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Fatty acid composition and sterol content of *Dictyostelium discoideum* cells at various stages of development

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During the development of *Dictyostelium discoideum*, fatty acid compositions of phospholipids and neutral lipids and sterol content gradually changed. The major fatty acid of phospholipids was octadecadienoic acid (18 : 2) and its amount gradually increased during the early developmental stage up to the late aggregation stage and then was maintained at a relatively high level during the later developmental stage. The contents of C_{18} acids and diunsaturated fatty acids also increased gradually. The similar trend of changes in fatty acid composition was also observed with neutral lipids. However, the changes in fatty acid composition of neutral lipids were always preceded by those of phospholipids.

The sterol isolated from *D. discoideum* cells was identified as stigmast-22-en-3β-ol (Δ^{22}-stigmastenol). The content of Δ^{22}-stigmastenol was 10% of total lipids at the vegetative stage and gradually increased to 12.5% by the early aggregation stage. The rate of the increase diminished after aggregation of cells and a rapid increase was again observed during the culmination.

Electron spin resonance (ESR) studies have shown no change in membrane fluidity during the development of *Dictyostelium discoideum* (VON DREELE and WILLIAMS, 1977; KAWAI and TANAKA, 1978; WEEKS and HERRING, 1980). In connection with it, WEEKS and HERRING (1980) reported that the compositions of phospholipids and fatty acids and sterol content of plasma membranes hardly changed. On the other hand, ELLINGSON (1974) and LONG and COE (1974) reported marked changes in phospholipid and neutral lipid compositions during the development of *D. discoideum*, respectively. However, they have not pointed out the significance of these changes of membrane lipid components. Therefore, the report by WEEKS and HERRING is only one indicating functional roles of lipids in the membranes of *D. discoideum*, although they concluded that gross changes in lipid

Abbreviations: In the designation of fatty acids, the first number used refers to number of carbon atoms in the fatty acid chain; the second number, to the number of double bonds in the molecule. Thus, octadecadienoic acid is designated as 18 : 2. 16 : ald, 17 : c, and 19 : c refer to palmitaldehyde, C_{17}-cyclopropanate (*cis*-9, 10-methylene hexadecanoic acid), and C_{19}-cyclopropanate (*cis*-11, 12-methylene octadecanoic acid), respectively.

composition were not involved in the establishment of cell interaction.

To ascertain the conclusion of WEEKS and HERRING, I examined lipid compositions of *D. discoideum* cells at various stages of development. And the effect of modified composition of lipids, especially phospholipids, caused by some agents on the development of *D. discoideum* was also examined. I found that there are no drastic changes in phospholipid and fatty acid compositions and in sterol content during the aggregation and differentiation of *D. discoideum*, but quantitative gradual changes occurred. This paper reports the changes in fatty acid composition and sterol content and the discrepancy between the results of WEEKS and HERRING and mine is discussed.

Materials and Methods

Materials

All reagent grade organic solvents were redistilled immediately before use in a glass apparatus. Silicic acid for column chromatography was purchased from Mallinckrodt Inc., St Louis, washed several times by suspending in methanol and filtration, and activated at 110°C before use. Silica gel G and H for thin-layer chromatography were purchased from E. Merck, Darmstadt. Cholesterol, ergosterol, and stigmasterol were purchased from Nakarai Chem., Kyoto, and purified by preparative thin-layer chroamtography before use.

Organism and culture conditions

Dictyostelium discoideum, strain NC 4, was used in the experiments. Amoebae cells were grown at 22-24°C in association with *E. coli* on SM agar plates (SUSSMAN, 1966).

Conditions for development

At the end of the exponential growth phase, cells were harvested in cold SM buffer, washed several times in the same buffer, once in BONNER's salt solution (BONNER, 1947) by centrifugation, and suspended in the BONNER's solution at a density of 4×10^8 cells/m*l*. Aliquots of 2.0×10^8 cells were spread onto Whatman No. 50 filters (7.0 cm in diameter) which were placed on sponge pads saturated with the BONNER's solution containing 0.2 mg/m*l* of streptomycin sulfate, and then incubated at 22-23°C in the dark. Under these conditions, the cells began to aggregate about 7 hr after the onset of incubation and finished their morphogenesis by 24 hr. The cells harvested at 4 hr intervals during the period from 0 hr to 24 hr of incubation were washed in cold distilled water, immediately frozen in liquid N_2, and lyophilized. The lyophilized cells were weighed and stored at -28°C in tubes free from

moisture.

For isolation of sterol, cells were incubated in association with *E. coli* on SM agar plates until late aggregation stage, then harvested and lyophilized.

Extraction and fractionation of lipids

Lipids were extracted from the dried cells by homogenization in chloroform-methanol (2 : 1, v/v) in a blender and centrifugation. The supernatants were washed by FOLCH's partition method (FOLCH *et al.*, 1957) and evaporated completely. The dry residues were dissolved in a small volume of chloroform-methanol (2 : 1, v/v) and insoluble materials were removed. The solution of purified lipids was evaporated completely, weighed, then redissolved in chloroform-methanol (2 : 1, v/v), and stored with N_2 in a deep freezer (−28°C). Phospholipids were separated by silicic acid column chromatography according to the method of ROUSER *et al.* (1967).

Determination of fatty acid composition

Phospholipid and neutral lipid fractions obtained at various stages of development were subjected to methanolysis with 5% anhydrous methanolic HCl for 5 hr at 100°C. After cooling, fatty acid methyl esters were extracted with hexane from the methanolysate and analyzed on either Diasolid ZT at 180°C or 10% EGSS-X on Chromosorb W (AW-HMDS, 60/80 mesh) linearly programmed from 100 to 140°C at a rate of 2°C/min. The column was a glass tube of 0.3×120 cm and the carrier gas was N_2. Characterization of fatty acid methyl esters was performed by gas-liquid chromatography-mass spectrometry with a Hitachi RMU-6MG instrument. The conditions of gas liquid chromatography were the same as mentioned above except the use of helium as carrier gas. Mass spectra were obtained under the following conditions : temperature of ion source, 180°C ; electron energy, 20 eV ; ion accerator voltage, 3.2 kV ; and ionizing current, 80 μA.

Isolation and characterization of sterol

The neutral lipid fraction from late-aggregation-stage cells was applied to a thin-layer plate (0.5 mm thick) of Silica gel H and chromatographed in petroleum ether-diethyl ether-acetic acid (80 : 20 : 1, v/v/v). The area containing sterol was detected by exposure to I_2 vapor and scraped from the plate and the sterol was eluted from silica gel by chloroform-methanol (2 : 1, v/v). The sterol fraction was then rechromatographed on the same plate for further purification. The purified sterol was washed with methanol, dried completely, and weighed. The purity of the sterol was checked by thin-layer chromatography on Silica gel G which were developed in following solvent systems. I. petroleum ether-diethyl ether-acetic acid (80 : 20 : 1, v/v/v) ;

II. chloroform-methanol-water (65 : 25 : 5, v/v/v); III. chloroform-methanol (90 : 10, v/v).

Infrared spectrum of the isolated sterol was taken in an infrared spectrophotometer (IR-G type, Nippon Bunko Kogyo, Ltd., Tokyo), using KBr pellet containing 3 mg of lipid.

The sterol was trimethylsilylated with pyridine-hexamethyldisilazane-trimethylchlorosilane (8 : 2 : 1, v/v/v) and analyzed with a Hitachi 063 Gas Chromatograph on a Diasolid ZT column packed in a glass tube of 0.3×100 cm at 220°C.

Mass spectrometric analysis was performed with a Hitachi RMU-6MG instrument using a direct inlet system. The conditions for mass spectrum were the same as mentioned above.

Determination of sterol content

Quantitative changes in sterol during the development of *D. discoideum* were determined by thin-layer chromatography and densitometry. Fifty μg of each of neutral lipid fractions from total lipid extracts which were prepared from whole cells at various stages of development was applied to a Silica gel G plate (0.25 mm thick). Five, 10, 15, and 20 μg of the purified sterol were also applied to the same plate as standard lipid. Then the plate was developed in solvent system I. The separated lipids were visualized on the thin-layer plate by spraying with 50% H_2SO_4 and heating at 150°C for 45 min. The visualized plate was scanned with a densitometer (Ozuma-82 types, Asuka Kogyo Co., Ltd., Tokyo). Areas under the peaks were measured and the contents of sterol were calculated from standard curves.

Results and Discussion

Fatty acid composition of D. discoideum

As Table 1 shows, major fatty acids present in *D. discoideum* cells were 18 : 1 and 18 : 2 which comprised 59-82% of total fatty acids and 16 : 0, 16 : 1, and 16 : 2 were also present. Other minor fatty acids detected and characterized by gas-liquid chromatography-mass spectrometry were 14 : 0, 15 : 0, 17 : 0, 18 : 0, and palmitaldehyde. Although some peaks which were supposed to correspond to some longer fatty acid methyl esters were observed in the gas-liquid chromatogram, they were not fatty acid methyl esters. Thus, our conclusion is that there is no fatty acid longer than C_{18}, in contrast to that of DAVIDOFF and KORN (1963).

As shown in Table 1, there were no drastic changes in fatty acid compositions of both phospholipids and neutral lipids during the development.

TABLE 1. Fatty acid composition of phospholipids and neutral lipids from *D. discoideum* cells at various stages of development[a)]

Time of development (hr)[c)]	Fatty acids[b)] (percent composition)									diunsat./ monounsat.	C_{18}/ C_{16}
	16:0	16:1	16:2	18:0	18:1	18:2	17:c	19:c	others[d)]		
phospholipids											
0	6.8	13.4	10.5	0.5	28.6	30.5	2.0	6.5	1.2	0.98	1.94
4	3.8	7.1	11.5	1.3	34.8	39.7	—	—	1.8	1.22	3.38
8	3.7	4.7	9.5	0.3	33.7	46.2	—	—	1.9	1.45	4.48
12	4.2	3.5	8.0	0.6	30.6	51.6	—	—	1.5	1.75	5.27
16	5.1	4.2	7.4	0.4	31.6	49.4	—	—	1.9	1.59	4.87
20	5.1	4.5	8.2	0.4	33.5	46.5	—	—	1.8	1.44	4.52
24	5.6	5.3	6.4	1.5	31.7	47.0	—	—	2.5	1.44	4.64
neutral lipids											
0	19.9	9.7	5.2	11.0	19.4	17.9	2.3	12.0	2.6	0.79	1.39
4	13.3	9.4	8.4	2.0	28.4	25.6	2.6	6.2	4.1	0.90	1.80
8	7.9	6.8	9.7	1.5	31.7	37.7	—	—	5.1	1.24	2.89
12	8.5	5.2	6.5	2.1	24.2	42.5	—	—	11.0	1.67	3.41
16	6.0	4.5	6.1	2.1	22.6	49.5	—	—	9.2	2.06	4.47
20	5.2	4.2	7.4	1.9	21.9	50.7	—	—	8.7	2.23	4.43
24	5.5	3.8	5.4	2.0	24.7	51.8	—	—	6.8	2.01	5.34

a) Fatty acid methyl esters prepared from each of phospholipids and neutral lipids of *D. discoideum* cells at various stages of development were analyzed by gas-liquid chromatography on a Diasolid ZT column at 180°C. On this column, the peak values of longer fatty acid methyl esters are always smaller than those on 10% EGSS-X column. Then, the data in the table were partially corrected by referring to the results on EGSS-X column.

b) Fatty acid designations are explained in Abbreviations of the text.

c) The indicated times correspond to the following stages of development: 0 hr, vegetative stage; 4 hr, interphase; 8 hr, early aggregation stage; 12 hr, late aggregation stage; 16 hr, preculmination stage; 20 hr, culmination stage; 24 hr, 1-day sorocarp stage.

d) 14:0, 15:0, 17:0, and 16:ald are included.

At the vegetative stage (0 hr development), 16:0, 18:0, and 19:c, major fatty acids of *E. coli* lipids, were also major constituents of fatty acids of neutral lipids present in *D. discoideum* cells and they were still detected at interphase (4 hr development). But in phospholipids from vegetative-stage cells, these fatty acids were minor components and 17:c and 19:c disappeared during the early development. These results showed that the fatty acids originated from digested *E. coli* cells remained for at least 4 hr after

the uptake by *D. discoideum* cells but they were not used for phospholipid synthesis in *D. discoideum* cells. During the development of *D. discoideum*, gradual changes in fatty acid composition occurred both in phospholipids and neutral lipids. That is, an increase of 18 : 2 content and corresponding decreases of 16 : 1 and 16 : 2 were observed. In phospholipids, 18 : 2 increased from 31% of total fatty acids to 52% during the early development (0 hr–12 hr development), then slightly decreased. Thus, the ratios of C_{18} acids (18 : 0, 18 : 1, and 18 : 2) to C_{16} acids (16 : 0, 16 : 1, and 16 : 2) and diunsaturated fatty acids (18 : 2 and 16 : 2) to monounsaturated fatty acids (18 : 1 and 16 : 1) increased from 1.94 to 5.27 and from 0.98 to 1.75, respectively, by the late-aggregation stage (12 hr development) and then slightly decreased. The developmentally regulated increase of diunsaturated fatty acids and that of longer chain molecules were also observed in neutral lipids, but the manner of changes was somewhat different from that observed for phospholipids. That is, these changes in neutral lipids occurred at least 4 hr later than the occurrence of such changes in phospholipids.

WEEKS and HERRING (1980) reported that the fatty acid composition of plasma membranes did not change appreciably during the early development (from the vegetative stage to the pseudoplasmodial stage), although there was a slight increase in amounts of diunsaturated fatty acids and the corresponding decrease of monounsaturated ones. These results agreed with mine. However, they have not observed the increase of C_{18} acids. The reason for this discrepancy may be due to the difference of the starting materials. They extracted lipids from purified plasma membranes but I extracted from dried whole cells.

The changes in fatty acid composition of phospholipids during the development were accompanied by those of neutral lipids but not *vice versa*. This suggests that newly synthesized fatty acids were quickly used for phospholipid synthesis and fatty acids for neutral lipids were supplied from the phospholipids after their degradation.

Characterization of the sterol from D. discoideum

Fig. 1 shows the result of thin-layer chromatography of the isolated lipid. Only one spot was detected on the thin-layer plate and it had the same mobility as cholesterol, ergosterol, and stigmasterol in all solvent systems used. As the infrared spectrum (Fig. 2) shows, this lipid exhibited some absorption bands characteristic of sterol (970, 1380, 1450 cm^{-1}) (LABARRER *et al.*, 1958). The absence of the peak at 800 cm^{-1} indicated that this lipid did not contain double bond in the steroid nucleus. As shown in Fig. 3 (a), the sterol has an apparent molecular weight of 414. No other mass peaks

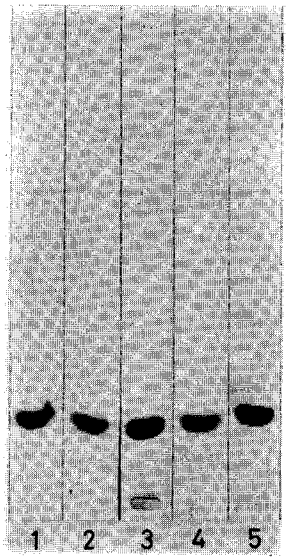

Fig. 1. Thin-layer chromatography of the sterol isolated from *D. discoideum* cells. Twenty-five μg of the purified *D. discoideum* sterol (lane 2 and 4) was applied to a Silica gel G plate. Cholesterol (lane 1), ergosterol (lane 3), and stigmasterol (lane 5) were also applied to the plate. The plate was developed in solvent system I and visualized by spraying with 50% H_2SO_4 and heating at 150°C for 30 min.

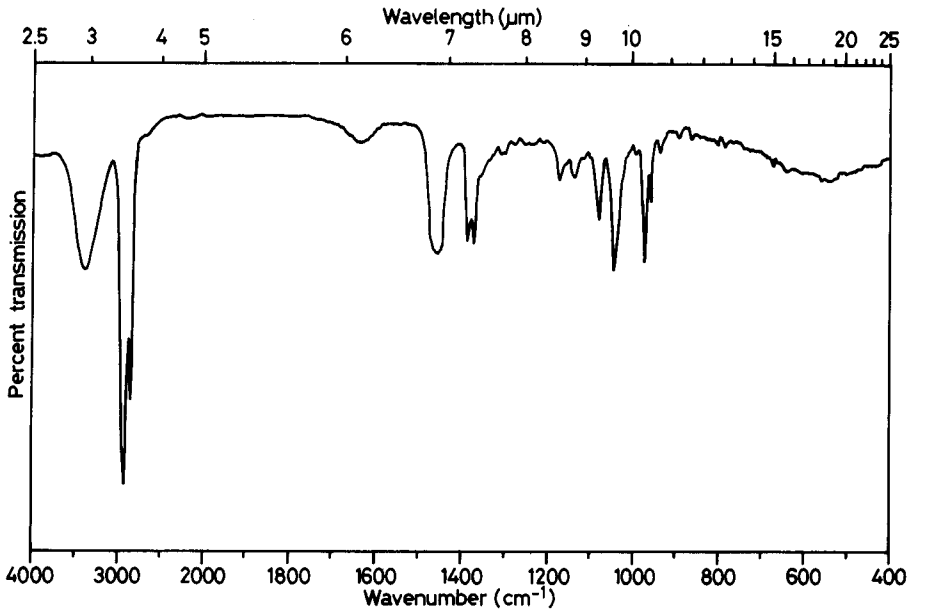

Fig. 2. Infrared spectrum of the sterol from *D. discoideum* cells.

which corresponded to other lipids with different molecular weight were detected. Characteristic ions for sterol at m/e 83, 257, 273, 302, 354, 371, and 399 were prominent and the ion at m/e 257 which was due to the

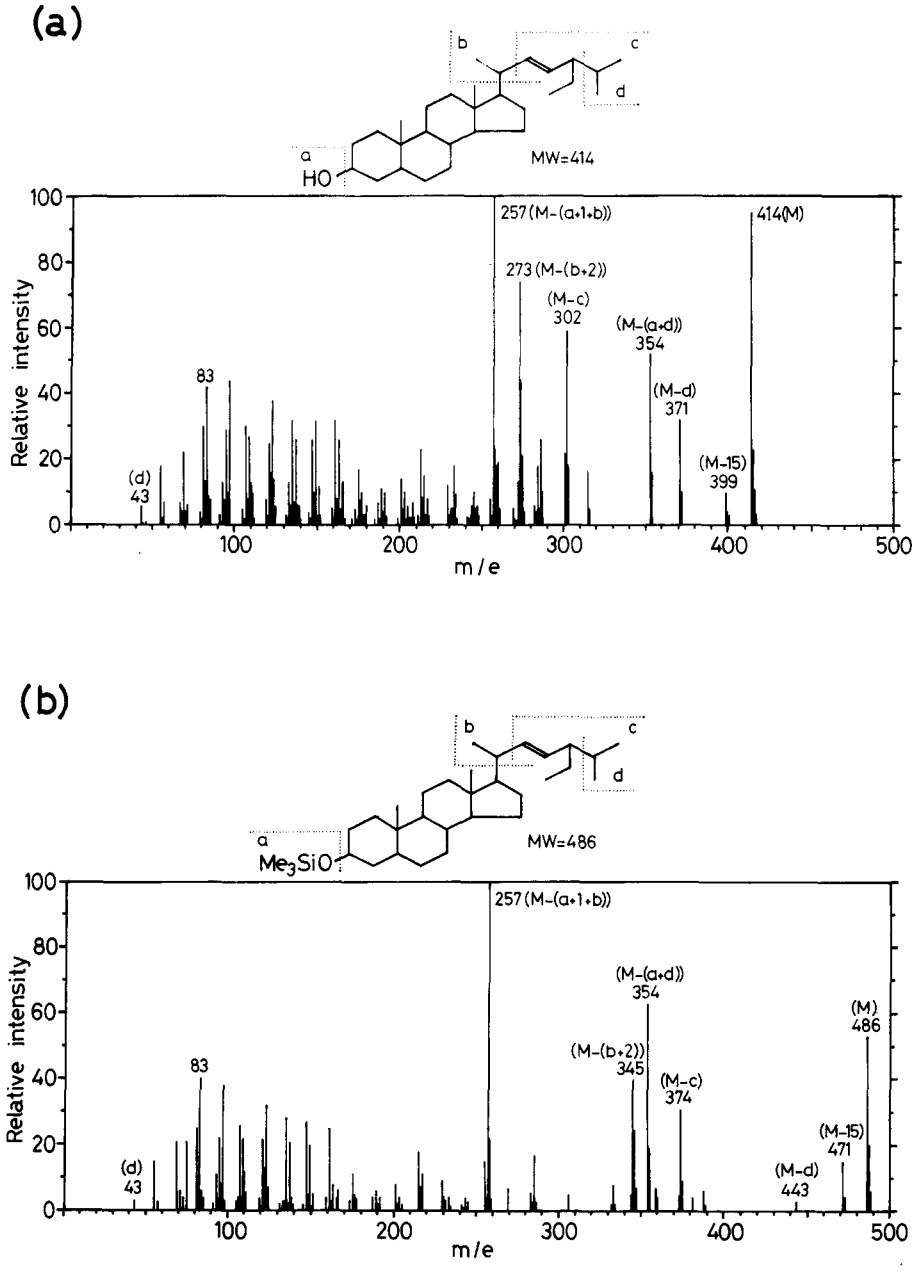

Fig. 3. Mass spectra of the intact sterol (a) and the trimethylsilylated sterol (b) from *D. discoideum* cells. The mass spectra were obtained using a direct inlet system. The conditions of analysis are given in Materials and Methods. The possible origin of fragment ions is illustrated in the figure.

steroid nucleus was the base peak. The sterol was then trimethylsilylated and its constituents were examined by gas-liquid chromatography on the Diasolid ZT column at 220°C. Mono peak was observed in the chromatogram. Therefore, the trimethylsilylated sterol was further analized by mass spectrometry using a direct inlet system and the mass spectrum (Fig. 3 (b)) was compared with that of a non-trimethylsilylated one. The 72 mass-unit increase of the molecular ions indicated the presence of one replaceable hydrogen. The absence of the ion at m/e 129 suggested that this compound did not contain the Δ^5-double bond (DIEKMAN and DJERASSI, 1967), which was further demonstrated by the presence of the base peak at m/e 257. Other ions had the same origin as those of the non-trimethylsilylated sterol as shown in Fig. 3.

From these results, I identified the isolated sterol as stigmast-22-en-3β-ol (Δ^{22}-stigmastenol) (HEFTMANN *et al.* 1960) and other sterol species were not included in the sterol fraction.

Changes in Δ^{22}-stigmastenol content during the development of D. discoideum

Since the purity of the isolated sterol was confirmed as mentioned above, it was used as a standard lipid for the determination of Δ^{22}-stigmastenol content in cells at various stages of development. The results are shown in Fig. 4. The content of Δ^{22}-stigmastenol was 10% of total lipids in vegetative-stage cells and gradually increased to 12.5% in early aggregation-stage cells, then the rate of increase declined and a constant level was maintained at the middle stages of development. The rate of increase was hastened again during the culmination and its content reached 14% of total lipids at the 1-day sorocarp stage (24 hr development). LONG and COE (1974) and WEEKS and HERRING (1980) already reported the developmental changes in sterol content. My results were somewhat different from the results by WEEKS and HERRING but almost agreed with those by LONG and COE. The possible origin of this discrepancy was the difference of parameters used to quantify sterol content. LONG and COE expressed the amount of sterol as per g dry cell weight and WEEKS and HERRING used the parameter as per mg plasma membrane proteins. As lipid contents in cells at various stages of development were relatively constant (10–12% of dry cell weight), I expressed the amount of sterol as per g total lipids. The parameter used by LONG and COE and me was different from that by WEEKS and HERRING. This difference might reflected on the results. However, which parameter was suitable for the expression of lipid contents is unknown.

As described above, there were gradual changes in the fatty acid com-

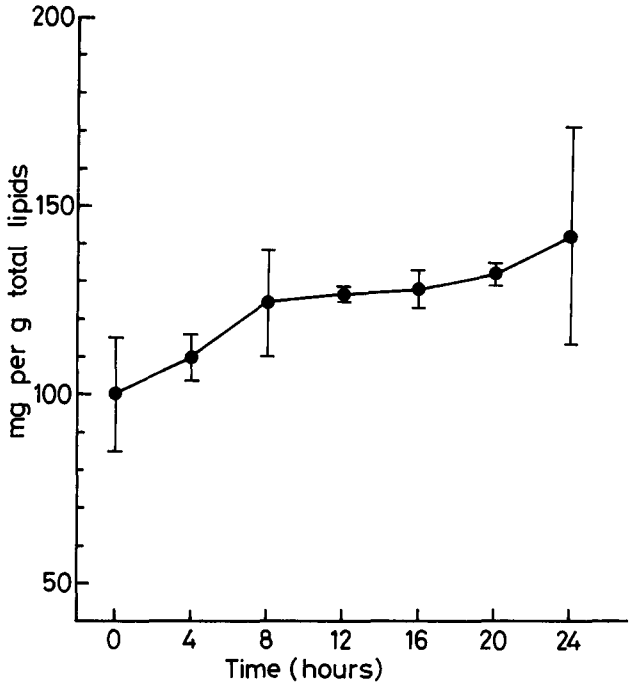

Fig. 4. Changes in Δ^{22}-stigmastenol content during the development of *D. discoideum*. Total lipids were extracted from dried cells at various stages of development. Then neutral lipids were prepared from the lipid extracts by silicic acid column chromatography. The contents of Δ^{22}-stigmastenol were determined by thin-layer chromatography of the neutral lipid fractions and densitometry. The values are means with the standard deviations of three determinants of each of three independents experiments. The stages corresponding to the indicated times are shown in the footnote of TABLE 1.

position and the sterol content during the development of *D. discoideum*. The fatty acid composition and the sterol content have been well known to affect membrane fluidity (SINGER and NICOLSON, 1972). However, the observed ratio of unsaturated fatty acids to saturated ones was high enough to maintain moderate membrane fluidity throughout all stages of development. Therefore, it is doubtful that the observed increase of diunsaturated fatty acids affected membrane fluidity, as described by HERRING and WEEKS (1979). Thus, the changes in fatty acid composition may be secondary phenomena which were accompanied with the changes in phospholipid composition. If some effects on membrane fluidity were brought about by changes in lipid composition, some changes in sterol content might give also any effects. However, since it has been shown that no changes in membrane fluidity

occurred during the development of *D. discoideum*, the direct contribution of sterol to membrane fluidity is obscure. As CULLIS *et al.* (1978) reported, sterol, when its content increased, might play some functional roles by affecting the bilayer configuration of biological membranes. Further investigations are required.

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