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STEROLS OF *CHLORELLA*. I. THE NATURALLY OCCURRING STEROLS OF *CHLORELLA VULGARIS*, *C. ELLIPSOIDEA*, AND *C. SACCHAROPHILA*[1,2]

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The characteristics of the sterols naturally occurring in three species of *Chlorella* were examined. The algae were grown heterotrophically on glucose. Sterols were extracted and isolated from the lipid fraction and were characterized by means of chemical and physical tests.

Chlorella vulgaris contained three sterols. Only the principal one, chondrillasterol, was identified. Chondrillasterol has been isolated previously from the genus *Scenedesmus*.

Chlorella ellipsoidea and *Chlorella saccharophila* were found to contain sterols with β-oriented alkyl groups at C-24 in contrast to the α-oriented groups commonly found in higher plants. Poriferasterol was identified as the principal sterol of both algae. Clionasterol and 22-dihydrobrassicasterol were identified as the two secondary sterols present. None of these sterols have previously been reported to occur in plants. The isolation of 22-dihydrobrassicasterol has not been previously reported from any natural source.

The presence of sterols in *Chlorella* was first reported by KLOSTY and BERGMANN (*1*), who found ergosterol to be the principal sterol in *Chlorella pyrenoidosa*. BERGMANN and FEENEY (*2*) reported the occurrence of chondrillasterol in *Scenedesmus obliquus*. KRAUSS and MCALEER (*3*) and IWATA et al. (*4, 5*) confirmed the principal sterol of *Chlorella* to be ergosterol while that of *Scenedesmus* is chondrillasterol. OTSUKA reported the occurrence of ergosterol and an unidentified Δ^5 sterol in *Chlorella ellipsoidea* (*6*). Since the sterols of *Chlorella* have been identified in only two species, it seems premature to conclude that ergosterol is the principal sterol of the genus. This paper provides additional data by reporting the nature of sterols in *Chlorella vulgaris*, *Chlorella ellipsoidea*, and *Chlorella saccharophila*.

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MATERIALS AND METHODS

Sterol extraction and isolation

Cells of *Chlorella vulgaris* Beyer., EMERSON's strain, *Chlorella ellipsoidea* Gerneck, and *Chlorella saccharophila* (Krüger) Migula, were grown heterotrophically on basal inorganic medium containing 0.5% glucose in fifteen-liter carboys equipped with bubbling tubes for air. The cells were harvested in a Sharples Super Centrifuge and dried in an oven at 70° for 24 hr. The average yield was 2.5 g dry weight per liter. The dried cells were ground to pass a 40-mesh screen, mixed with enough glacial acetic acid to make a thin paste, and heated to 70° with stirring for one hour. The acetic acid was removed under vacuum and the dry cells extracted with acetone in a Soxhlet apparatus for 24 hr. The acetone was evaporated under vacuum and the liquid material was saponified with a 20% solution of KOH in 80% aqueous ethanol under an atmosphere of nitrogen. The lipid material was then extracted with ether for 12 hr in a liquid-liquid extraction apparatus (Fig. 1). The non-saponifiable fraction was obtained by

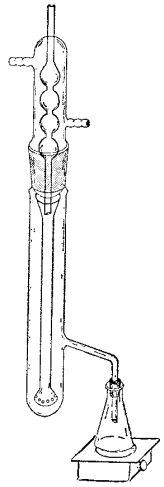

Fig. 1. A liquid-liquid extraction apparatus for the extraction of unsaponifiable matter from an alkaline lipid solution.

evaporating the ether under nitrogen. The non-saponifiable lipid was fractionated as described by HEFTMANN et al. (7) on Woelm grade III neutral alumina. The sterol was eluted in the 50% benzene-Skellysolve F fraction. The sterol fraction was acetylated and rechromatographed for higher purity. The mixture of sterol acetates were subjected to column chromatography on Anasil B[3] which was added to the 3 cm×40 cm column in a slurry with *n*-hexane (8). The sterol acetates were added to the column in a minimum amount of *n*-hexane, and were eluted from the column with 2% ethyl ether in *n*-hexane. Fifteen ml fractions were collected with an ISCO

[3] Analabs, Hamden, Connecticut.

model A fraction collector. Fractions containing unresolved mixtures were rechromatographed; those containing a pure sterol acetate were combined. Fractions were analyzed by gas chromatography. Repeated recrystallizations with methanol and acetone yielded pure sterols which were used in obtaining physical and chemical data for complete identification.

RESULTS AND DISCUSSION

Sterol identification in Chlorella vulgaris

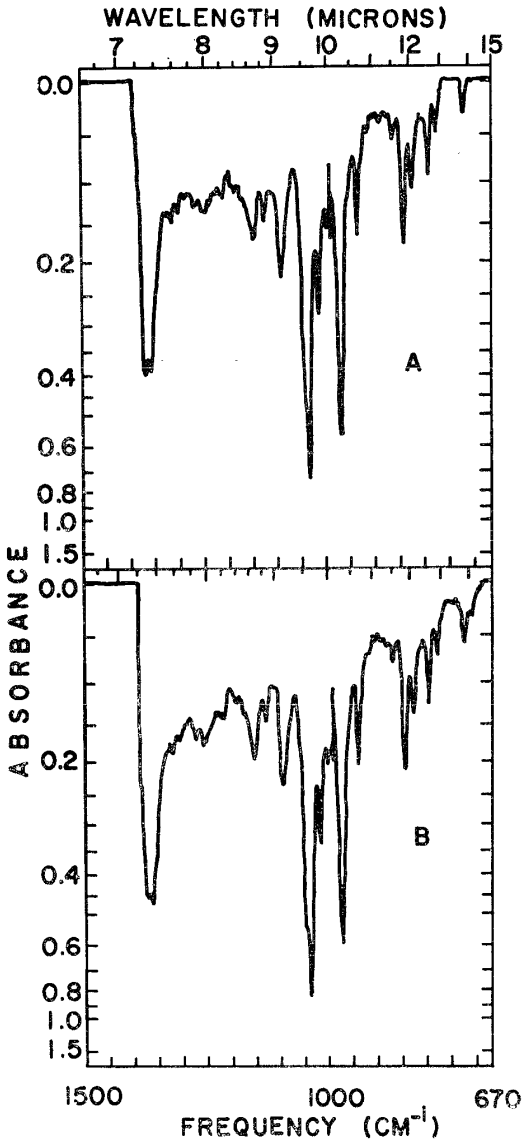

Fig. 2. A comparison of the infrared spectrum of α-spinasterol (A) with that of the #1 sterol of *C. vulgaris* Beyer (B). Spectra were obtained from a 0.06 M solution in CS_2.

TABLE I

A comparison of the retention times on gas chromatographic columns of certain known sterols with those sterols isolated from Chlorella

Compounds	% of total algal sterols	Relative retention time[a]		
		SE-52[b]	QF-1[c]	DEGS[d]
α-Spinasterol		3.16	4.43	14.8
Chondrillasterol		3.16	4.43	14.8
C. vulgaris sterol	65	3.16	4.43	14.8
Stigmasterol		2.80	3.82	12.7
Poriferasterol		2.80	3.82	12.7
C. ellipsoidea #2 sterol	56	2.80	3.82	12.7
Campesterol		2.55	3.75	12.5
22-Dihydrobrassicasterol[e]		2.55	3.75	12.5
C. ellipsoidea #1 sterol	28	2.54	3.76	12.5
β-Sitosterol		3.20	4.59	14.8
Clionasterol		3.20	4.59	14.8
C. ellipsoidea #3 sterol	16	3.19	4.56	14.8

[a] Relative to cholestane.

[b] Column 1.8 m×3.4 mm I.D., 3% SE-52 on 100–120 mesh Gas Chrom P, 20 p.s.i., 240 degrees, cholestane time 9 minutes.

[c] Column 1.8 m×3.4 mm I.D., 1% QF-1 on 100–120 mesh Gas Chrom P, 20 p.s.i. 217 degrees, cholestane time 4 minutes.

[d] Column 1.8 m×3.4 mm I.D., 1% diethylene glycol succinate on 100–120 mesh Gas Chrom P, 20 p.s.i., 205 degrees, cholestane time 2 minutes.

[e] Putative values based on campesterol.

TABLE II

Optical rotation and melting point data of Chlorella *sterols compared to those of known sterols*

Sterol	M.P. (sterol)	M.P. (acetate)	Specific[a] rotation
α-Spinasterol (12)	174	187	− 5
Chondrillasterol (12)	169	175	− 1
C. vulgaris sterol	168−9	173−4	± 0
Stigmasterol (12)	170	144	−46
Poriferasterol (12)	156	147	−49
C. ellipsoidea #2 sterol	156−7	147−8	−55
Campesterol (12)	163−4	141	−34
22-Dihydrobrassicasterol (14)	158	145	−42
C. ellipsoidea #1 sterol	159−60	146−8	−46
β-Sitosterol (12)	137	127	−37
Clionasterol (13)	140	140	−42
C. ellipsoidea #3 sterol	143−4	139−41	—

[a] All determinations on algal sterols were conducted at 25° with chloroform as solvent.

Gas chromatography indicated the presence of three sterols in *C. vulgaris*. The major sterol from this mixture was the last one to be eluted from the silica gel column. It gave a rapid, positive, LIEBERMANN-BURCHARD color test, but did not have ultraviolet absorption above 220 mμ. The infrared spectrum of the unknown compound was identical to that of chondrillasterol and α-spinasterol (Fig. 2). The strong absorption at 10.3 μ is specific for a trans double bond at C-22 in the steroid side chain (*9*). Gas chromatographic retention times of the unknown sterol were also identical those of chondrillasterol and α-spinasterol (Table I.) The characteristic melting points of the unknown sterol and its derivatives, as well as the optical rotation of the known sterol are compared with those of chondrillasterol and α-spinasterol in Table II. The optical rotation of the unknown is slightly less levorotatory than α-spinasterol. The melting points of the unknown sterol and those of its derivatives were significantly lower than those of α-spinasterol while agreeing closely with those of chondrillasterol. The unknown sterol is concluded to be 24β-ethyl-$\Delta^{7,22}$ cholestadienol, or chondrillasterol (Fig. 3). Studies are now in progress to determine the identity of the two minor sterols in *C. vulgaris*.

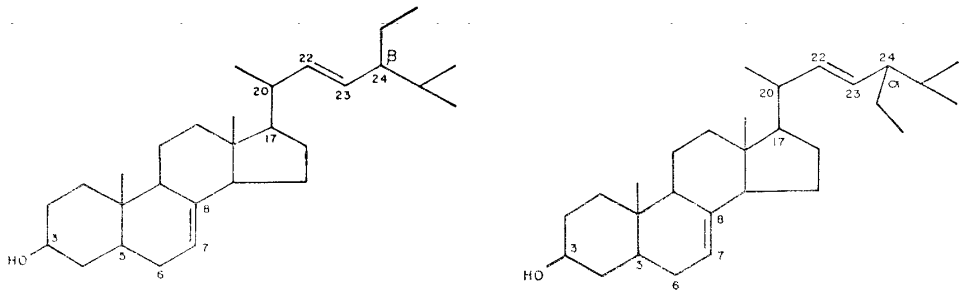

Fig. 3. Structural formulae for chondrillasterol and α-spinasterol.

Sterol identification in Chlorella ellipsoidea *and* C. saccharophila

Gas chromatography indicated the presence of three sterols in *C. ellipsoidea*. The sterols were numbered in the order of their elution on gas chromatography. All three sterols gave a slow, positive LIEBERMANN-BURCHARD color test. The major sterol of *C. ellipsoidea* was sterol #2. It was the last sterol, however, to be eluted from the silica gel column. An infrared spectrum of this sterol revealed the strong band at 10.3 μ specific for the Δ^{22} double bond. The infrared spectrum (Fig. 4) and gas chromatographic retention times (Table I) of the unknown were identical to that of 24-α-ethyl-$\Delta^{5,22}$ cholestadienol, or stigmasterol. However, neither gas chromatography (*10*) nor infrared spectrum (*11*) can distinguish between stigmasterol and its 24β isomer, poriferasterol, since they differ only in configuration about C-24. Comparison of melting points of the unknown

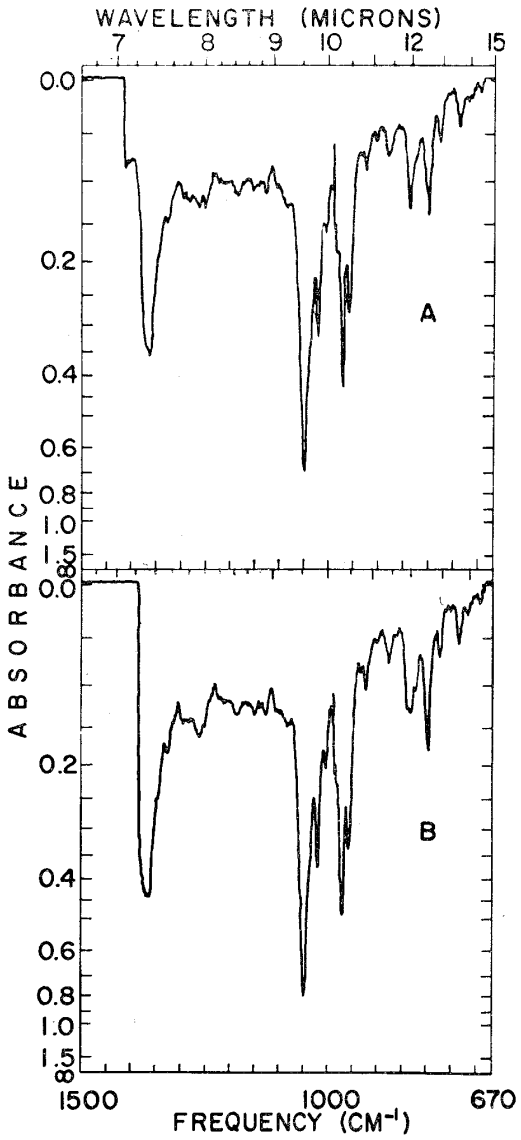

Fig. 4. A comparison of the infrared spectrum of stigmasterol (A) with that of the #2 sterol of *C. ellipsoidea* Gerneck (B). Spectra were obtained from a 0.06 M solution in CS_2.

sterol and its derivatives with those of stigmasterol and poriferasterol is shown in Table II. The melting points of the unknown were very close to those of poriferasterol but were much lower than those of stigmasterol, yet higher than that of stigmasterol acetate. The major sterol of *C. ellipsoidea* is thus concluded to be poriferasterol (Fig. 5), a sterol not previously isolated from any plant.

The first sterol eluted from the gas chromatographic column was eluted second from the silica gel column. The infrared spectrum (Fig. 6) and gas

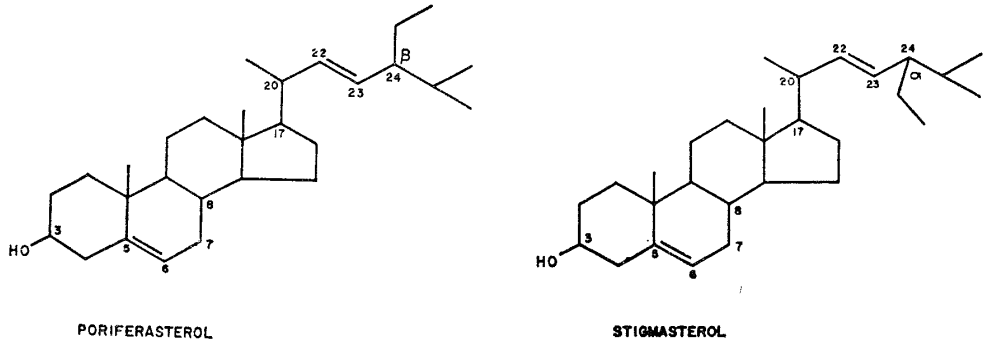

Fig. 5. Structural formulae for poriferasterol, and stigmasterol.

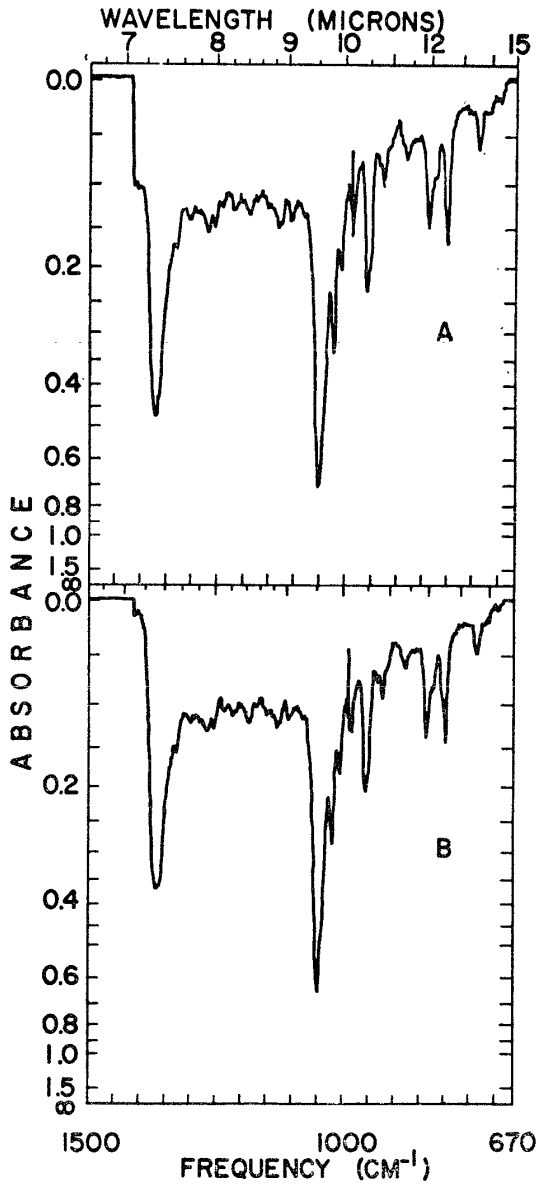

Fig. 6. A comparison of the infrared spectrum of campesterol (A) with that of the #1 sterol of *C. ellipsoidea* Gerneck (B). Spectra were obtained from a 0.06 M solution in CS_2.

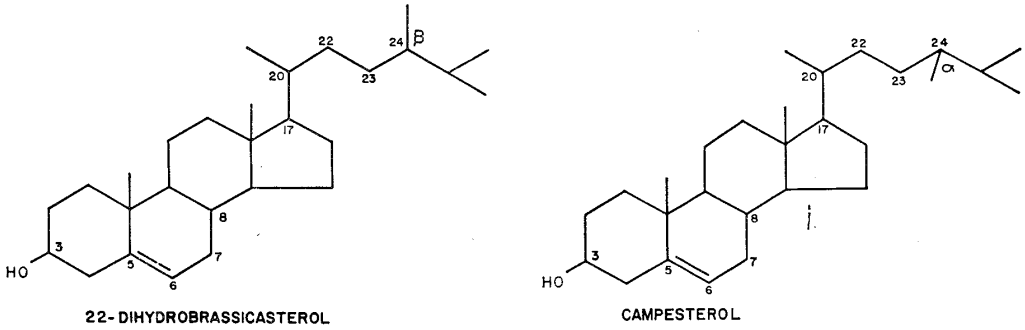

Fig. 7. Structural formulae for campesterol and 22-dihydrobrassicasterol.

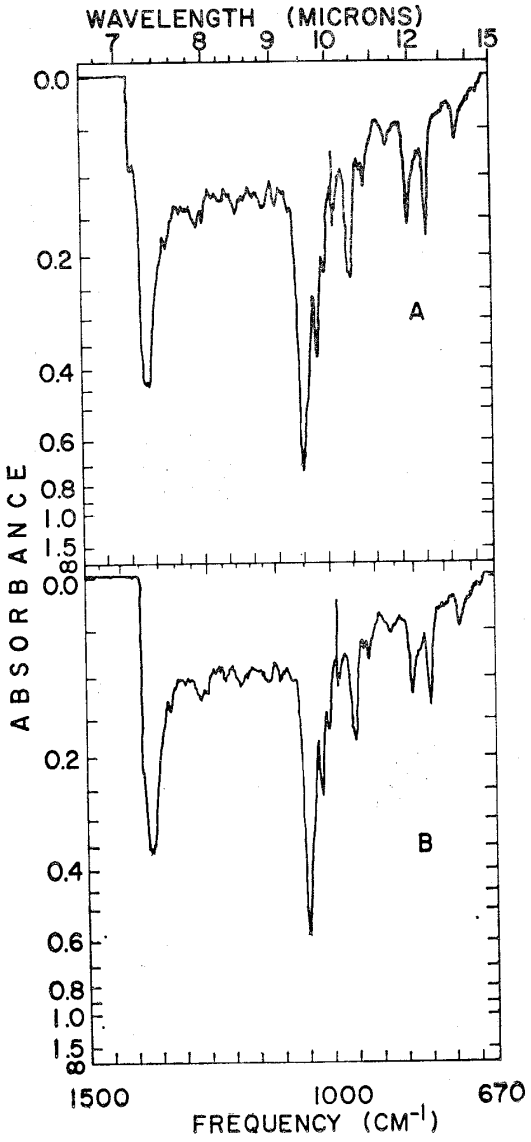

Fig. 8. A comparison of the infrared spectrum of β-sitosterol (A) with that of the #3 sterol of *C. ellipsoidea* Gerneck (B). Spectra were obtained from a 0.06 M solution in CS_2.

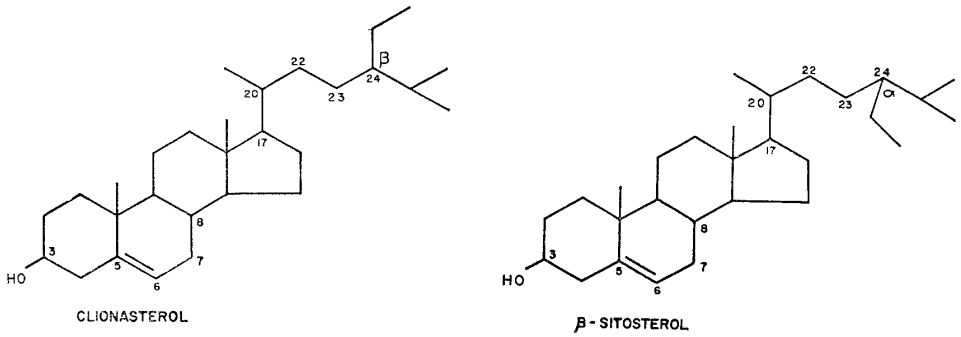

Fig. 9. Structural formulae for clionasterol and β-sitosterol.

chromatographic retention times (Table I) were identical tothat of 24α-methyl cholesterol or campesterol. Again, however, these data cannot distinguish between campesterol and 22-dihydrobrassicasterol, the C-24β isomer of campesterol. Melting point comparisons showed good agreement with the standard values for 22-dihydrobrassicasterol but were significantly different from those of campesterol. The optical rotation of the unknown sterol was more levorotatory than that of campesterol, although gas chromatographic analysis showed the complete absence of the more levorotatory poriferasterol. The #1 sterol from *C. ellipsoidea* must be concluded to be 22-dihydrobrassicasterol (Fig. 7), a sterol whose occurrence in nature has been suspected (*12*) but never demonstrated.

The last sterol eluted from the gas chromatographic column was eluted first from the silica gel column. The infrared spectrum (Fig. 8) and gas chromatographic retention times (Table I) were identical to that of 24α-ethyl cholesterol or β-sitosterol. Although a pure sample of 24β-ethyl cholesterol has never been isolated, melting point data indicate that the unknown sterol is the 24β isomer of β-sitosterol, clionasterol (Fig. 9). The fact that the other two sterols from *C. ellipsoidea* also contain β-oriented alkyl groups at C-24 support this conclusion.

C. saccharophila was found to contain the same three sterols as *C. ellipsoidea* and in approximately the same proportions. A very small amount of a fourth sterol was detected as a result of ultraviolet absorption at 282 mμ. This is indicative of a $\Delta^{5,7}$ sterol, possibly ergosterol. Although OTSUKA found ergosterol to be the principal sterol in *C. ellipsoidea*, no trace of it was found in *C. ellipsoidea* in our study and there is present only trace amounts of a $\Delta^{5,7}$ sterol in *C. saccharophila*. Since $\Delta^{5,7}$ sterols such as ergosterol are relatively easy to detect by examination of their ultraviolet absorption spectra, it is likely that OTSUKA's strain of *C. ellipsoidea* is different from ours with respect to ergosterol content, but that the Δ^5 sterol described by her may actually be several Δ^5 sterols identical to those described in this paper.

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