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Studies on Fungicides. XIII.[1] Lipids Compositions of Conidia of *Cochliobolus miyabeanus* and Their Changes during Spore Germination

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The conidia of *Cochliobolus miyabeanus* were extracted repeatedly with $CHCl_3$–MeOH mixture (2 : 1) and the contents of their lipids, fatty acid composition of these lipids, and the changes of the fatty acid content with advance of germinating processes in conidia were examined. The results of these examinations were as follows. (1) Total lipid compositions: The extractable lipid and bound lipid contents were 6.3—7.5% and 0.3—1.2% of conidial weight, respectively, and the extractable lipid was composed of 1.54—2.85% triglyceride, 0.12—0.36% diglyceride, 0.22—0.66% monoglyceride, 0.72—1.17% free fatty acid, 0.09—0.10% steroid ester, 1.08—2.49% steroid, and 0.99—1.24% phospholipid of conidial weight. (2) Fatty acid composition: The pattern of fatty acid composition in each fraction of the extractable lipids was almost identical and $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ fatty acids were found in each fraction as dominant constituent. These 5 kinds of fatty acids constituted 85% of total conidial lipids. (3) Changes in fatty acid composition during the germination processes: Although a slight change in fatty acid compositions was found in each lipid fraction during all stages of conidial germination, the content of $C_{18:2}$ in the fraction of phospholipid was inclined to increase with the advancement of germinating process.

Keywords——lipid composition; *Cochliobolus miyabeanus*; spore; germination; fatty acid; fungi; phospholipid

Introduction

When the dormant conidia of *Cochliobolus miyabeanus*, which is a typical pathogenic fungus in rice plants, are incubated under suitable conditions, they commence the germinative action accompanied by some rapid morphological changes. Although many reports have been presented on the metabolisms of reserved carbohydrates,[3] ribonucleic acids,[4] and proteins[5] in germinating spores, little is known on their lipids. In bacteria, McElhaney and Tourtellotte[6] have discussed the change of physiological characters caused by variations in fatty acid composition of membrane lipids of *Mycoplasma laidlawii*, while in fungal spore even the fatty acid composition of lipid fraction has not been elucidated. The present study was carried out to clarify the change of lipid composition that occurred during the period of shift of life phases from dormant stage to germinative stage.

Materials and Methods

1) Harvesting of the Conidia and Germination

The strain of fungus (*Cochliobolus miyabeanus*), the harvesting procedure of the conidia, and germinating conditions were described previously.[7] The conidia in various germinative stages were harvested by centrifu-

1) Part XIII: H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1576 (1976).
2) Location: *Motoyama-kita-machi, Higashinada-ku, Kobe.*
3) H. Kuroda and M. Tokuhiro, *Yakugaku Zasshi*, **88**, 308 (1968).
4) E.M. Philip, *J. Bacteriol.*, **117**, 196 (1974).
5) M.B. Robert and L.E. James, *Arch. Biochem. Biophys.*, **137**, 442 (1970).
6) R.N. McElhaney and M.E. Tourtellotte, *Science*, **164**, 433 (1969).
7) A. Kumano, S. Okamoto, and H. Kuroda, *Eisei Kagaku*, **10**, 165 (1964).

gation (3000 rpm 5 min) and, after checking on a part of them for germinative ratio by lactophenol-Cotton Blue staining test, the conidia were lyophilized.

2) **Extraction and Fractionation of the Lipid**

(1) **Extraction of the Lipid**——The lyophilized conidia (500 mg) were homogenized with a mortar and pestle in 20 ml of cold acetone and extracted twice with 20 ml each of $CHCl_3$–MeOH mixture (2: 1) and once with 20 ml of $CHCl_3$–MeOH (1: 1) in succession. The combined extract was washed with 0.73% NaCl solution and evaporated to dryness. This residue was termed the "extractable lipid." The residue of extracted conidia homogenate was refluxed with 20 ml of 2 N KOH for 2 hr and acidified with H_2SO_4. The $CHCl_3$ extract of this hydrolysate was termed the "bound lipid." These preparations were treated in N_2 stream.

(2) **Fractionation of the Extractable Lipid by Thin-Layer Chromatography (TLC)**——The extractable lipids were developed on a silica gel G plate (0.5 mm thick) with (1) ether–benzene–EtOH–AcOH (40: 50: 2: 0.2) and (2) hexane–ether (94: 6) as solvent systems according to the so-called double developing method. The lipid areas were located with the aid of iodine vapor or Rhodamine 6G solution in EtOH stained guide plate and were scraped off. The collected parts corresponding to authentic steroid ester and steroid were extracted with petroleum ether and the parts corresponding to authentic triglyceride, diglyceride, monoglyceride, free fatty acid and phospholipid were extracted with $CHCl_3$–MeOH mixture (2: 1). The extracts were evaporated to dryness and their weight was determined.

3) **Identification of the Fatty Acid**

(1) **Methylation of the Fatty Acid**——The fatty acid moieties of extracted lipids were methylated by the BF_3–MeOH method.[8] In the case of steroid ester, the fatty acid moiety was released previously by saponification with 50% KOH and resultant free fatty acid was methylated under the condition to be described below. The fatty acid moiety of triglyceride, diglyceride, monoglyceride or phospholipid was methylated without previous saponification. To the sample dissolved in MeOH, BF_3–ether complex salt was added to give 13% BF_3 and the solution was refluxed at 80° for 15 min (for free fatty acid) or 120 min (for others). The solution was poured into H_2O, the produced methyl ester of fatty acids was extracted with petroleum ether, and the petroleum ether extract, after being dried over anhyd. Na_2SO_4, was submitted to gas–liquid chromatographic analysis.

(2) **Hydrogenation or Oxidation of the Unsaturated Fatty Acid Methyl Ester**——In order to confirm the presence of some unsaturated fatty acids in the methyl esters obtained as above, hydrogenation or oxidation of these methyl esters was carried out as follows.

(i) Hydrogenation: A mixture of the fatty acid methyl ester dissolved in MeOH and Raney Ni was shaken in a current of H_2 at room temperature and atmospheric pressure for 24 hr. After filtration and removal of the solvent, the residue was dissolved in a small quantity of petroleum ether and submitted to gas–liquid chromatographic analysis.

(ii) Oxidation: The fatty acid methyl ester dissolved in 30 ml of *tert*-BuOH was added to 20 ml of 0.0025 M $KMnO_4$ containing 0.42 g $NaIO_4$ and 25 mg K_2CO_3, and the whole volume was brought to 100 ml with H_2O. The solution was allowed to stand for 6 hr at room temperature. After bleaching with $Na_2S_2O_5$, the solution was saponified with 2 g KOH at 50° and evaporated to 20 ml, followed by acidification with H_2SO_4. The acidic solution was extracted 5 times with 2 volumes each of ether and the combined ether extract was washed with NaCl-saturated water, dried over anhyd. Na_2SO_4, and evaporated to dryness. The residue was dissolved in a small quantity of petroleum ether and submitted to gas–liquid chromatographic analysis.

(3) **Gas–Liquid Chromatography (GLC) of Fatty Acid Methyl Ester**——Fatty acid methyl ester dissolved in petroleum ether was analyzed by GLC (Shimadzu Model GC–1C) under the condition given in Table I.

Table I. Condition of GLC

Column:	Stainless steel (3 mmϕ X 1.8 m)
Packing:	15% Diethylene glycol succinate (on 60—80 mesh Celite)
	5% SE-30 (on 60—80 mesh Shimalite)
Injector temperature:	230°
Detector temperature:	250°
Column temperature:	180°
Nitrogen flow:	42 ml/min
Detector:	FID

8) W.R. Morrison and L.M. Smith, *J. Lipid Res.*, **5**, 600 (1964).

Result

Lipids Contents of Dormant Conidia

The contents of extractable lipid and bound lipid in the dormant conidia were 6.3—7.5% and 0.3—1.2% of the conidial weight, respectively. Since the extractable lipid was fractionated to steroid ester, triglyceride, diglyceride (1,3-diglyceride and 1,2-diglyceride), monoglyceride, free fatty acid, steroid, and phospholipid by TLC analysis, these fractions were extracted and their weight was determined. Typical results are given in Fig. 1 and Table II. More than 20% of total lipid consisted of triglyceride.

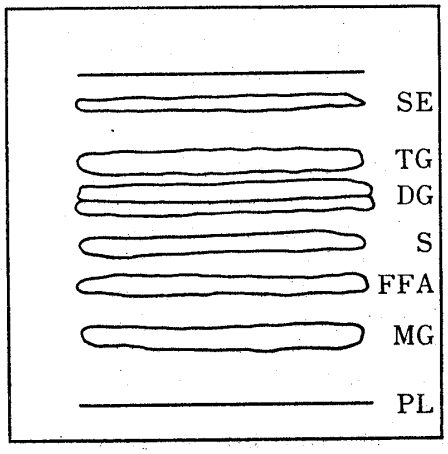

Fig. 1. Thin-Layer Chromatogram of Extractable Lipid

Plate: Silica gel G, 500μm.
Solvent: (1) ether–benzene–EtOH–AcOH (40:50:2:0.2).
(2) ether–hexane (6:94).

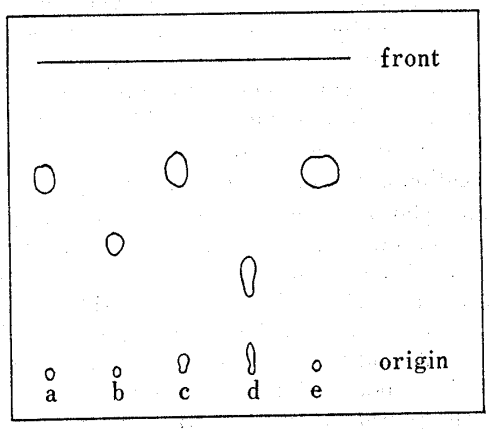

Fig. 2. Thin-Layer Chromatogram of Methylated Lipids

Plate: Silica gel G, 250μm.
Solvent: hexane–ether (94:6).
a: authentic methyl palmitate.
b: authentic tripalmitin.
c: methylated triglyceride of conidia.
d: authentic lecithin.
e: methylated phospholipid of conidia.

Table II. Lipid Composition of Conidia

	mg/100 mg conidia	% for total lipid
Steroid ester	0.10	1.4
Triglyceride	1.54	21.9
Diglyceride	0.36	5.1
Steroid	1.08	15.4
Free fatty acid	1.17	16.6
Monoglyceride	0.66	9.4
Phospholipid	1.24	17.6
Bound lipid	0.88	12.5

Fatty Acid Composition of the Extractable Lipid

The fatty acid composition of each fraction obtained from the extractable lipid was examined. The TLC analysis of the methylated lipids indicated that triglyceride and phospholipid fraction dissolved in MeOH was completely methylated by refluxing for 120 min with BF_3–ether complex salt (Fig. 2) and, in the case of free fatty acid fraction, refluxing for 15 min with BF_3–ether complex salt gave the completely methylated compound.

By comparison of GLC analyses on fatty acid methyl esters obtained from triglyceride fraction and authentic fatty acid methyl esters, the presence of some fatty acids was clarified, as shown in Fig. 3 a,b.

Further, based on the fact that an almost linear relation is obtained between the logarithm of retention time and the number of carbon atoms in homologous series of fatty acids, and from the result of GLC analysis given Fig. 3, the presence of acids, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ was presumed. Catalytic hydrogenation with Raney Ni or $KMnO_4$–HIO_4 oxidation of fatty acid methyl esters was carried out and their product was submitted to GLC analysis, because the presence of some unsaturated acids was presumed. As shown in Fig. 4, by catalytic hydrogenation of methyl esters, the peaks corresponding to those of unsaturated acids disappeared and the same disappearance of the peaks was also recognized by oxidation. From these results, the presence of unsaturated acids was confirmed.

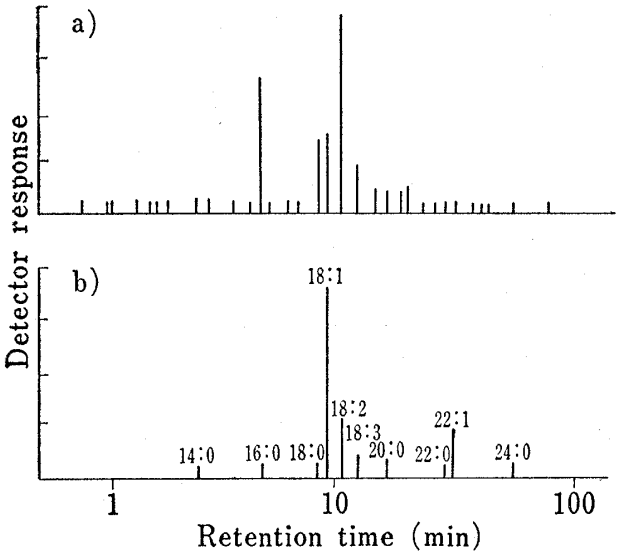

Fig. 3. GLC of Fatty Acid Methyl Esters in TG and Authentic Fatty Acid Methyl Esters

a) fatty acid methyl esters of conidia.
b) authentic fatty acid methyl esters.

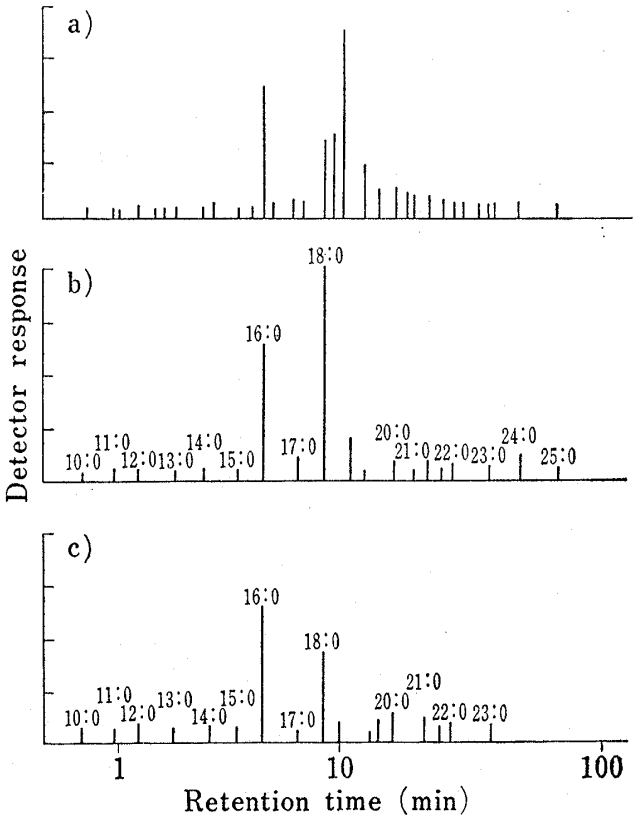

Fig. 4. GLC of Fatty Acid Methyl Esters in TG

a) before hydrogenation or oxidation.
b) after hydrogenation.
c after oxidation.

The same analyses were carried out on steroid ester, diglyceride, monoglyceride, free fatty acid, and phospholipid fractions and the bound lipid. The content ratio of each fatty acid was calculated from the peak areas obtained by GLC analyses and these results are shown in Table III.

Changes of Fatty Acid Contents in Conidia during Germination

Changes in the content of some major fatty acids in each fraction from conidia with the advancement of germinating processes are shown in Fig. 5. Since almost no $C_{21:0}$ fatty acid was found in conidia, the quantitative GLC analyses of fatty acids were performed by use of this acid ($C_{21:0}$) as an internal standard. Under this experimental condition, the formation of germ tube was initiated after 60 min of incubation and almost all of conidia formed their tubes during further 60-min incubation. As shown in Fig. 5a,b, the contents of steroid ester and monoglyceride fractions in conidia were very small and the fatty acid

Table III. Fatty Acid Composition of Each Fraction

Fatty acid carbon chain[a]	Relative retention time	Composition (%)[b]						
		SE	TG	DG	MG	FFA	PL	BL
10:0	0.16	t	t			t		t
10:2	0.20	t				t	t	t
11:0	0.23	t	t	t	t	t		0.3
12:0	0.31	t	t	t	2.0	0.1	t	t
12:1 or 12:2 ?	0.35		t	t	t	t		t
13:0	0.44	0.9	t	t	0.2	0.1	t	0.5
13:1 or 13:2 ?	0.48				t	t	t	
14:0	0.55	0.4	t	t	0.4	0.3	t	0.2
14:1 or 14:2 ?	0.61	t	t	t	1.2	t		t
15:0	0.74	t	t	t	0.2	0.4	t	0.3
16:0	1.00	11.5	21.6	15.6	25.9	29.7	11.4	14.5
17:0	1.37	t	0.6	0.5	0.8	1.0	0.4	0.4
18:0	1.89	20.4	14.2	2.6	3.9	15.6	8.5	3.8
18:1	2.00		14.3	11.2	5.0	12.8		6.7
18:2	2.29	61.4	42.6	55.3	45.9	26.7	67.1	59.5
18:3	2.73	2.2	4.0	5.6	4.1	1.6	8.1	5.0
19:2	2.94	t			1.7	0.2		1.8
20:0	3.43	1.9	0.2	0.7	1.2	0.3	0.3	1.7
19:3 or 20:1 ?	3.60				t	0.8	t	t
20:2	4.10	t	0.5	0.5	1.7	0.3	1.7	t
20:3	4.33		0.4	t		t		
21:0	4.62	t	t	0.5	0.6	0.2		t
21:1	5.30		0.1	0.7		0.6	0.3	
21:2	5.87		0.1	0.7			0.3	t
22:0	6.52	1.3	t		1.9	0.3		2.7
22:2	8.31	t	0.2			3.4	1.0	
23:0	8.82		0.4	2.5		4.7	0.3	
22:3 or 23:1 ?	9.63	t	0.2	3.2	3.3		0.7	
24:0	12.12	t	0.3	0.3	t	0.8	t	1.8
25:0	17.59		0.2					0.7

a) Number of carbon atoms: number of double bonds.
b) Calculated as percentage of total peak area.
t: trace (less than 0.1%).
Abbreviations used in all Tables and Figures: SE=steroid ester, TG=triglyceride, DG=diglyceride, MG=monoglyceride, FFA=free fatty acid, PL=phospholipid, BL=bound lipid.

composition of their fractions was almost constant through all stages of conidium germination. In the case of triglyceride fraction, the content of $C_{18:0}$ and $C_{18:3}$ was almost constant and the others decreased gradually. In diglyceride fraction, the content of $C_{18:2}$ decreased at the germ tube forming stage (90—120 min), while this acid content began to increase at the germ tube elongating stage (after 120 min). In free fatty acid and bound lipid fractions, the content of $C_{16:0}$ and $C_{18:2}$ increased at an early stage of germ tube formation and then decreased after this stage, especially the content of $C_{18:2}$ in bound lipid fraction. In phospholipid fraction, which was the dominant conidial lipid, almost constant increase of the content of $C_{18:2}$ was observed through all stages of conidium germination.

Discussion

Since the metabolic activities of all kinds of living matter are affected by their growing conditions, it is anticipated that alterations of lipid contents and their composition in conidia are caused by the difference in their storage period or germinating stages. Although, by difference of storage period, even in the case of samples stored in a refrigerator, some fluc-

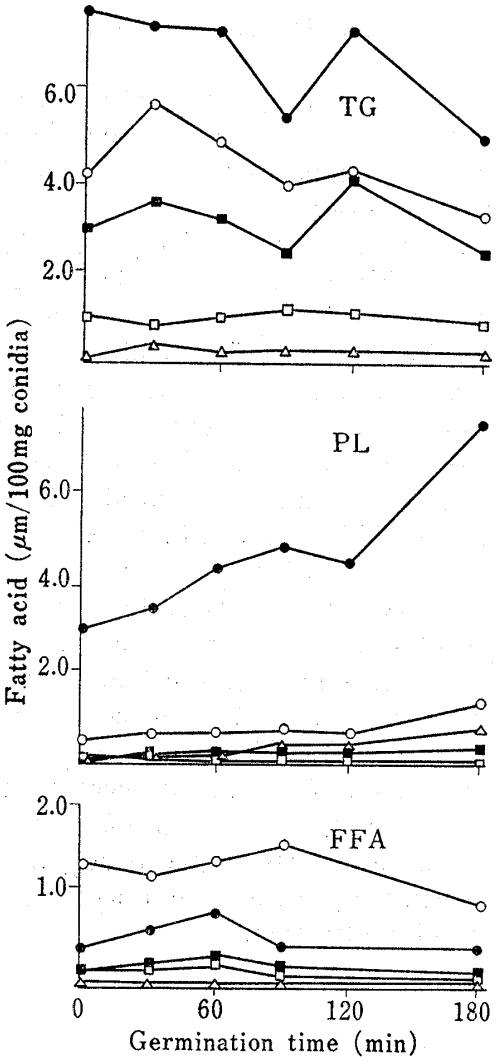

Fig. 5a. Variation of Fatty Acid Composition in Germinating Conidia

○, 16:0. □, 18:0. ■, 18:1. ●, 18:2. △, 18:3.

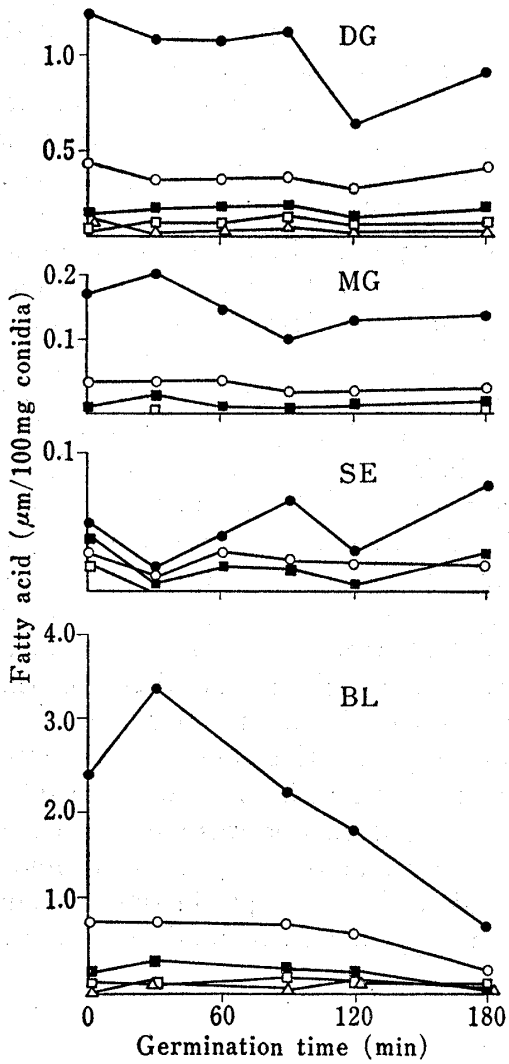

Fig. 5b. Variation of Fatty Acid Composition in Germinating Conidia

○, 16:0. □, 18:0. ■, 18:1. ●, 18:2. △, 18:3.

tuations were found in analytical results, the typical data in several repeated experiments were presented in this paper.

Total lipid content of fungal spores varies with species, *e.g.*, 9.6% in *Trichoderma viride*,[9] 20.0% in *Candida albicans*,[10] 19.0% in *Neurospore crassa* Lindegren,[11] 10.0% in *Sphaerotheca humili* var. *fuliginia*,[12] 12.0% in *Erysiphe graminis*,[12] and 1.4% in *Pithomyces chartarum*.[13] Judging from many reports, 1–35% of dried weight of spore was occupied by lipids. In the case of our fungal conidia, total lipid content was 7—9%. Tsuda, et al.[14] reported that the lipid content of the same fungal mycelia was about 10% and this value is very close to that of the conidia.

The fatty acid composition of the conidial lipid was examined and its results (Table III) indicated that $C_{16:0}$ was predominant in saturated fatty acid groups and $C_{18:1}$ and $C_{18:2}$ were predominant in unsaturated acids. In the mycelial lipids of the same fungus, major fatty acids were constituted by $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ and especially $C_{18:2}$ was a predominant

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acid.[14] These data show that there is little difference in the fatty acid composition between conidial and mycelial lipid.

As shown in Fig. 5 a,b, there were few fatty acids indicating a marked change in the content in each conidial fraction during the all germinating processes. It is considered that almost all of phospholipid content is located in the membraneous fraction which showed marked morphological changes during the germinating processes.

In this phospholipid fraction, the data showed that the dominant fatty acid ($C_{18:2}$) increased constantly through all stages of germination. McElhaney and Tourtellotte[6] reported that *Mycoplasma laidlawii* cells grown in a medium containing several fatty acids with saturated and long hydrocarbon chain ($C_{17:0}$, $C_{18:0}$, $C_{19:0}$, $C_{20:0}$) swelled and finally lysed, while the cells grown in a medium containing acids with unsaturated or short hydrocarbon chain did not swell. This lytic reaction of the *Mycoplasma*, which possesses saturated and long carbon-chain acids in membraneous lipids might be due to the formation of "crystalloid" with some hydrophobic interactions of lipids. On the other hand, it is considered that the enrichment of some unsaturated acids in membraneous lipids will lead to the formation of an "expanded film" by hydrophilic character given by the double bonds existing in these unsaturated acid structures. The data in Fig. 5b showed that unsaturated acid $C_{18\,2}$ in phospholipid fraction increased constantly through all stages of germination. This result suggests that the membrane of germinating conidia becomes more elastic than that of ungerminated conidia and it becomes possible for morphological alternations to occur with the progress of germinating stages. The content of $C_{18:2}$ in bound lipid fraction decreased with the progress of germination (2.7 μmol/100 mg of conidia, Fig. 5a), but the increase of $C_{18\,2}$ in phospholipid fraction was more violent (4.5 μmol/100 mg of conidia, Fig. 5b). Therefore, it is unlikely that the $C_{18:2}$ in bound lipid was transported directly to phospholipid. Since the location of bound lipid in spore remained unknown, we can't assume a special relation between the germination and decrease of $C_{18:2}$ in bound lipid fraction.

14) M. Tsuda, A. Ueyama, M. Nakano, and Y. Fujino, *Nippon Shokubutsu Byori Gakkai Ho.*, 38, 60 (1972).