr. Glenn W. Patterson for supplying relative retention data which was useful in identifying the sterols.

REFERENCES

1. Heftmann, E., ANN. REV. PLANT PHYSIOL. 14, 225-248 (1963).

2. Groover, R. D., and Bold, H. C., SOUTHWESTERN NATURALIST 13, 129-138 (1968).

3. Richardson, B., Orcutt, D. M., Rosner, L., and Miller, R. L., Technical Report 69-3, USAF School of Aerospace Medicine, Brooks AFB, Texas (1969).

4. Miller, R. L., Rose, G. W., Kilian, H. J., Wickline, H. E., and Martinez, C. L., Technical Report 69-64, USAF School of Aerospace Medicine, Brooks AFB, Texas (1969).

5. Richardson, B., Orcutt, D. M., Schwertner, H. A., Martinez, C. L., and Wickline, H. E., APPL. MICROBIOL. 18, 245-250

6. Brooks, C. J. W., Horning, E. C., and Young, J. S., LIPIDS 3, 391-402 (1968).

7. McCloskey, J. A., METHODS IN ENZYMOLOGY, Vol, XIV, Editor J. M. Lowenstein, Academic Press, Inc., New York, 1969, pp. 382-450.

8. Knights, B. A., J. GAS CHROMATOG. 5, 273-282 (1967).

9. Patterson, G. W., and Krauss, R. W., PLANT AND CELL PHYSIOL. 6, 211-220 (1965).

10. Ikekawa, N., STEROIDS 12, 41-49 (1968).

11. Heftmann, E., LLOYDIA 31, 293-317 (1968).

12. Ritter, F. J., and Wientjeus, W. H. J. M., TNO-NIEUWS 22, 381-392 (1967).

13. Patterson, G. W., PLANT PHYSIOL. 42, 1457-1459 (1967).

14. Krauss, R. W., Technical Report 1017, Department of Botany, University of Maryland, College Park, Md. (1968).

15. Goad, L. J. TERPENOIDS IN PLANTS, Editor J. B. Pridham, Academic Press, Inc., New York, 1967, pp. 159-190.

16. Kemp, R. J., and Mercer, E. I., BIOCHEM. J. 110, 119-125 (1968).