Studies on Lipoamino Acids in *Streptomyces sioyaensis*

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CONTENTS

INTRODUCTION

Needless to say, progress of biochemistry has shown that the amino acid is one of the important natural elements in the metabolism of microorganisms as well as animals and plants. On the other hand, another important element: lipid has been remained behind others, because of the difficulty of its treatment. However by recent development of techniques of isolation, purification and analyses, a great many knowledges were obtained.

Previous reports taught the author that there existed some bound type amino acids in the phospholipid fraction [1,2], but no one has shown what kinds of amino acids they were.

With the opinion that there might be some new compounds in the boundary region between amino acid and lipid, the author tried the screening to select strains which produce ninhydrin-positive lipid. [3]

Streptomyces sioyaensis, isolated from Shioya district in Kobe, and named after the place [4], had three ninhydrin-positive lipids [5], one of which was a new lysine-containing lipid. [6] It was named "siolipin A" [7]. Other two substances were two kinds of phosphatidylethanolamine, from one of which α-hydroxy

fatty acid (2-hydroxy-13-methyl-tetradecanoic acid) was isolated [8]. It suggested the possibility of α-oxidation by the microorganism.

Studies on the chemical structure of the lysine-containing lipid showed that it was a new ester of lipo-amino acid [7].

Further studies of the siolipin revealed that Streptomyces sioyaensis produced an ornithine-containing lipid (siolipin B) besides lysine-lipid (siolipin A) [9]. The ratio of these two siolipins varied according to the culture conditions [9]. This siolipin B was the same type substance as the ornithine-lipid reported in Myco-bacterium [10,11], Rhodopseudomonas spheroides [12], Rhodospirillum rubrum [13]. The chemical structure of the ornithine-lipid from Rhodopseudomonas spheroides was determined by Gorchein [14] at almost the same time as the author determined the structure of siolipin.

Siolipin showed three biological activities [15]; (1) hemolytic action for rabbit erythrocyte, (2) acceleration of fibrin clot formation, (3) antibacterial activity on Bacillus subtilis PCI-219 grown on a synthetic medium.

In this paper, will be described all detailed data about siolipin — detection, isolation, purification,

determination of the structure, condition for biosynthesis, and biological activities etc.

The author thinks that studies on the lipoamino acid[16] have just begun, and that they should be developed in the future.

CHAPTER I. DISTRIBUTION OF NINHYDRIN-POSITIVE LIPIDS

Phospholipid compositions in bacteria have been investigated by many workers. Phospholipids of *Streptomyces* group, however, have never been characterized to our knowledge except the study on lipid composition of *Actinomycetales* (*Nocardia polychromogenes*, *Streptomyces griseus*, *Microbisporangium chromogenes*) reported by Kataoka and Nojima.[20] They found that this group of microorganism contains three phospholipids, i.e., cardiolipin, phosphatidylethanolamine and phosphatidyl-inositolmannoside.

In the present work the author have shown that *Streptomyces sioyaensis*[4] (*Streptomycetaceae*) produces three ninhydrin-positive lipids (named substances A, B and C, tentatively) under the conditions used, whereas 25 other strains of *Actinomycetales* produce only one ninhydrin-positive phospholipid (substance A) and 5 strains produce two phospholipids (substances A and B). The present chapter deals with a comparison of the ninhydrin-positive lipids not only among various strains of *Streptomycetaceae*, but also among some of other families such as *Actinomycetaceae* (*Nocardia*) and *Actinoplananceae*

(Actinoplanes).

1. MATERIALS AND METHODS

(1) Organisms. Streptomyces sioyaensis H-690-3[4], which produces a chromopeptide antibiotic called "siomycin"[4,17,18,19], was aerobically precultured for forty-eight hours in the Bennett's medium. The medium consisted of 1.0% of glucose, 0.1% of yeast extract, 0.1% of beef extract and 0.2% of NZ-amine. pH was adjusted to 7.3 with 10% aqueous sodium hydroxide. Five millilitres of the precultured broth was transferred into 150 ml of the same medium (Sakaguchi flask), which was incubated aerobically on a reciprocating shaker at 28°C (115 reciprocations per minute, 7.0 cm amplitude). The mycelium of St. sioyaensis was harvested by centrifugation (3,000 r.p.m. for ten to fifteen minutes), before pH of the broth reached at 7.0 (4 - 5 days culture) to prevent decomposition of lipids on alkaline pH. The mycelium was then washed 3 to 4 times with deionized water and dehydrated with acetone. The washed mycelium was extracted with a mixed solvent of chloroform-methanol (2:1, v/v). The extraction was repeated several times until no more lipids were afforded. Once or twice, the mycelium was suspended in one volume of methanol and the

suspension was heated at 60 - 70°C. Then into the suspension, two volumes of chloroform was added. The mycelial suspension was stirred for two to four hours at room temperature and was filtered through Büchner funnel. In cases of the other microorganisms mycelia were also harvested before pH of the brothes reached at 7.0.

(2) Materials. Silica gel G (E.Merck, A.G., Darmstadt, Germany) was used for thin-layer chromatography without pretreatment. Phosphatidylethanolamine was purchased from Mann Res., New York, U.S.A.

(3) Detection of lipids on thin-layer chromatography. The chloroform-methanol extracts were evaporated in vacuo almost to dryness and applied on a thin-layer plate of silica gel G. The sample on the plate was developed with the solvent system of chloroform-methanol-water, 65:25:4 or 70:22:4 (ascending technique). After development, lipids were detected with ninhydrin-, and Dittmer[21] reagents, respectively. Each reagent was prepared by the way of Wagner et al.[22]

(4) Determination of phosphorus. After development of the thin-layer chromatogram, lipid sample was taken from the plate. Then it was put into a micro-kjeldahl flask with silica gel G, into which 1 ml of perchloric acid and a small piece of porous glass were added. The

flask was heated for three hours over an electric digestor. After cooling, water was added and the contents were heated again in boiling water for twenty minutes. Then they were transferred to centrifuge tubes and were centrifuged (3,000 r.p.m. for five minutes) to remove silica gel G, which was washed 2 to 3 times with deionized water. The supernatants and the washings were transferred quantitatively to a volumetric flask to equal 8 ml. After addition of 1 ml of amidol reagent and 1 ml of ammonium molybdate reagent[23], optical density of the color developed was measured at 830 mμ.

2. RESULTS

(1) Strains in Actinomycetales. The distribution of the three ninhydrin-positive lipids was studied with 31 strains in Actinomycetales. Table I lists the strains tested together with antibiotics produced and morphological properties. Amount of each lipid based on phosphorus content was also shown in Table I, which shows that only St. sioyaensis among 31 strains (3%) contains three ninhydrin-positive lipids and that 5 strains (16%) contain two ninhydrin-positive lipids, while many other strains (81%) contain only one ninhydrin-positive lipid. The result is summarized in two ways in Table II. In the

TABLE I DISTRIBUTION OF NINHYDRIN-POSITIVE PHOSPHOLIPIDS AMONG STRAINS IN ACTINOMYCETALES

	Strains tested	Antibiotic produced	Morphological properties	Ninhydrin-positive phospholipid (mg P/g cell)		
				A	B	C
1.	*St. griseus*	streptomycin	straight	375		
2.	*St. virginiae*	cycloserine	straight	209		
3.	*St. albiverticuli*	?	whorl	78		
4.	*St. toyocaensis*	toyocamycin	spiral	36	190	
5.	*St. showdoensis* C-224	showdomycin	straight	158		
6.	*St. showdoensis* C-209-A	showdomycin	N.A.M.*	121		
7.	*St. sioyaensis*	siomycin	spiral	162	272	++
8.	*St. aburaviensis*	aburamycin	straight	53		
9.	*St. minoensis* 1-523	minomycin	spiral	132		
10.	*St. erythreus* C-233-6	erythromycin	spiral	140		
11.	*St.* M-88	M-88 substances	whorl	22		
12.	*St. bostroemi*	?	Nocardia type	89		
13.	*Nocardia asteroides*	?		40		
14.	*Nocardia erythropolis*	?		82		
15.	*Actinoplanes philippinensis*	?	N.A.M.*	53		
16.	*Micromonospora* T-12	T-12 substance	N.A.M.*	197		
17.	*St. orchidaceus*	cycloserine	straight	435		
18.	*St. echinatus*	echinomycin	spiral	68		
19.	*St. netropsis*	netropsin	whorl	148		
20.	*St.* G-193	actinomycin C	straight	629	159	
21.	*St.* N-329	nonaction & valinomycin	straight	585	140	
22.	*St.* N-946	amphomycin	straight	94		
23.	*St.* N-58	rufomycin	straight	++	++	
24.	*St.* Z-242	amphomycin	straight	355		
25.	*St. rubrireticuli*		whorl	160		
26.	*St. reticuli*		whorl	573		
27.	*St. luteoverticillatus*		whorl	185		
28.	*St. olivoverticillatus*		whorl	204	124	
29.	*St. thioluteus*		whorl	289		
30.	*St. roseoverticillatus*	?	whorl	37		
31.	*St. erythreus* C-233-4	erythromycin	spiral	233		

* N.A.M.=no aerial mycelium.
++=not estimated, though detected with ninhydrin reagent.

Table II. Distribution of Ninhydrin-Positive Phospholipids (Substances A, B, C) among Various Strains in Actinomycetales

Morphological group	Total strain	Number of strains which contain phospholipid		
		A	A & B	A,B, & C
Streptomyces				
straight	11	8	3	0
spiral	6	4	1	1
whorl	10	8	1	0
Nocardia	2	2	0	0
Micromonospora	1	1	0	0
Actinoplanes	1	1	0	0
Total	31	25	5	1
		(81%)	(16%)	(3%)
Peptide antibiotic-producing strain	9	5	3	1
Others	22	20	2	0
Total	31	25	5	1

upper part, distribution of ninhydrin-positive lipids is presented from the morphological point of strains, but not any remarkable relationship is seen between the lipid

content and morphological properties. On the other hand, in the lower part, peptide-antibiotic-producing strains seem to have a tendency to contain more than one ninhydrin-positive lipid (4 out of 9 strains; 44%) while most of the other strains do not seem to have the tendency (2 out of 22 strains; 9%). Final conclusion about their relationship requires further work and remains to be elucidated.

(2) Streptomyces sioyaensis and its auxotrophs

(a) Detection of ninhydrin-positive lipid. The mycelium was extracted with chloroform-methanol (2:1, v/v). The extracts were concentrated and subjected to a thin-layer chromatography. Various detection tests were done to see what kinds of lipid were contained (Fig. 1). Among several spots of lipid found on thin-layer chromatogram, substances A,B and C were of interest. As they were positive for ninhydrin (amino group)-and rhodamine (lipid)-reagents at the same time, they might be lipids combined with amino acid or at least lipids with amino group.

(b) Amino acid-requiring mutants of St. sioyaensis. There were four mutants of St. sioyaensis which required lysine, histidine, arginine or trypophan, respectively, for their growth. Of particular interest was the lysine-requiring mutant which could not biosynthesize lysine in

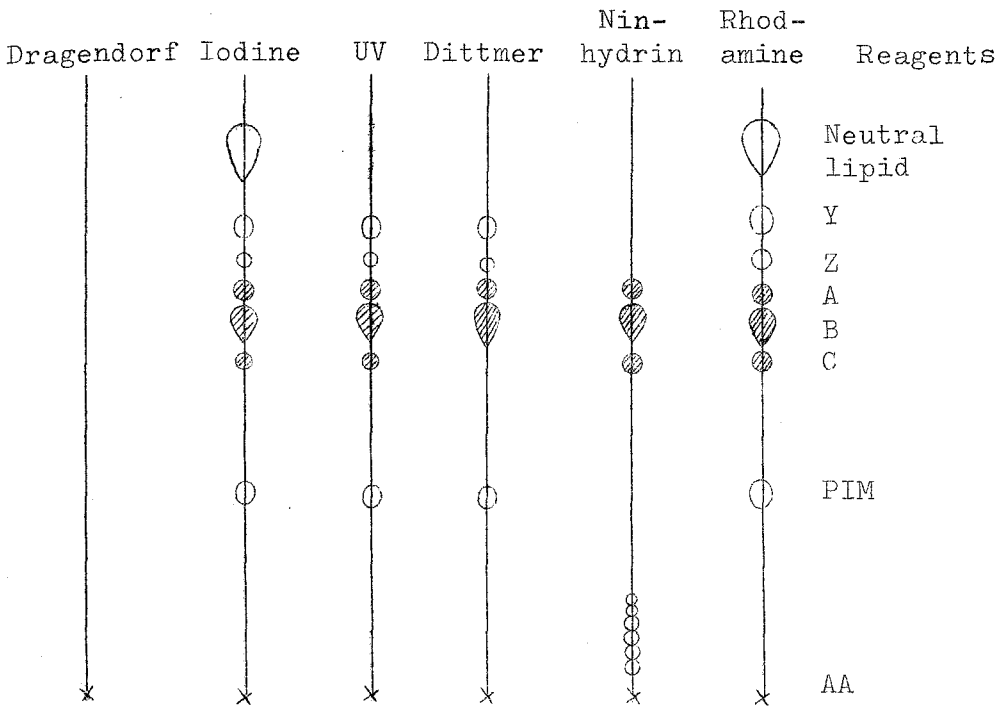


Fig. 1. Detection Test of Lipids from St. sioyaensis on Thin-Layer Chromatogram.

Solvent system; $CHCl_3/MeOH/H_2O$=65:25:4. Y=cardiolipin, Z=unidentified, A,B = phosphatidylethanolamine, C = a new lysine-containing lipid, PIM = phosphatidylinositolmannoside, AA = free amino acids.

itself. As one of the three lipids described above (substance C) was proved to be a new lysine-containing lipid, the mutant was considered to lack the substance C. Contrary to the expectation, the lysine-requiring mutant

contained three ninhydrin-positive lipids as its prototroph did, and it indicated the possibility that lysine of the medium was incorporated directly into substance C.

3. DISCUSSION

In 1962 MacFarlane[24] reported the presence of O-amino acid esters of phosphatidylglycerol in Clostridium welchii. Gale[25] and van Deenen[26] also recognized lysyl-phosphatidyl glycerol in Staphylococcus aureus. The author was interested in these lipoamino acids in which amino acid and lipid are combined. In his studies on St. sioyaensis, three ninhydrin-positive lipids (substances A, B and C) were found. The study on distribution of these lipids showed that only St. sioyaensis out of 31 strains contained three ninhydrin-positive lipids and that 5 out of 31 strains contained two ninhydrin-positive lipids corresponding to substances A and B from St. sioyaensis. Not any spot corresponding to substance C was found in all strains investigated, although it was commonly found in four mutants of St. sioyaensis.

As the lipid pattern of St. sioyaensis was found quite interesting, the individual lipids were isolated and characterized. Substances A and B were found to contain ethanolamine as ninhydrin-positive part, and

substance C was found to contain lysine. Further investigation revealed that the substances A and B were two phosphatidylethanolamines with different fatty acid composition, and that the substance C was a new lysine-containing lipid. At first substance C was thought to be a lysyl-phosphatidyl glycerol described above. But IR-absorption spectrum of substance C proved it to be different from lysyl-phosphatidyl glycerol.

CHAPTER II. LIPID COMPOSITION OF *STREPTOMYCES SIOYAENSIS*

The pattern of ninhydrin-positive lipids of *Streptomyces sioyaensis* was shown to be quite different from those of the other strains of *Actinomycetales*. The lipids of *St. sioyaensis* contained three ninhydrin-positive lipids which were designated as substances A, B and C in the decreasing order of R_f values on thin-layer chromatography. Substance C was proved to be a new lysine-containing lipid. The present chapter deals with the isolation and identification of lipids from *Streptomyces sioyaensis*.

1. MATERIALS AND METHODS

(1) Organism. *Streptomyces sioyaensis* H-690-3 was used throughout this study. It was precultured in Bennett's medium containing 1.0% of glucose, 0.1% of yeast extract, 0.1% of beef extract and 0.2% of NZ-amine (the pH was adjusted to 7.2 with 10% NaOH aqueous solution). Five ml of precultured broth was inoculated into Sakaguchi flasks containing the same medium. The flasks were incubated at 28°C for 4 to 5 days on a reciprocal shaker (115 reciprocations per minute, 7.0 cm amplitude). A jar

fermenter was operated as follows: temperature, 28°C; aeration, 1.0 v/v/m (20 liters per minute); agitation, 150 r.p.m.; inner pressure, 0.5 kg/cm^2.

(2) Materials. Silicic acid (Mallinckrodt, 100 mesh) for column chromatography was processed as described by Rouser[27]. Diethylaminoethyl (DEAE)-cellulose (Serva 0.76 meq./g) was used in the acetate form according to Rouser's method[28]. Silica gel G and H (E. Merck, AG, Darmstadt, Germany) for thin-layer chromatography were used without pretreatment. Phosphatidylethanolamine and cardiolipin were prepared from tumor tissues[29] and bovine heart,[30] respectively. Phosphatidylglycerol was prepared from spinach leaves according to the method of van Deenen[31]. Methyl esters of branched chain fatty acids used as standards for gas chromatography were obtained from Applied Science Lab. Inc., State College, Pa., U.S.A.

(3) Preparation of lipids. After harvest by centrifugation, the mycelia were washed with deionized water, dehydrated with acetone, and extracted with chloroform-methanol (CM 2:1, v/v). Extraction was repeated several times at room temperature until no more lipids were detected in the solvent layer. The mycelia were finally extracted at 70°C. Extracts were dried <u>in vacuo</u>, and

subjected to Folch's partition dialysis[32] to remove water-soluble substances. Per cent recovery by Folch's procedure was almost 100%. The chloroform layer was concentrated to 100 ml and treated with 20ml of an aqueous solution of 1% $CaCl_2$ to prepare the calcium salt. After standing overnight at room temperature, the chloroform layer was separated and evaporated to dryness.

(4) Detection of individual lipids. The extracted lipid fraction was applied to a thin-layer plate of silica gel G and developed with chloroform-methanol-water (65:25:4 or 70:22:2, v/v/v) as solvent system. Individual lipids were detected by Dittmer reagent[21] and the ninhydrin reagent.

(5) Mild alkaline hydrolysis. The deacylated product of lipid was obtained by Dawson's method.[33] The hydrolysis products thus obtained were compared with those of authentic lipids by paper chromatography (phenol/water/acetic acid/ethanol=80:20:10:12, v/v/v/v; i-propanol/NH_4OH=3:1, v/v).

(6) Silicic acid column chromatography. Total phospholipid (300 to 350 mg) was applied to a silicic acid column (20g) and elution was carried out with chloroform-methanol. The concentration of methanol in chloroform was increased stepwise. One-ml aliquots were removed

from each fraction (10g) and used for the ninhydrin reaction and for phosphorus determination.

(7) Gas-liquid chromatography. The methyl esters of fatty acids were prepared by transesterification of lipids with boron trifluoride-methanol.[34]

The phosphatidylinositolmannoside fraction (Fraction IV) was methanolyzed in a sealed tube with 5% HCl in methanol at 105°C overnight. The glycerol, inositol and mannose in the methanolyzate were converted to trimethylsilyl derivatives and detected by gas-liquid chromatography with a Shimadzu Gas Chromatograph IB equipped with a hydrogen flame ionization detector. Separation was made under the following conditions: (a) on a 3-m stainless steel column packed with 15% diethyleneglycol succinate polyester on Chromosorb W (acid washed, siliconized) at 190°C, for the methyl esters of fatty acids; and (b) on a 1.5-m stainless steel column packed with 5% SE-52 on Chamelite CS at 214°C according to the methods of Sweeley[35] for trimethylsilyl derivatives of methylglycosides.

(8) Analytical methods. Melting point was determined on a Kofler block (Yanagimoto micro-melting point apparatus) without correction. Optical rotation was

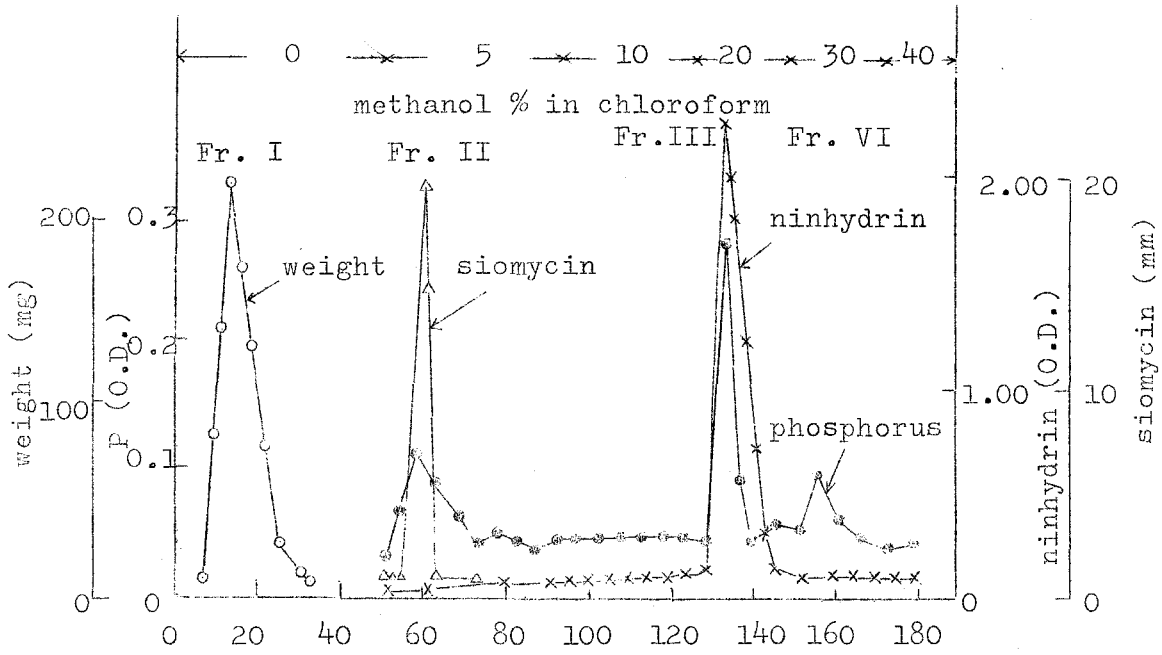


Fig. 2. Separation of Lipids on Silicic Acid Column Chromatography.

measured in chloroform with a Rudolf Photoelectronic Polarimeter, model 200, and infrared absorption spectrum was taken in chloroform and on KBr pellets with a Koken IR spectrophotometer, model 301. Phosphorus was estimated by the method of Allen.[23] Ninhydrin-positive materials, ethanolamine and lysine, were determined by paper chromatography (n-butanol/acetic acid/water = 4:1:2, v/v/v) and by an automatic amino acid analyzer (Hitachi

Table III. Separation of Lipids on Silicic Acid Column Chromatography

Fraction	Weight (mg)	Phosphorus (μg)	Ninhydrin	Hexose	Antibacterial activity	Components
I	2105.5	-	-	-	-	Neutral lipids (triglyceride)
II	159.6	2500	-	-	+	Cardiolipin, Substance Z, Siomycin
III	42.6	750	+	-	-	PE* (A and B), Substance C
IV	42.6	460	-	+	-	PIM**

* PE = Phosphatidylethanolamine. ** PIM = Phosphatidylinositolmannoside.

KLA-2). Amino-nitrogen was estimated by the method of Moore and Stein[36]. Fatty acids were estimated as esters by the method of Rapport,[37] in which egg-yolk lecithin was used as a standard. Siomycin[4,17,18] was estimated by pulp disc diffusion method against Bacillus subtilis PCI-219.

2. RESULTS

(1) Separation and Identification of Individual Lipids.

The lipid fraction of St. sioyaensis prepared as described above was subjected to silicic acid column chromatography, and separated into four fractions (Fig. 2,

Table III).

Fraction I. Neutral lipids. Fraction I was eluted with chloroform from the silicic acid column. It gave main spot with an R_f value identical to that of tristearian and other minor spots on a thin-layer chromatogram (n-hexane/diethyl ether/acetic acid = 6:4:0.1, v/v/v).

Fraction II. Substance Y (cardiolipin) and Substance Z. Substances Y,Z and siomycin[4,17,18,19] were eluted with CM (95:5) from the silicic acid column. They were separated by thin-layer chromatography, and each of them was eluted from the plate. Although substance Z could not be identified, substance Y was identified to be cardiolipin. The ratio of fatty acid to phosphorus in substance Y was 2.0 (Table IV). The deacylated product of substance Y gave a spot with the same R_f value as deacylated cardiolipin by paper chromatography. Substance Z was found only in trace and appeared to be a decomposed substance of cardiolipin. Its deacylated product behaved like phosphatide II of Coulon-Morelec[38] on paper chromatography. The ratio of fatty acid to phosphorus in substance Z was 1.9 (Table IV).

Fraction III. Three ninhydrin-positive lipids. Fraction III eluted with CM (80:20) from the silicic

Table IV. Molar Ratio of Fatty Acid and Phosphorus

Substances	Fatty acid/phosphorus
Lecithin	2.0 (as standard)
Substance Y (cardiolipin)	2.0
Substance Z (unidentified)	1.9
Substance A (PE*)	2.1
Substance B (PE*)	2.0
Substance C (a new lipid)	——
PIM**	1.9
Cardiolipin (bovine heart)	1.8

* PE = phosphatidylethanolamine.

** PIM = phosphatidylinositolmannoside.

acid column contained three ninhydrin-positive lipids designated as substances A, B and C. Substance C was rechromatographed and successfully separated from a mixture of A, B and C by elution with different concentrations of methanol in chloroform. Substance C was eluted with CM (87:13), whereas substances A and B were eluted with CM (90:10). Substance C fraction was chromatographed again over Florisil column to remove concomitant substance B (phosphatidylethanolamine). Substance C was eluted with CM (50:50 to 20:80) from the Florisil column, which

decomposed some amount of substance C. Fraction of substance C thus obtained was again chromatographed on silicic acid column to remove the decomposed substance. The substance C thus purified was submitted to chemical analyses. Substances A and B were separated from each other on preparative thin-layer chromatography with silica gel H and extracted with CM (2:1) from the area where they were detected by iodine vapor. The separated substances A and B were purified by repeated chromatography on silicic acid column, respectively. When acid hydrolysis with 6N HCl was performed at 105°C for 12 hours, paper chromatography and analysis with an automatic amino acid analyzer revealed that substances A and B contained only ethanolamine as the ninhydrin-positive component, whereas substance C contained only lysine (Fig. 3). Thin-layer chromatography showed that authentic phosphatidylethanolamine was quite similar to substance A from St. sioyaensis. Substances A and B were subjected to mild alkaline hydrolysis.

The deacylated products of purified substance A, purified substance B, and a mixed sample of substances A and B gave one spot, respectively, on paper chromatography, the R_f values of which were identical with that of deacylated

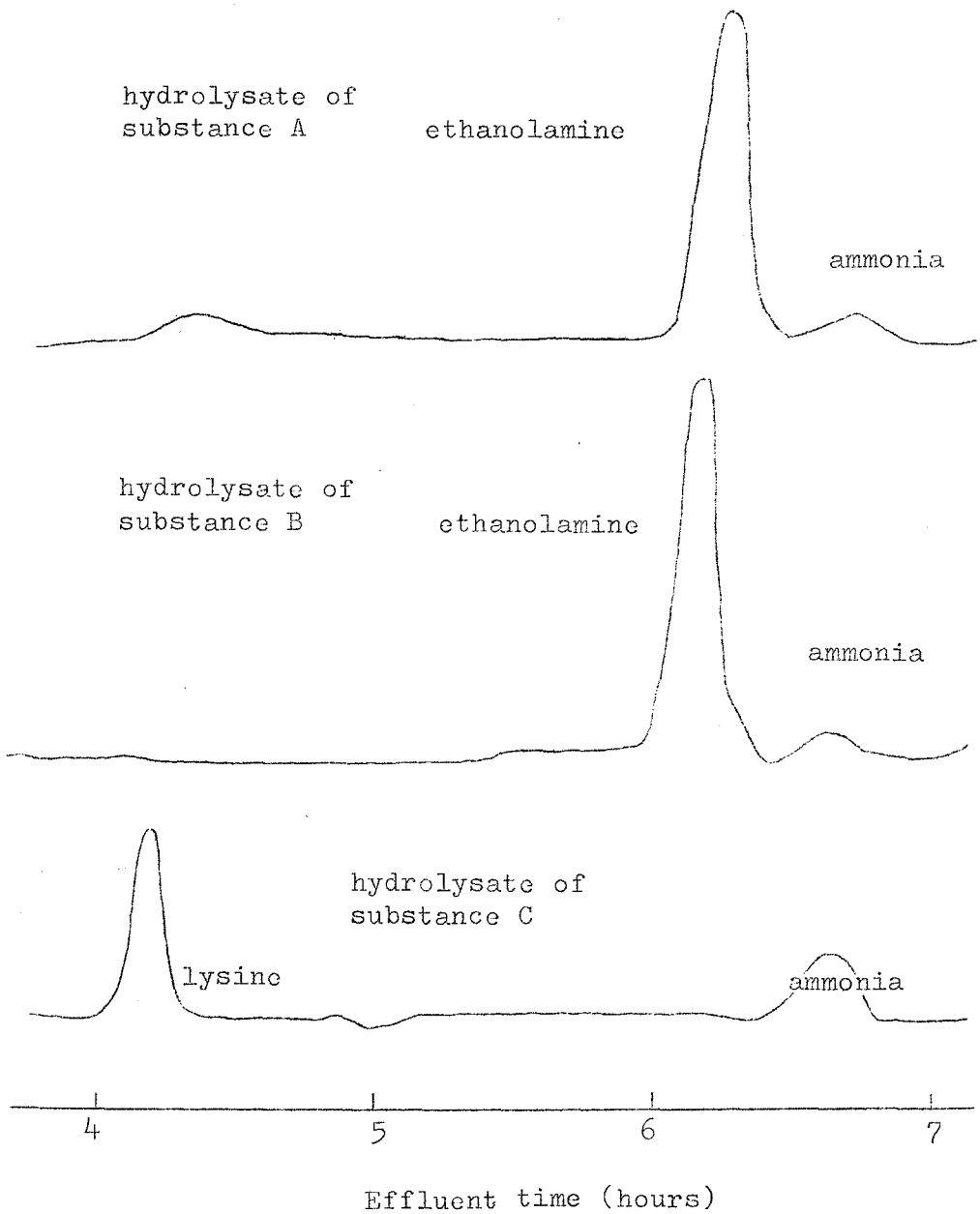


Fig. 3. Analyses of Hydrolysates of Ninhydrin-Positive Substances with Amino Acid Analyzer.

phosphatidylethanolamine. The ratio of fatty acid to phosphorus was 2.1 for substance A, and 2.0 for substance B (Table IV). These results suggested that substances A and B had the same skeleton, and that they were two kinds of phosphatidylethanolamine with a different fatty acid composition (Figs. 5, 6).

Substance C contained lysine as described above (Fig. 3), but it did not possess phosphorus in its molecule. Its physicochemical properties will be described in chapter IV. The infrared absorption spectrum of the substance is shown in Fig. 11. It has characteristic absorptions of the amide group at 1643, and of the primary amine at 1596 cm^{-1} which were not found in the IR-spectrum of lysyl-phosphatidyl glycerol reported by MacFarlane.[24)]

Fraction IV. Phosphatidylinositolmonomannoside (PIM). A lipid separated from fraction IV was hydrolysed with 5% HCl in methanol at 105°C overnight. After removal of the fatty acid methyl esters, the hydrolysates were led to trimethylsilyl derivatives and analysed by gas-liquid chromatography as shown in Fig. 4, in which inositol and mannose were found in the ratio of 1:1. The ratio of fatty acid/phosphorus/hexose (as mannose) was 2:1:1. Therefore, it contained fatty acid, phosphorus, inositol and mannose in the molar ratio of 2:1:1:1.

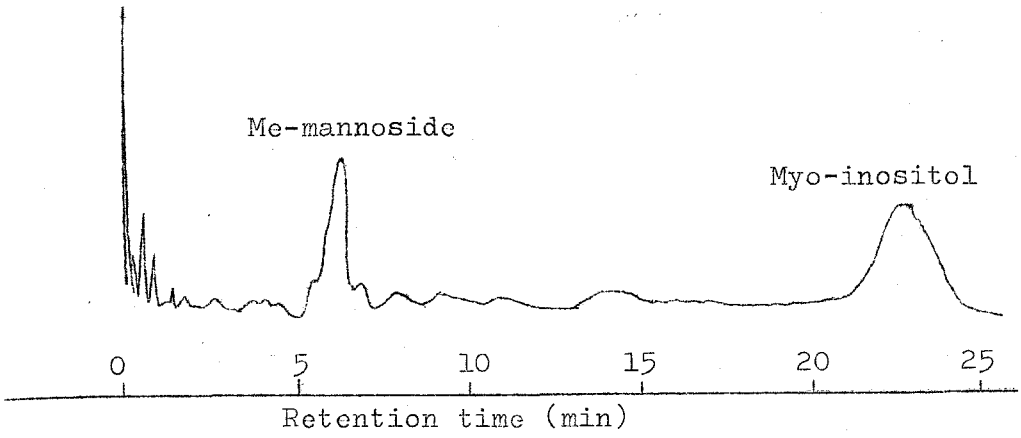


Fig. 4. Gas-Chromatographic Analysis of Phosphatidylinositolmannoside from Streptomyces sioyaensis.

Condition; 5% SE-52 on Chamelite CS, at 180°C.

(2) Fatty acid composition. The fatty acid composition of each lipid was determined by gas-liquid chromatography and the results are shown in Figs. 5 and 6. The patterns were similar with each other. Hydrogenation of these fatty acids did not cause any remarkable changes in their patterns, indicating that these fatty acids were saturated. Analysis revealed that these fatty acids were branched ones, so-called iso- and anteiso-acids which were generally found in the lipids of microorganisms. The fatty acid compositions of substances A and B were quantitatively

different from each other. Individual fatty acid methyl esters from the neutral lipids were separated by an Aerograph Autoprep A-700 (25% DEGS on chromosorb W, at 190°C, flow rate 100ml He/min.). They were then subjected to an identification of structure with a Hitachi RMU-6 Mass Spectrometer (ionizing voltage; 80V) which gave M minus 15($M-CH_3$) and M minus 65($M-CH_3OH-H_2O-CH_3$) peaks characteristic of iso-fatty acids.[39] As a result, it was verified that fatty acid methyl esters from the neutral lipids consisted of an iso-14, an iso-15 and an iso-16 fatty acid. Besides them an anteiso-17 fatty acid was contained, though it was not subjected to Mass Spectrometer.

3. DISCUSSION

St. sioyaensis contained three ninhydrin-positive substances (substances A, B and C), whereas many other strains contained only one ninhydrin-positive substance, i.e., phosphatidylethanolamine. The interest in the lipid composition of this microorganism led the author to the separation of each lipid. The lipid of St. sioyaensis was separated by silicic acid column chromatography into four fractions.

Fraction I contained neutral lipids. Fraction II

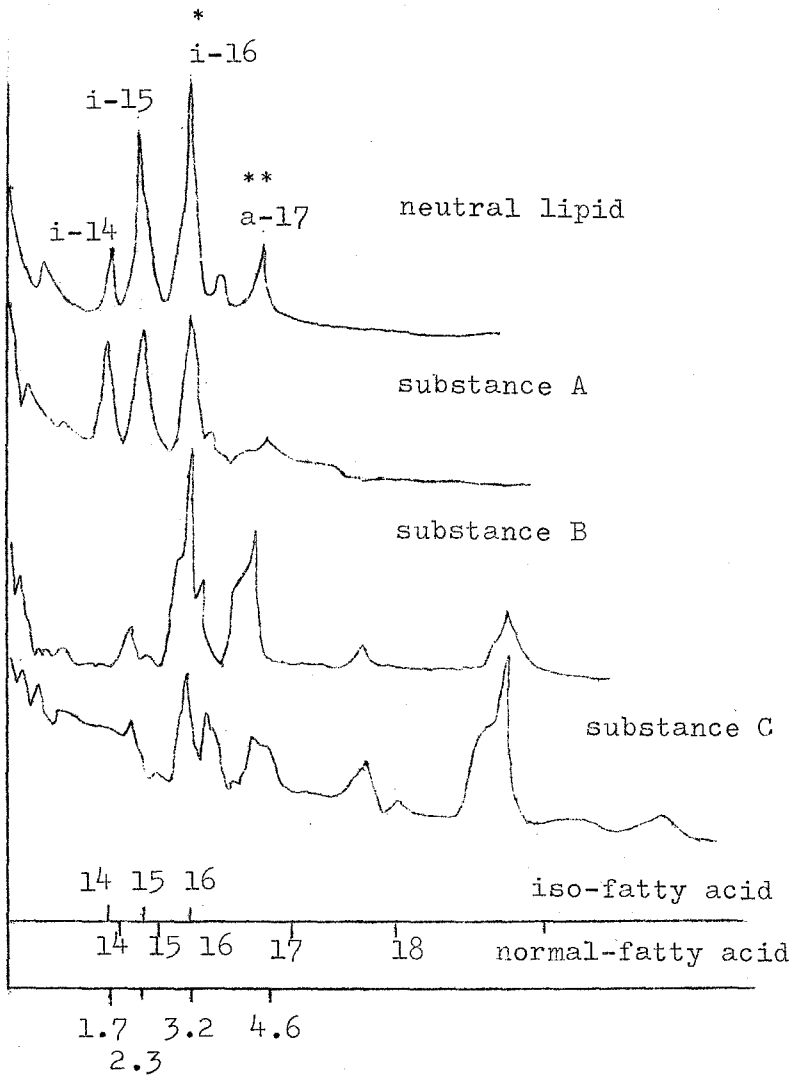


Fig. 5. Fatty Acid Composition of Lipids from St. sioyaensis.

* i= iso-fatty acid.

** a=anteiso-fatty acid.

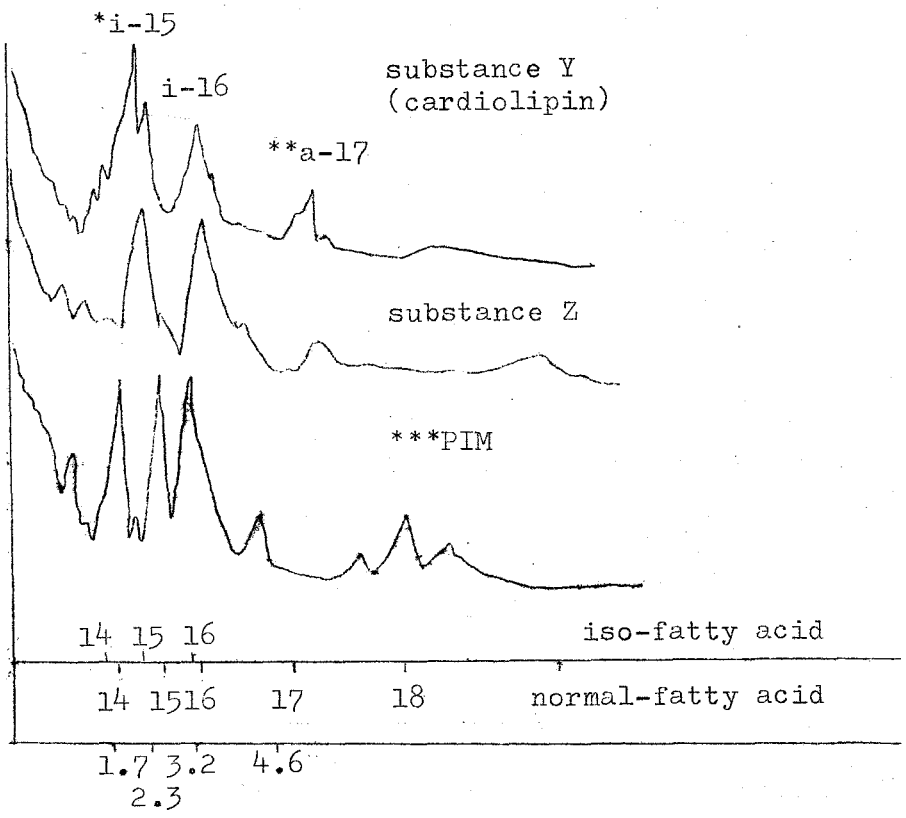


Fig. 6. Fatty Acid Composition of Lipids from St. sioyaensis.

* i = iso-fatty acid.

** a = anteiso-fatty acid.

*** PIM = phosphatidylinositolmonomannoside.

contained cardiolipin, substance Z, and siomycin which is a chromopeptide antibiotic produced by this microorganism. Fraction III gave three ninhydrin-positive spots by thin-layer chromatography, two of which (substances A and B) were proved to be phosphatidylethanolamines and another one (substance C) was shown to be a new lysine-containing lipid. Fraction IV contained phosphatidylinositolmono-mannoside.

Substances A and B were found to be two types of phosphatidylethanolamine with different fatty acid composition.

Substance C containing lysine appeared, at first, to be similar to lysyl-phosphatidyl glycerol reported by MacFarlane[24] and other workers,[25,26] but it did not contain phosphorus. The IR-spectrum of substance C showed the presence of amide linkage (Fig. 11) which was not found in the spectrum of lysyl-phosphatidyl glycerol.[24] The structure and biological activities of substance C will be described in other chapters.

Chapter III. HYDROXY FATTY ACID FROM PHOSPHATIDYL-ETHANOLAMINE

Phosphatidylethanolamine from St. sioyaensis afforded a double spot on a thin-layer chromatogram, typical of most glycosphingolipids from animal tissues. They were phosphatidylethanolamines, one of which had only non-hydroxylated fatty acids and the other hydroxy fatty acids in addition to non-hydroxylated fatty acids, respectively.

The distribution of the fatty acids was studied by hydrolysis with snake venom phospholipase A (E.C.3.1.1.4.). Hydroxy fatty acids were located in the β-position of the glycerol moiety, differing from the results for Brucella abortus[50] phospholipids in which location in the α-position has been reported.

The main hydroxy fatty acid was purified by preparative gas-liquid chromatography. The structure of the hydroxy fatty acid was analyzed by oxidation with lead tetraacetate, proton magnetic resonance, and mass spectrometry, etc. From these results, it was assumed that the main acid was 2-hydroxy-13-methyltetradecanoic acid.

1. Materials and methods

(1) Reference hydroxy fatty acids

α-hydroxy stearic and α-hydroxy-hexacosanoic acid, and 12-hydroxy stearic acid were purchased from Cabiochem (Los Angeles, U.S.A.) and purified by column chromatography. Pure 10-hydroxy stearic acid[40] was a kind gift of Dr. K. Saito (Kansai Medical School, Osaka).

(2) Phosphatidylethanolamine

This lipid had been isolated from St.sioyaensis as described previously. The phosphatidylethanolamine moved as a double spot on thin-layer chromatograms, from which the upper and the lower spots were scraped off separately.

(3) Hydrolysis by phospholipase A

About 110 mg of the phosphatidylethanolamine was dissolved in 10 ml of freshly distilled ether. Twenty mg of snake venom (Habu, Trimeresurus flavoviridis, Hallowell) in 10 ml of 0.1 M borate buffer (pH 7.0) and 0.25 ml of 0.0025 M calcium acetate were added to this ethereal solution. This mixture was shaken for 5 hr, with the aid of a shaker (Taiyo incubator K-II). Thin-layer chromatography coated with silica gel G was used to establish if degradation was complete. The resultant lyso-phosphatidylethanolamine and fatty acids were separated by thin-layer chromatography on silica plates (20 X 20 cm), or by silicic acid column chromatography,

using chloroform-methanol-water (70:20:5, v/v/v) as a deve developer.

(4) Hydroxy fatty acids

It was revealed by thin-layer chromatograpny that the fatty acids released by the hydrolysis of the lower phosphatidylethanolamine with snake venom phospholipase A were composed of both non-hydroxylated and hydroxylated fatty acids. Thin-layer chromatography was performed with dichloromethane as a developer.[41] The bands corresponding to the authentic methyl α-hydroxy stearate (Calbiochem) were visualized after being sprayed with water, scraped off from the chromatoplate and extracted immediately with chloroform. The methyl esters of the hydroxy fatty acids obtained were analyzed by gas-liquid chromatography and were found to consist of one main peak and two minor peaks. Accordingly, this mixture was further purified by preparative gas-liquid chromatography (an Aerograph Autoprep. A-700) to give one major peak. The methyl ester of the hydroxy fatty acid thus purified was analyzed by thin-layer chromatography, infrared spectroscopy, mass spectroscopy and so on.

(5) Analytical methods

Optical rotation of the methyl ester of the hydroxy

acid was measured in chloroform with a Perkin-Elmer Polarimeter, type 141. An infrared spectrum was taken in CCl_4 with a Nihon Bunko DS-201 B Spectrophotometer. Proton magnetic resonance spectra were measured in carbon tetrachloride, containing tetramethylsilane as an internal standard using a Varian Model A-60 Spectrometer.

(6) Mass spectroscopy (Y.N.)

Mass spectra were obtained with a Hitachi RMU 6E single focus mass spectrometer with an energy of 70 eV and 80 μamp, using a direct inlet system at 250°C. The sample treated with deuterium oxide was introduced in a slurry.

(7) Gas liquid chromatography

The methyl esters of the released fatty acids were prepared by treatment with diazomethane in ether. The methyl esters of the fatty acids from lyso-phosphatidyl-ethanolamine were prepared by transesterification with hydrochloric acid in methanol. The methyl esters were analyzed by means of a Shimadzu gas chromatograph, model 1B, equipped with a flame ionization detection system and 15% polydiethyleneglycol succinate column as previously described[5, 42]. The fatty acids were identified by comparison with the retention times of standards. Preparative gas-liquid chromatography was accomplished by an

Aerograph Autoprep, A-700, using 25% polydiethyleneglycol succinate on a chromosorb W column, at 190°C, flow rate 100 ml He/min.

(8) Oxidation with lead tetraacetate

Treatment of the hydroxy acid (10 mg) with lead tetraacetate (16.3 mg) in glacial acetic acid (2 ml) at 50°C for 5 hr resulted in the formation of an aldehyde and a carboxylic acid. The aldehyde was isolated from the neutral fraction, while the carboxylic acid was extracted from the basic fraction after acidification with hydrochloric acid, respectively. The aldehyde and the methyl ester of the carboxylic acid were subjected to gas-liquid chromatography.

2. RESULTS

In chapter I and II, it was reported that the phosphatidylethanolamine of Streptomyces sioyaensis was composed of two groups which afforded a double spot on a thin-layer chromatogram as in the glycolipids from animal tissues. It was previously assumed that the upper spot has shorter chain fatty acids and the lower spot longer ones[5].

This chapter describes a more detailed study on the fatty acid moiety of the phosphatidylethanolamine from

St. sioyaensis. The cephalin fraction was separated from other lipid classes by DEAE-cellulose or silicic acid column chromatography. The cephalin fraction was further separated into each phosphatidylethanolamine by preparative thin-layer chromatography. Each phosphatidylethanolamine was hydrolysed separately with 5% methanolic hydrochloric acid to give methyl esters of the component fatty acids. Each fatty acid methyl ester obtained was analyzed by thin-layer chromatography, respectively, using dichloromethane as a developer[41] and by gas-liquid chromatography (Fig. 5), in which the lower phosphatidylethanolamine contained acids giving rise to shifts in the retention times after trimethylsilylation[42], indicating that they were hydroxy fatty acids. Accordingly, it can be stated that the less polar (upper) phosphatidylethanolamine has only non-hydroxylated fatty acids, while the more polar (lower) one has monohydroxy fatty acids in addition to a series of non-hydroxylated fatty acids. Such a finding has often been observed in the glycosphingolipids from animal organs[42], but no information was available for bacterial phospholipids. On the other hand, it was found that the hydroxy fatty acids in the more polar phosphatidylethanolamine were located in the β-position of the

glycerol moiety by a study of hydrolysis[43] with snake venom phospholipase A (E.C. 3.1.1.4.).

Furthermore, in order to elucidate the structure of the hydroxy fatty acids, they were purified as follows: after the removal of siolipin A[5] from the cephalin fraction, a mixture of phosphatidylethanolamine were subjected to hydrolysis with methanolic hydrochloric acid. The methyl esters of the hydroxy fatty acids were separated from the methyl esters of the non-hydroxylated fatty acids by silicic acid column chromatography[44]. It was revealed by gas-liquid chromatography that the methyl esters of the hydroxy fatty acids were composed of a main acid and two minor ones. As shown in Fig. 7, the retention times of these methyl esters are on a straight line drawn through the retention times of the methyl esters of saturated straight chain α-hydroxy fatty acids. This fraction was further purified by preparative gas-liquid chromatography to give one peak. The methyl ester of the hydroxy acid thus purified had the following physico-chemical properties; $[\alpha]_D^{22} = -3.2 \pm 0.5$ (C, 0.805 in chloroform). Anal. found: C, 70.50; H, 11.74. Calcd. for $C_{16}H_{32}O_3$, C, 70.59; H, 11.76%. It was analyzed by a variety of techniques.(1) As shown in Fig. 8, its infrared spectrum indicated this methyl ester

of a hydroxy fatty acid. (2) Gas-liquid chromatographic analysis indicated that the methyl ester had a retention time corresponding to that of the methyl ester of α-hydroxy pentadecanoic acid. (3) This methyl ester corresponded to the methyl 2-hydroxy stearate, but not the

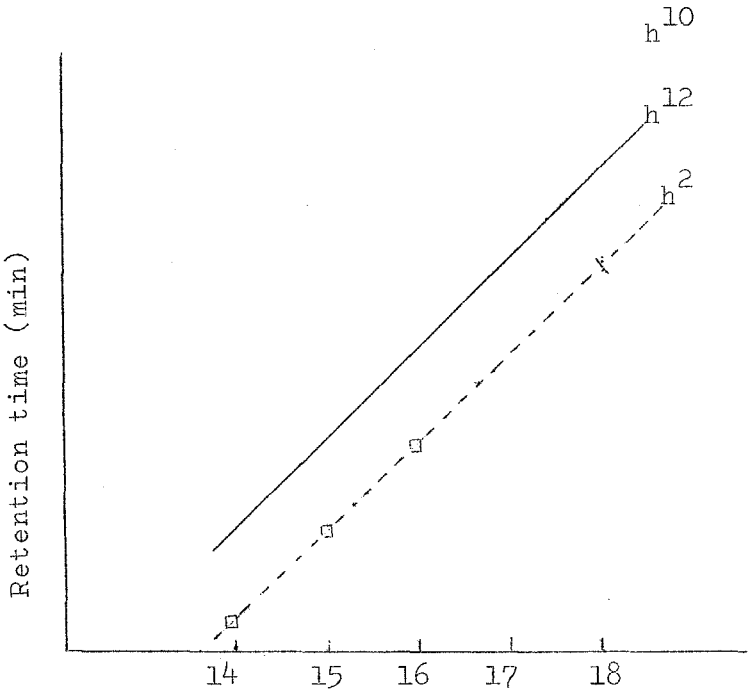


Fig. 7. Relation between Carbon Chain Length and Retention Times of Methyl Esters of Hydroxy fatty acids.

——·—— Methyl esters of 10- and 12- hydroxy fatty acids.

--- □ --- Methyl esters of the hydroxy fatty acids from the phosphatidylethanolamine.

---×--- Methyl esters of normal α-hydroxy fatty acids.

methyl 10- and 12-hydroxy stearate on the basis of its mobility on thin-layer chromatography. (4) Although the reaction of this acid with copper nitrate[45] afforded a white precipitate, this reaction was not specific for α-hydroxy fatty acids in the control experiment. (5) treatment of this hydroxy acid with lead tetraacetate gave a peak corresponding to that of a branched (iso or anteiso) tetradecanal by gas-liquid chromatography in which the retention time of this aldehyde was on a straight line almost parallel but lower than the line drawn through the retention times of the saturated straight chain aldehyde. (6) A mass spectrum of the methyl ester of this hydroxy fatty acid is shown in Fig. 9, together with that of the ester treated with deuterium oxide. These spectra are consistent with that of a methyl α-hydroxy pentadecanoate as follows: the molecular ion peak at m/e 272 corresponds to $C_{16}H_{32}O_3$ which correlates with the result by elementary analysis. This molecular ion peak was somewhat larger than those of the methyl esters of normal fatty acids and of such hydroxy fatty acids as methyl 10- and 12-hydroxy stearate. Relatively intense peaks were observed at m/e 90 (89+1) and 213 (M-59), characteristic for the methyl esters of α-hydroxy acids.[46,47]

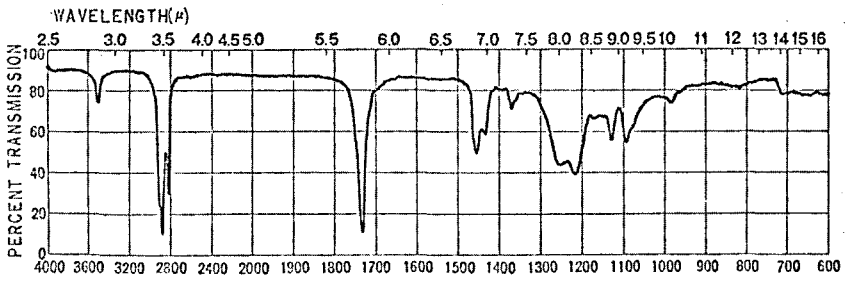


Fig. 8 Infra-red spectrum of the purified methyl ester of the hydroxy fatty acid from the phosphatidylethanolamine.

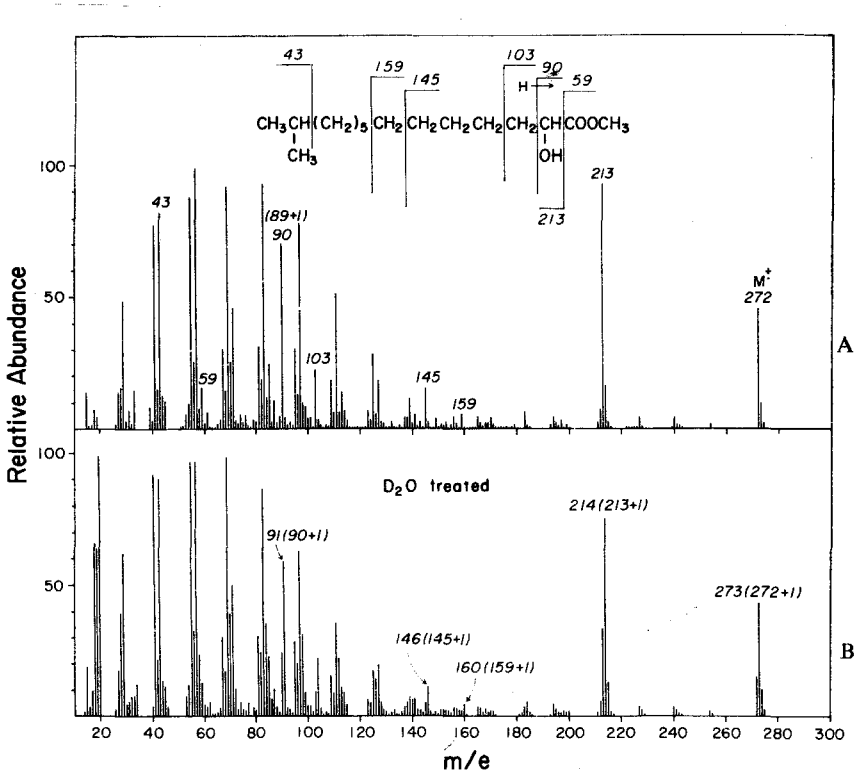
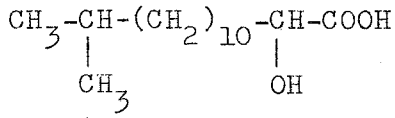


Fig. 9 Mass spectra of methyl ester of the hydroxy fatty acid obtained from the phosphatidylethanolamine. A, Methyl ester of the hydroxy fatty acid. B, Methyl ester of the deuteroxy fatty acid.

In addition, relatively intense peaks were also observed at m/e 145 and 159, appearing to give rise in an analogous way through simple 6,7 and 7,8 cleavage, respectively. Other peaks were very similar to those from the isostearoyl alcohol[47]. As shown in Fig. 9 B, the spectrum of the deuterated compound showed peaks owing to deuterium exchange of the active hydrogen. However, mass spectrometry could not reveal the position of the methyl branching in the hydroxy acid, while the methyl esters of non-hydroxylated iso fatty acids gave a peak at m/e (M-65) due to the loss of ($CH_3OH + CH_3 + H_2O$). Hence, a study by proton magnetic resonance spectrometry was undertaken. As seen in Fig. 10, it showed the presence of a doublet signal (J= 7 cps, 6H) at 9.14 τ due to an isopropyl group.

From these results, it is most probable that this hydroxy fatty acid is 2-hydroxy-13-methyl-tetradecanoic acid.



3. DISCUSSION

The overall fatty acid composition of the phosphatidyl-ethanolamine from St. Sioyaensis was already reported[5].

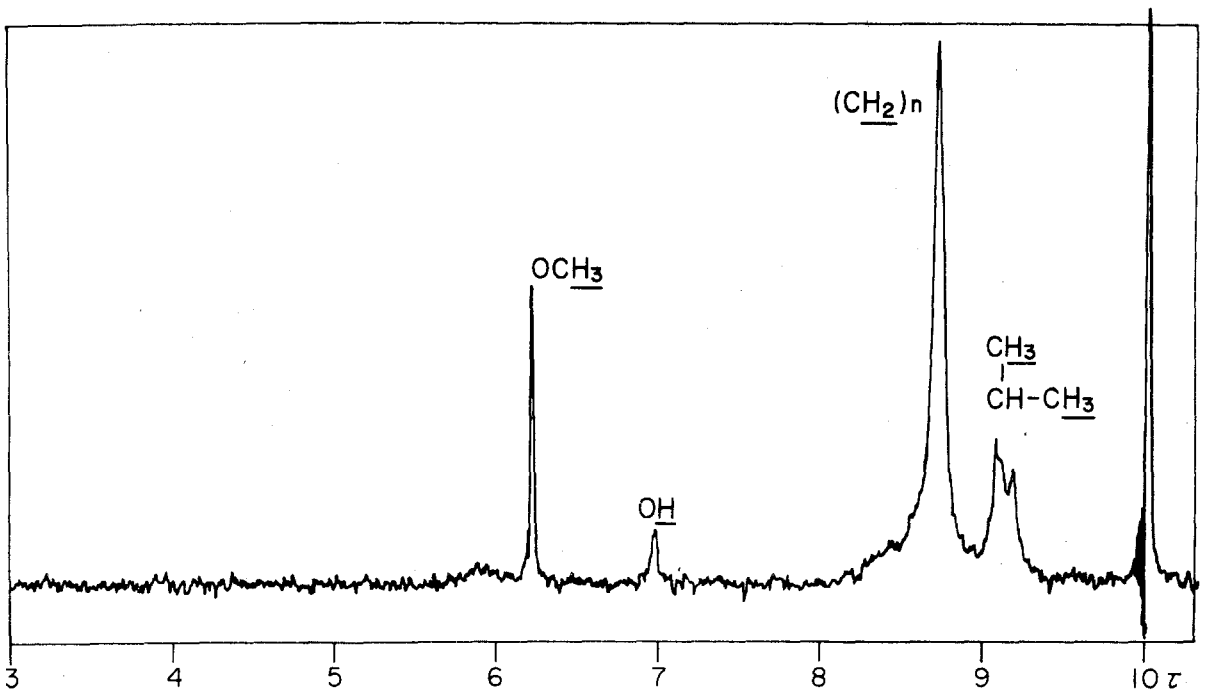


Fig. 10 Proton magnetic spectrum of methyl ester of the hydroxy fatty acid obtained from the phosphatidylethanolamine.

The upper phosphatidylethanolamine contained only non-hydroxylated fatty acids and the lower one contained hydroxy fatty acids in addition to non-hydroxylated fatty acids. On the other hand, hydrolysis with snake venom phospholipase A, an enzyme which releases specifically the fatty acids from the 2-ester position[48,49], indicated that the hydroxy fatty acids occupied exclusively the 2-position, this being in contrast to the findings on the phospholipid fatty acids from Brucella abortus[50].

It is clear that the isolated fatty acid was a hydroxy fatty acid on the basis of elementary analysis, gas-liquid chromatographic study, thin-layer chromatography and the infrared spectrum. Mass spectrometry by deuterium exchange of the active hydrogen indicated the hydroxy acid to be a mono-hydroxy fatty acid. Further, the localization of the hydroxy group on the skeleton of the fatty acid was determined by mass spectroscopy and by the oxidation of this acid with lead tetraacetate; that is, the oxidation with lead tetraacetate gave a tetradecanal, while as seen in Fig. 9, its mass spectrum afforded ion peaks at m/e 90, 145, 213 (M-59), 272 (molecular ion) characteristic for a methyl ester of α-hydroxy fatty acids. However, with the results of the above experiments, a methyl branching on the fatty acid skeleton could not be

confirmed clearly. Hence, proton magnetic resonance spectroscopy was undertaken in order to resolve this question. As seen in Fig. 10, a doublet at 9.14 τ was observed for the terminal methyl protons (six protons), indicating the hydroxy acid to have an isopropyl group. Therefore, the hydroxy fatty acid obtained here may be defined as 2-hydroxy-13-methyl-tetradecanoic acid.

The occurrence of α - and β -hydroxy fatty acids have been reported in several bacteria, including species of Serratia[51], E. coli[52], Pseudomonas[53,54], Brucella[50] and Mycobacterium[55]. However, no information was available on the β -localization and the branched chain hydroxy fatty acids. It may be assumed that the occurrence of this acid in St. sioyaensis shows that such branched chain fatty acids as iso and anteiso acids also might be degradated by α -oxidation as in animal brain fatty acids[56] and the localization of this acid at the β -position appears to indicate that this acid actively might turn over as the highly unsaturated fatty acids at the β -position of glycerophosphatides.

CHAPTER IV. A NEW LYSINE-CONTAINING LIPID (SIOLIPIN A)

1. Isolation, Purification and Chemical Structure of Siolipin A

The author wishes to report the method of isolation and chemical characterization of a new lipoamino acid ester from Streptomyces sioyaensis, in which the presence of a lysine-containing lipid was previously described[5,6]. The substance, for which the author proposed the name siolipin A, is a lipoamino acid ester and defined as a fatty polyalcohol ester of Nα-acyl lysine (I). The wet mycelium of this microorgaism was washed with acetone. The dried mycelium was extracted with chloroform-methanol (2:1, v/v) to give lipid extracts (100 to 200 mg per g cell, the yield depending on the culture conditions such as pH and the age of growth). The extracts were fractionated by chromatography on silicic acid to give a cephalin fraction (about 1.8% of total lipid recovered) containing phosphatidylethanolamine and siolipin A, as described in chapter II [5,6]. This fraction could also be obtained by elution of the extracts with chloroform-methanol (7:3, v/v) on DEAE-cellulose column chromatography[27]. This shows that siolipin A is neutral, similar to phospha-

tidylethanolamine. In order to separate siolipin A from phosphatidylethanolamine, this cephalin fraction (493.5 mg) was submitted to hydrolysis by snake venom (Habu, Trimeresurus flavoviridis, Hallowell) phospholipase A[43], by which the phosphatidylethanolamine was degradated to its lyso-form, while siolipin A remained intact. The reaction products could easily be separated into each component by silicic acid chromatography. Siolipin A thus purified was recrystallized from methanol to afford a white amorphous powder (about 0.1% of total lipid), with m.p. 132-134°C after previous sintering. It was ninhydrin-positive. The substance contained neither glycerol nor phosphorus[23], while paper chromatography (i-propanol-acetic acid-water, 3:2:1, v/v/v) of the hydrolysate with 6 N HCl for 24 hr showed the presence of lysine and fatty materials such as fatty acids and fatty alcohols. Quantitative estimation by an automatic amino acid analyzer (Hitachi KLA-2) revealed the presence of 1 mole of lysine per mole of siolipin A, on the basis of the molecular weight (630) determined by the vapour pressure method (1.36 μmoles of lysine was found in 1 mg of siolipin A). Siolipin A was submitted to dinitrophenylation,[57] followed by acid hydrolysis with 6 N HCl at 105°C for 24 hr. A DNP-

lysine so obtained was identified as Nε-DNP-lysine by paper electrophoresis (pH 6.5: pyridine-acetic acid buffer and 9.0: $NaHCO_3$-Na_2CO_3 buffer) and paper chromatography (upper phase of n-butanol-butylacetate-1% aq. ammonia, 1:2:3, v/v/v .). This shows that siolipin A is a Nα-protected lysine derivative. As seen in Fig. 11 A, the infrared spectrum of this compound shows the presence of a primary amine (3426 and 1596 cm^{-1}), hydrocarbon chain (2930, 2870, 1470, 1386 and 725 cm^{-1}), ester function (1734, 1265 and 1250 cm^{-1}), and an amide group (1643 cm^{-1}). $[\alpha]_D^{23} = +5.7 \pm 0.3$ (c, 1.150 in chloroform). Anal. found: C, 67.64; H, 11.47; N, 4.09. Calcd. for $C_{38}H_{78}N_2O_7$: C, 67.66; H, 11.57; N, 4.15%.

Siolipin A (10.3 mg) was subjected to mild alkaline hydrolysis[58] in order to cleave the ester linkage. Silicic acid chromatography of the hydrolysis products (5.5 mg) afforded a fatty alcohol (1.7 mg) by eluting with chloroform and chloroform-methanol(95:5 to 85:15) and a lysine-containing lipid (IIa) (0.9 mg) by eluting with chloroform-methanol (1:1). This substance (IIa) moved as a ninhydrin-positive spot with an $R_{\text{siolipin A}}$ value of 0.3 on thin-layer chromatography. The same substance (IIa) was obtained in about 5% yield during the course

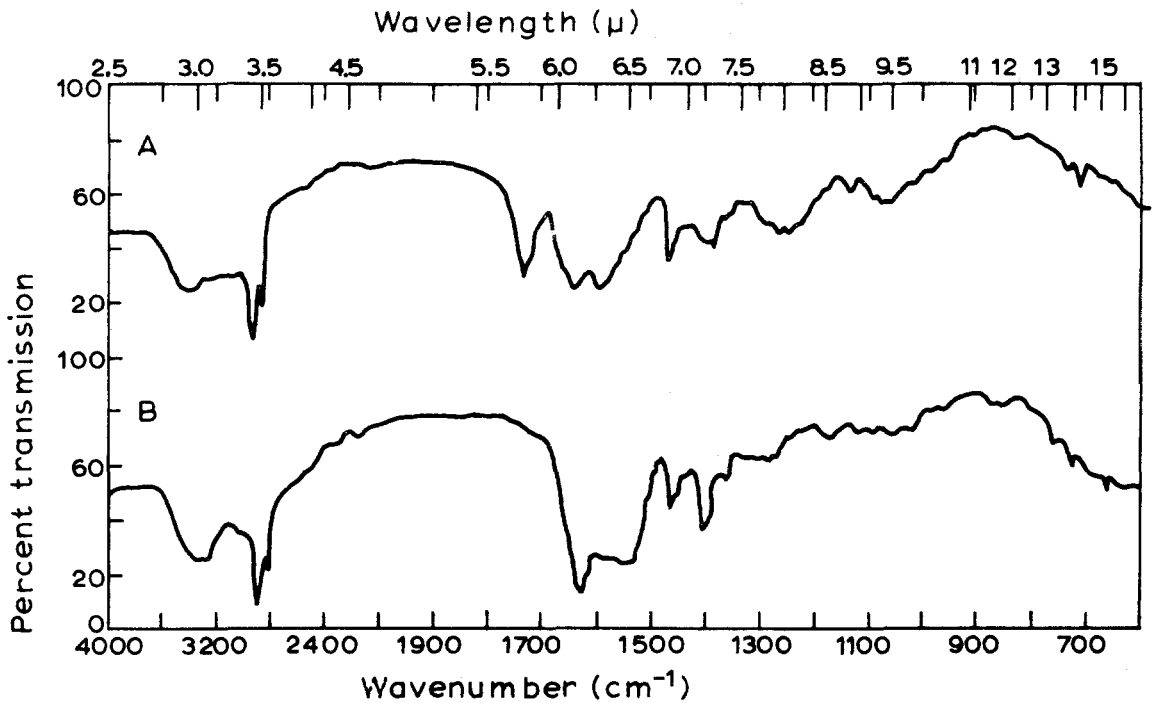


Fig. 11 Infrared spectra of siolipin A (A) and its derived lipid (IIa) (B) from *S. sioyaensis* (KBr).

of the purification of siolipin A by Florisil column chromatography. This compound (IIa), so purified, melted at 162-163.5°C after previous sintering. The molecular weight (vapour pressure method) was 434. Anal. found: C, 60.42; H, 10.28; N, 6.32. Calcd. for $C_{22}H_{44}O_4N_2 \cdot 2\ H_2O$: C, 60.51; H, 11.08; N, 6.42%. As seen in Fig.11 B, the infrared spectrum of this compound (IIa) no longer exhibited the presence of an ester linkage, while the primary amine (3360 and 1408 cm^{-1}), hydrocarbon chain (2930, 2870, 1470 and 1365 cm^{-1}) and the amide group (1633 cm^{-1}) remained unattacked. On the other hand, the esterification of this lipid (IIa) with diazomethane in ether recovered the ester linkage. The methyl ester of this lipid (IIb) moves with an R_f value similar to the original siolipin A on thin-layer chromatography (chloroform-methanol-water, 70:22:5, v/v/v.). This fact suggests that the carboxyl group in siolipin A is esterified by a radical, R_2.

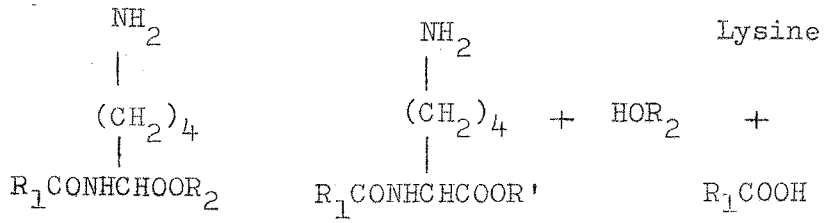
On the other hand, the fatty material (R_2OH) eluted with chloroform and chloroform-methanol (95:5 to 85:15) has a molecular weight of about 240. Gas-liquid chromatographic analysis indicated that this residue did not contain fatty alcohols such as those derived from iso and

anteiso methyl esters with $LiAlH_4$, but less volatile fatty polyalcohol. Structural studies on this fatty polyalcohol (R_2OH) are under way in cooperation with Dr. Lederer's group.

The derived lipid (IIa) was hydrolysed with 2 N HCl for 18 hr to give lysine and fatty acids (R_1COOH). Lysine was identified by an amino acid autoanalyzer and gas-liquid chromatography (Perkin-Elmer model 881)[59] (0.75% HIEFF-IBP-0.25% EGSS-X on Gas-chrom P), while the fatty acids (R_1COOH) were esterified with diazomethane in ether followed by thin-layer chromatography in dichloromethane. Thin-layer chromatography revealed the presence of normal, and monohydroxy fatty acids in a ratio of 1:4 on visual estimation. It was shown by gas-liquid chromatography[5] (15% DEGS on Chromosorb W) that the fatty acid residue (R_1COOH) consisted of both non-hydroxylated (i-16, n-16, a-17, n-18 and n-19) and unidentified hydroxylated fatty acids which are converted to those having lower retention time by trimethylsilylation[42].

The structure of this derived lipid (IIa) was further established by the following synthetic approach. Nε-Carbobenzyloxy-L-ysine was acylated with palmitoylchloride by Schotten-Bauman's method to give Nα-palmitoyl-Nε-carbobenzyloxy-lysine and, following the removal of the protected

group with hydrogen bromide in acetic acid, afforded N^{ε}-palmitoyllysine (IIc). Infrared spectrum and the thin-layer chromatographic behaviour of this synthetic analogue (IIc) were identical with those from the natural product (IIa).



I

IIa, R' = H
b, R' = CH_3
c, R_1 = $C_{15}H_{31}$, R' = H

Based on these results, the author proposed structure I as the structure of a new lysine-containing lipid from St. sioyaensis. Moreover, he found that an ornithine-containing lipid, for which he proposed the name siolipin B, also occurred in this organism under the different culture conditions. The mycelium at an early period of the culture contains only siolipin A and mycelium at the stationary phase contains much siolipin B. It is of interest that siolipin B is related to the ornithine-containing lipid in Mycobacterium[10,11], Rhodopseudomonas spheroides[12,14] and Rhodospirillum rubrum[13].

2. Distribution of Siolipin in the Mycelium of *Streptomyces sioyaensis*

Streptomyces sioyaensis contained siolipin[4,17,18,19] together with various phospholipids.[5,6] As a first step to investigate the physiological role of siolipin, its distribution was studied in comparison with phospholipids. Though siolipin has the properties similar to those of phosphatidylethanolamine, its physiological meaning has not been elucidated. It might take a part in the biosynthesis of peptideglycan of the cell wall or in the transportation of amino acids through the membrane. In the former case, siolipin might be localized in the cell wall fraction. To see the relative content of siolipin and phospholipids in the cell, the author tried to compare the distribution of these lipids.[60] The mycelium was disrupted and divided into supernatant and precipitated residue which showed the properties of the cell wall. The isolation of the latter fraction was confirmed with an electron microscope and biochemical analyses (amino acids and glucosamine). The siolipin was distributed in the cell in almost the same ratio as phospholipids.

St. sioyaensis H-690-3 was grown on Bennett's medium as previously described.[5,17,18] The mycelium, after 4 day-cultivation, was centrifuged at 3000 rpm. for 5 min and

washed three times with deionized water and once with 0.3M sucrose solution. It was then resuspended in 0.3M sucrose solution and disrupted by a sonicator-150 (Ohtake) at 10 kc for 30 min.[61] After breaking the mycelium, the suspension was centrifuged at 2000 rpm. for 15 min to remove the unbroken mycelium. The supernatant was then centrifuged at 10,000 rpm. in a refrigerated centrifuge UV-90 (Tominaga) for 15 min to collect the precipitate which showed the characteristic properties of the cell wall, and the remaining supernatant was lyophilized. The precipitated material was then washed twice with deionized water, five times with M/15 phosphate buffer (pH 7.5) and finally eight times with deionized water. It was then resuspended in deionized water and lyophilized. The material was shadowed with chromium and checked by an electron microscope (JEM 6C).

The purity of the material was further checked by analyses of its components, i.e. amino acids (Table V) and hexosamine (Table VI). The amino acid composition of the hydrolysate (6N-HCl, 110°C, 20 hr) of the material was determined by an automatic amino acid analyzer (Hitachi KLA-2). Hexosamine[62] and lipids[5] were determined and estimated, respectively. Glycine, glutamic acid, alanine, diaminopimelic acid (DAP) and lysine were present about

Table V. Amino Acid Composition of the Cell Wall Material of *Streptomyces sioyaensis*

	μmole/mg	Ratio to Ala 1.00
Asp	0.051	0.08
Thr	0.038	0.06
Ser	0.032	0.05
Glu	0.578	0.95
Pro	0.026	0.04
Gly	0.546	0.90
Ala	0.607	1.00
Val	0.049	0.08
DAP*	0.368	0.61
Ileu	0.018	0.03
Leu	0.046	0.08
Tyr	0.382	0.04
Phe	0.011	0.02
MuA**	0.422	0.70
Lys	0.416	0.69
His	0.014	0.02
Arg	0.038	0.06
Amm***	1.11	1.83

* DAP=diaminopimelic acid

** MuA=muramic acid

*** Amm=ammonia

ten times as much as other amino acids (Table V). These amino acids have been usually found to be constituents of the cell wall of *Streptomycetes* [63-67]. The

material was also susceptible to lysozyme (E.C.3.2.1.17). These results showed that the material had a property characteristic of the cell wall. It is quite interesting that the cell wall of most Streptomycetes contain lysine and DAP at the same time[67,68] while those of most bacteria[69] and Actinomycetes[70] have either lysine or DAP, but not both.

The cell walls of Gram-positive bacteria contain 1 to 3% of lipid, while those of Gram-negative bacteria do much more (10 to 30%).[71,72]

Studies on the distribution of lipids showed that 5.0% of siolipin, 6.1% of lipid-phosphorus, 7.0% of lipid-ninhydrin-positive substances were contained in the cell wall material of St. sioyaensis (Table VI), and that most of siolipin (95%) as well as phospholipid (93 to 94%) was contained in the cytoplasm. Lipid-phosphorus means the phosphorus which is extractable with chloroform-methanol (2:1, v/v), and which originates from phospholipids (cardiolipin, phosphatidylethanolamine, phosphatidylinositol-mannoside etc.). Lipid-ninhydrin-positive substances cover two kinds of phosphatidylethanolamines[5,8] and siolipin. Phospholipids were generally believed to be contained in the cell membrane. Distribution study, as

Table VI. Distribution of Lipids in Fractionated Materials of St. sioyaensis

	Cell wall material		Supernatant	
Hexosamine (μg/mg) $Glc\text{-}NH_2/Gal\text{-}NH_2$=1.3	187 (as $Gal\text{-}NH_2$)		trace	
Lipid-phosphorus (μg)	217	(6.1%)	3340	(93.9%)
Lipid-ninhydrin-positive substances (as Leu. eq.) (μmoles)	2.5	(7.0%)	33.4	(93.0%)
Siolipin (μmoles)	0.343	(5.0%)	6.55	(95.0%)

described above, showed a possibility that most siolipin is distributed in the cytoplasm (membrane) just as other phospholipids.

The changes[9] of siolipin content and its biological activities will be reported later.

CHAPTER V. THE OCCURRENCE OF AN ORNITHINE-CONTAINING LIPID (SIOLIPIN B)

In the studies on siolipin A, an ornithine-containing lipid named "siolipin B", was also found in the same microorganism. Both lipids had similar properties and showed the same R_f value on thin-layer chromatography. Siolipin B is the same substance as that obtained by Gorchein from Rhodopseudomonas spheroides[12,14]. Similar kinds of ornithine-lipid have been reported also in Mycobacterium[10,11], and Rhodospirillum rubrum[13] etc.

Siolipin A was isolated from the young mycelium of Streptomyces sioyaensis, while siolipin B was obtained from the aged mycelium of the same organism. Studies of the relations between siolipins A and B in prototroph of Streptomyces sioyaensis revealed that the amounts of them depend on the age of the mycelium, pH of the broth, and culture conditions (Sakaguchi flask or jar fermenter). Both lipids were also found in auxotrophic mutants[3,9] of Streptomyces sioyaensis, though the ratio of them were different with each strain tested.

1. MATERIALS AND METHODS

Prototroph strain of Streptomyces sioyaensis H-690-3

was cultivated in Bennett's medium as previously described.[5,17,18] After certain period of time, the mycelium was harvested by centrifugation and subjected to extraction of lipids. When the effect of pH was studied (Table VII. Exp. 2, 3, Table VIII. Exp. 5, 6, 7), the mycelium was divided into 2 to 3 fractions, one of which was subjected to extraction of siolipins A and B as standard sample. The other fractions were further incubated for 5 hr in citrate buffer (M/5 Na_2HPO_4-M/10 Na-citrate) at pH 3.0 or 8.0, respectively. Then the mycelium was treated with chloroform-methanol (2:1, v/v) for the extraction of lipids as in the foregoing experiments.[5] The chloroform-methanol extracts as total lipid fraction were treated with deionized water containing 1% $CaCl_2$, 0.9% NaCl to remove water soluble materials (free amino acids etc.). The lipid fraction was subjected to acid hydrolysis with 6N-HCl for 20 hr. The hydrolysate was treated several times with n-hexane to extract free fatty acids. The water layer containing deacylated amino acids (lysine and ornithine) was analyzed by an automatic amino acid analyzer[73] (Hitachi KLA-2). Hitachi spherical resin No. 3105 was equilibrated with 0.7 M Na-citrate buffer, pH 5.28, and packed into a column of 0.9

x 50 cm. Elution was carried out with the same buffer at a flow rate of 30 ml/hr (50°C). Lysine and ornithine emerged at the effluent volume of 91.5, 86.5 ml, respectively (Fig. 12). The amounts of these amino acids were shown as the amounts of siolipins A and B.

2. RESULTS

(1) Prototroph of Streptomyces sioyaensis

(a) Culture by jar fermenter. Young mycelium contains only siolipin A, while older one does more siolipin B. Ratio of siolipin A to siolipin B (A/B) decreased with the culture period (Table VII. Exp. 1). Culture age is, as well known, closely connected with pH of the broth, so the change of the ratio of siolipin A to siolipin B could not necessarily attributed only to the age of the mycelium. When St. sioyaensis was cultivated in Bennett's medium, pH of the broth decreased with the growth of the mycelium to reach pH 4.3, then it began to increase when the mycelium ceased to grow[17,18]. Young mycelium is in the broth of lower pH, while older mycelium in that of higher pH. Effect of pH was studied in Exps. 2 and 3 (Table VII). At acidic pH side almost the same amount of siolipins A and B were present, but at basic pH side only siolipin B was estimated. When the

Table VII. The Changes of Siolipins A and B in the Mycelium Cultured by Jar Fermenter

	Age (hr)	pH	Siolipin (μ mole/g cell) A	B	Ratio (A/B)
Exp. 1	22	5.5	0.509	0	—
	65	4.3	0.183	0.149	1.22
	95	8.4	0.134	0.147	0.91
Exp. 2	96	2.0*	1.43	1.54	0.92
	96	5.2	trace	0.92	—
	96	9.0*	trace	1.82	—
Exp. 3	96	3.0**	2.29	2.22	1.03
	96	6.0	trace	2.74	—
	96	8.0**	trace	2.22	—

* pH of broth was adjusted by HCl or NaOH and incubation was carried out for another 5 hr.

** Mycelium, after harvest, was further incubated in citrate-buffer for 5 hr.

mycelia were incubated in citrate buffer (pH 3.0 or 8.0), the effect of pH was also recognized. These results have shown that pH of the environment have an influence on the relative amount of siolipins A and B.

(b) Culture in Sakaguchi flasks. The mycelium cultivated in Sakaguchi flasks showed the same tendency as that grown in jar fermenter. Table VIII (Exp. 4) shows that even young mycelium contained siolipin B and

Table VIII. The Changes of Siopilins A and B in the Mycelium Cultured by Sakaguchi Flask

	Age (hr)	pH	Siolipin (μ mole/g cell) A	B	Ratio (A/B)
Exp. 4	48	4.7	0.881	0.037	21.9
	168	6.5	1.774	0.274	6.5
Exp. 5	48	4.7	0.811	0.037	21.9
	48	8.0*	0.820	0.034	24.1
Exp. 6	168	3.0*	3.231	0.215	15.0
	168	6.5	1.774	0.274	6.5
Exp. 7	168	3.0*	1.30	0.72	1.8
	168	6.4	0.30	0.24	1.2
	168	8.0*	0.64	0.62	1.0

* Mycelium, after harvest, was further incubated in citrate-buffer for 5 hr.

that ratio of siolipin A to siolipin B also decreased with culture age. Effect of pH was studied as follows; young mycelium, harvested from the broth of low pH (pH 4.7), was further incubated in basic citrate buffer (pH 8.0) (Exp. 5), on the other hand, old mycelium, harvested from the broth of rather higher pH (pH 6.5), was further incubated in acidic citrate buffer (pH 3.0) (Exp. 6). These results show that amounts of siolipins A and B in younger mycelium were not affected by environmental pH, but in older mycelium they changed according to pH.

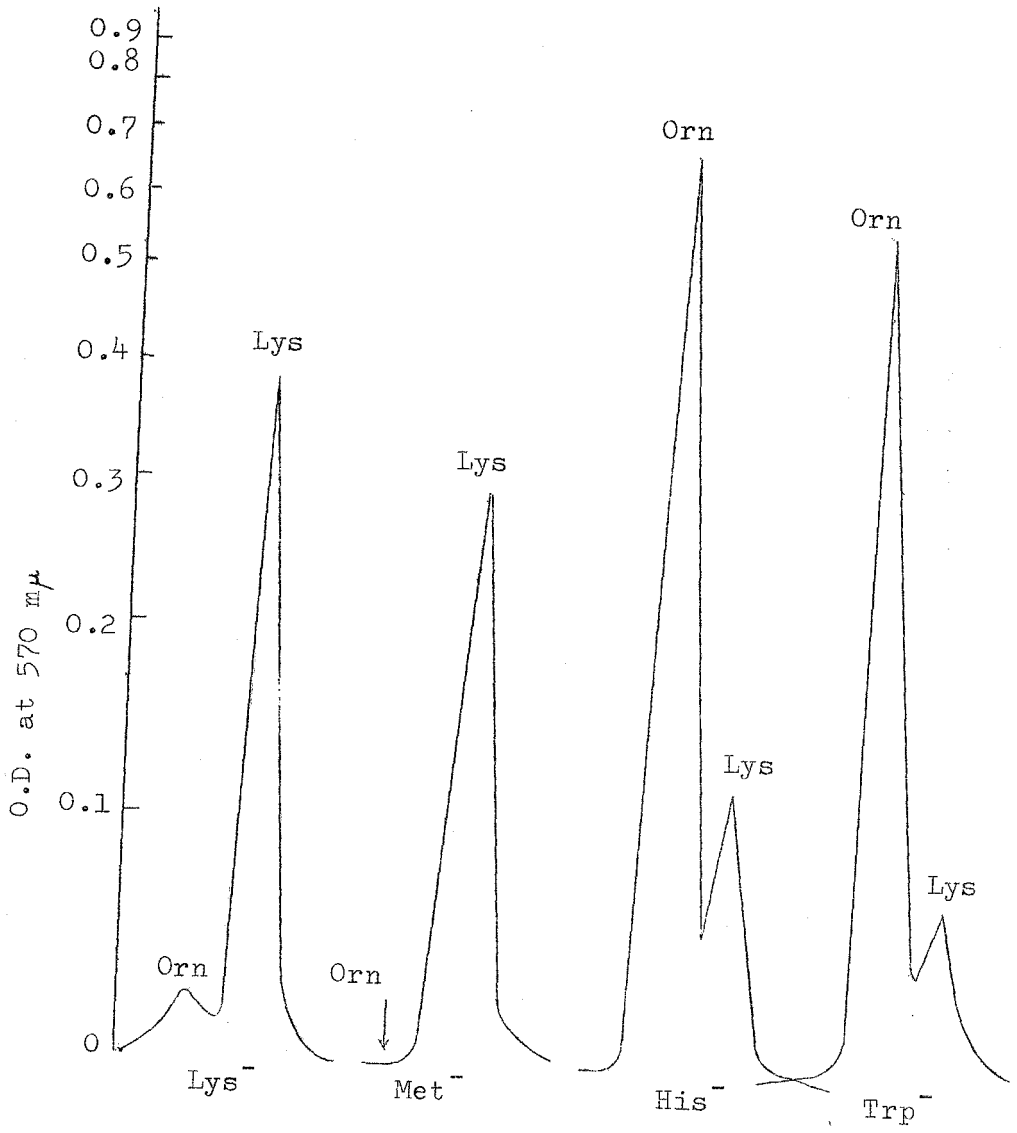


Fig. 12. The Ratio of Lysine- and Ornithine-lipids in St. sioyaensis and Its Mutants.

Conditions: column, 0.9 x 50 cm; resin, Hitachi spherical resin No. 3105; buffer, 0.7 M Na-citrate, pH 5.28; temperature, 50°C; rate, 30 ml/hr; apparatus, Hitachi automatic amino acid analyzer.

Using the older mycelium, the author further confirmed the effect of pH (Exp. 7). As a whole, the mycelium grown in Sakaguchi flasks did not show so striking results as that cultured in jar fermenter.

(2) Amino acid-requiring mutants

In chapter I[3], the author reported that amino acid-requiring mutants of St. sioyaensis contain siolipin (substance C). In this section content of the siolipin (ratio of siolipins A to B) was analyzed (Fig. 12). Lysine-less mutant contained siolipins A and B in almost the same ratio as those of prototroph of St. sioyaensis. Histidine-less (Aux-16) and tryptophan-less mutants contained much siolipin B than siolipin A, especially the later contained larger amount of siolipin B per mycelium (μmole/g). The most distinguished strain is the methionine-less mutant, which contained only siolipin A. The results were summarized in Table IX with those obtained as to prototroph, but they do not give any clear explanations on the relation between biosynthesis of siolipins and amino acid requirements.

3. DISCUSSION

Streptomyces sioyaensis contains lysine-lipid (siolipin A) and ornithine-lipid (siolipin B) at the same time under

Table IX. The Ratios of Siopilins A to B in the Autotrophic Mutants of *Streptomyces sioyaensis*

Strains	Amino acid required (40 mg/ml)	Age (hr)	Final pH	Siolipin (μmole/g cell) A	B	Ratio (A/B)
Prototroph (H-690)		48	4.7	0.811	0.037	21.9
		168	6.5	1.774	0.274	8.3
Auxotroph 146-16	Met	72	4.6	0.244	0	-
		168	7.8	0.409	0	-
Auxotroph 150	Lys	96	5.7	1.234	0.053	23.3
		168	7.0	0.553	0.038	14.5
Auxotroph 80-91	His	72	4.8	0.534	0.788	0.68
		144	6.6	0.636	0.430	1.48
Auxotroph 16	His	72	6.0	0.310	1.960	0.16
		144	6.8	0.170	0.884	0.19
Auxotroph 107-18	Trp	72	4.8	1.365	4.190	0.33
		144	6.8	0.254	2.544	0.10

some conditions. Siolipin B have been found in *Rhodopseudomonas spheroides*[12,14], *Mycobacterium* sp.,[10,11] *Rhodospirillum rubrum*,[13] etc. Siolipin A could possibly be found in these microorganisms, for in the phospholipid fraction of *Mycobacterium*[11] small amount of lysine was reported together with ornithine, ethanolamine etc.

Ratio of siolipin A to siolipin B in *St. sioyaensis*

varied with various conditions (age, pH etc.). Under some conditions the author can get siolipin A without containing siolipin B, but he has not yet succeeded in getting siolipin B free of siolipin A. Addition of L-tryptophan (1 mg/ml) disturbed the balance of the biosynthesis of siolipins A and B. In this case only siolipin A was biosynthesized, while siolipin B was not detected. These results suggested that the biosynthesis of siolipins A and B is regulated by many complicated factors, and that it might have some relation with the metabolism of amino acids.

Depinto, based on the isotopic experiment[13] in which the lack of turn over of ornithine-lipid was reported, suggested a structural role of the ornithine-lipid. However, changes of the ratio of siolipins A to B according to the environment led the author to the consideration that siolipins might take a role other than structural role.

The author is expecting that besides these two esters of lipoamino acids (lysine, ornithine), similar derivatives of other amino acids might be found in microorganisms.

CHAPTER VI. BIOLOGICAL ACTIVITIES OF SIOLIPIN

Biological activities of these esters of lipoamino acids have never been reported. In the survey for the activities of siolipin, three biological activities were found[15], (1) hemolytic activity, (2) acceleration of coagulant activity in a fibrin clotting system, (3) antibacterial activity for Bacillus subtilis PC1-219 grown on a synthetic medium.

The present chapter deals with these biological activities of siolipin. The sample used in these experiments was the pure siolipin A.

1. MATERIALS AND METHODS

(1) Hemolytic activity. Hemolytic activity of siolipin was tested for 2.5 and 5.0% of rabbit erythrocyte in 0.85% saline solution. After incubation overnight at 37°C, the activity was visually determined. Then the reaction mixture was centrifuged at 3,000 rpm for 5 min and optical density of the supernatant was measured at 580 mμ.

(2) Coagulation test. Fibrin clotting system used was as follows[74]; fibrinogen 0.0956% (bovine containing approximately 96% clottable protein), thrombin 1.612 units/ml, 0.02 M Tris+0.145 M NaCl (pH 7.3). Opacity development

was followed at 21 - 23°C by reading absorbance at 600 mμ.

(3) Antibacterial activity. The in vitro antimicrobial activity of siolipin was determined by the pulp disc method on the synthetic medium, which contained 1% glucose, 0.1% KH_2PO_4, 0.1% $(NH_4)_2HPO_4$, 0.5% NaCl, 0.04% $MgSO_4 \cdot 7H_2O$, 1.5% agar. The pulp disc was dipped in a chloroform-methanol solution of siolipin and then, after drying, was put on the agar plate. The concentration of spore suspension of Bacillus subtilis PCl-219 was 10^7 cell/ml. The inhibitory zone of siolipin was measured after incubation overnight at 37°C.

2. RESULTS AND DISCUSSION

(1) Hemolytic Activity

Siolipin showed the hemolytic action for rabbit erythrocyte at the concentration more than 0.9 μg/ml by visual estimation, but by colorimetric estimation its minimal hemolysis concentration (MHC) was 0.11 μg/ml (Table X). The hemolytic activity corresponded to the concentrations of siolipin.

Lyso-siolipin (Nα-acyl-lysine)[7] showed the stronger hemolytic activity than siolipin at higher concentrations (more than 40 μg/ml) (Fig. 13), though its MHC was almost the same as that of siolipin. The reason of this strong

Table X. Hemolytic Activity of Siolipin

(2.5% rabbit erythrocyte)

Concentration (μg/ml)	Optical density (at 580 mμ)	Visual Estimation
1000	> 1.4	+++
500	> 1.4	+++
250	> 1.4	++
125	> 1.4	++
62.5	> 1.4	++
31.2	1.190	+
15.6	0.950	+
7.8	0.500	+
3.9	0.347	+
1.9	0.264	+
0.9	0.142	±
0.45	0.044	−
0.22	0.023	−
0.11	0.017	−
0.05	0	−
0.01	0	−
(Control) 0	0	−
MHC* (μg/ml)	0.11	0.9-1.9

* MHC=Minimal Hemolysis Concentration.

activity of lyso-siolipin is supposedly based on its solubility. As it is more polar than siolipin and more soluble in water, the velocity of hemolytic action might be accelerated. In the case of lyso-siolipin, hemolysis

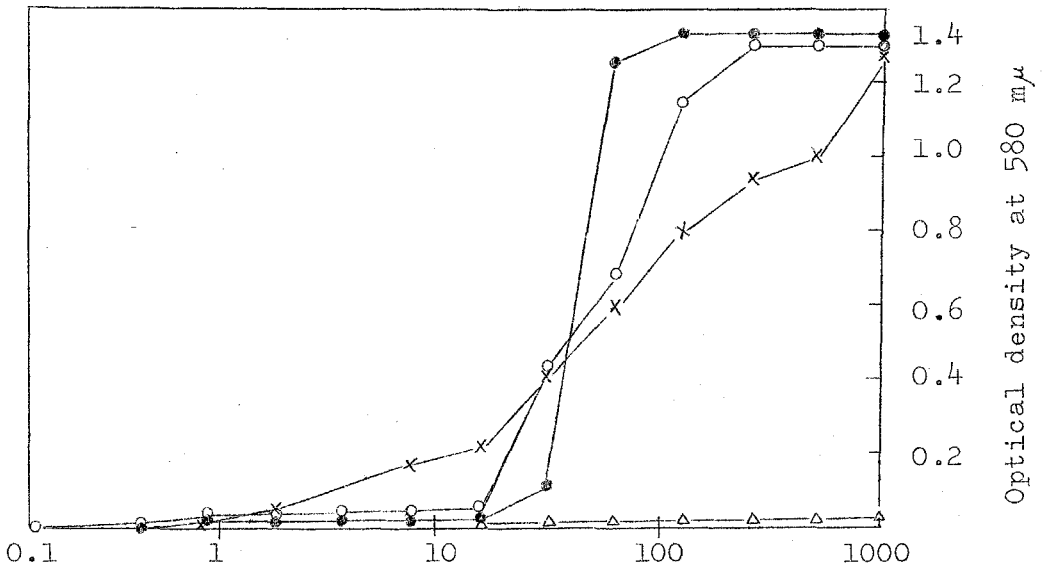


Fig. 13. Hemolytic Activities of Siolipin and Its Related Substances (5% Rabbit erythrocyte).

○—○ Siolipin ×—× Fatty acid

●—● Lyso-siolipin △—△ L-Lysine, L-Ornithine

occurred in a short time, and a red pigment spread all over the tube, whereas in the case of siolipin, which is insoluble in water, hemolysis was seen only after 16 to 20 hr, and a red pigment was observed only at the bottom of the tube. By the thin-layer chromatographic analysis, the possibility was denied that siolipin, after being hydrolysed to lyso-siolipin by erythrocyte enzymes, might

have shown the hemolytic activity. Siolipin was detected from the reaction mixture even after 24 and 48 hr incubations, but lyso-siolipin was not.

L-Lysine and L-ornithine did not show the hemolytic activity even at the concentration of 1000 μg/ml (Fig. 13).

Branched fatty acids obtained from Streptomyces sioyaensis, as anticipated, showed the hemolytic activity (Fig. 13). Though it was weaker than those of siolipin and lyso-siolipin, the MHC was almost the same as those of siolipin and lyso-siolipin. Lyso-siolipin is an effective substance which combines the hemolytic activity of fatty acid with the solubility of amino acid.

As the hemolytic activity is related to the cytolytic action for various cells[75], siolipin may also have effects on tissue culture cells, protoplast of various bacteria, mitochondria, lysosomes etc. This property may be concerned with the antibacterial action of siolipin which will be described later.

(2) Acceleration of Fibrin Clot Formation

Besides hemolytic activity, siolipin has an accelerating effect on the fibrin clot formation. When siolipin was added to the thrombin-fibrinogen system, as shown in Fig. 14, the formation of fibrin clot was accelerated even at

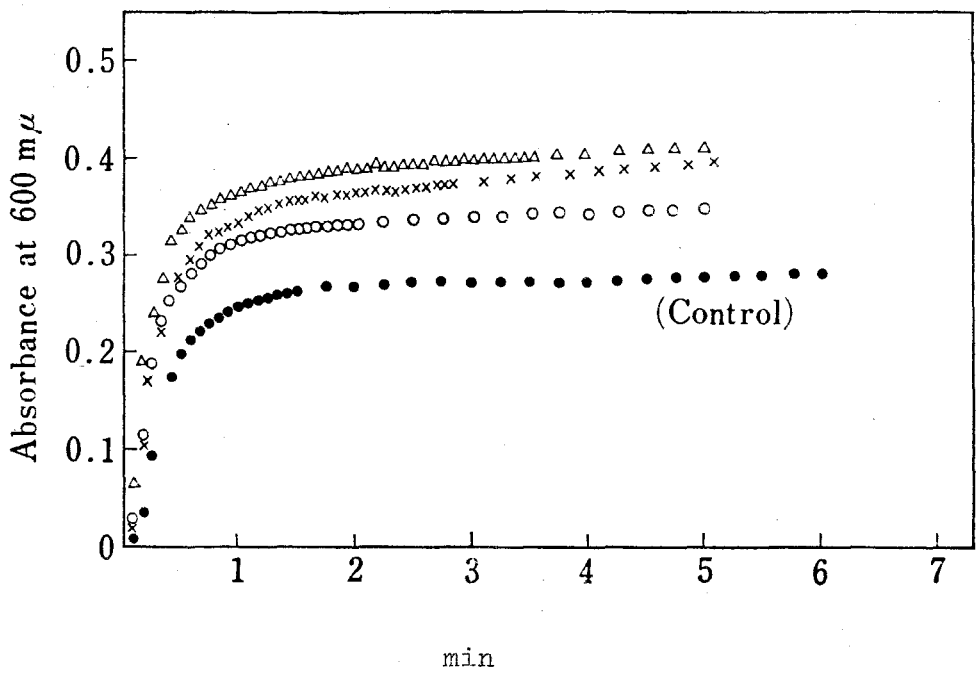


Fig. 14. Effect of Siolipin on Fibrin Clot System.

Concentration of siolipin.

● 0 × 121.0

○ 24.2 μg/ml △ 241.9

the concentration of 24.2 μg/ml of siolipin. The degree of acceleration was a function of the concentration of siolipin.

(3) Antibacterial Activity

Another activity of siolipin was the inhibition of the growth of Bacillus subtilis PCI-219 grown on a synthetic medium (Table XI), but it was inactivated when an organic component was added to the medium (Table XII).

Table XI. Effect of Siolipin on Various Microorganisms

Microorganisms	Inhibitory zone diameter (mm)	
	Nutrient medium	Synthetic medium
Aerobacter aerogenes	0	
Pseudomonas aeruginosa	0	
Serratia marcescens	0	
Aspergillus niger	0	
Mycobacterium tuberculosis	0	
Staphylococcus aureus 209p	0	
Proteus vulgaris	0	—
Escherichia coli Umezawa	0	0
Bacillus subtilis PC1-219	0	26.3
" megaterium	0	0
" cereus	0	—
" anthracis	0	—
" agri	0	—

—; no growth.

The fact that casein hydrolysate inactivated the antibacterial activity of siolipin suggested the author the reversal effect of amino acids. Experiments about individual amino acids showed that L-histidine and L-cysteine strongly reversed the activity of siolipin (Table XIII). L-Cysteine was effective at the concentration more than 1 μg/ml, whereas L-histidine at more

Table XII. Reversal Effect of Nutrient Component on the Antibacterial Action of Siolipin

Nutrient component added (0.5%)	Inhibitory zone diameter (mm) SL* 500 (μg/ml)	SL* 1000 (μg/ml)
GS**-medium	22.5	28.6
" + Yeast extract	0	0
" + Casamino acid	0	trace
" + NZ-amine	0	trace
" + Polypeptone	0	0
" + Beef extract	0	0

* SL = Siolipin. ** GS-medium = Glucose-Simmon's medium (1.0% glucose, 0.1% KH_2PO_4, 0.1% $(NH_4)_2HPO_4$, 0.5% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 1.5% agar).

than 100μg/ml (Table XIV). Some analogues of these amino acids (D-histidine, D-cysteine, L-homocysteine, S-methyl-L-cysteine, S-ethyl-L-cysteine etc.) had also the reversal effect, whereas imidazole, 2-amino thiazole (L-histidine analogues) did not show the effect. These experimental results suggested the possibility that incorporation of siolipin was antagonized by some amino acids and vice versa. Studies on the inhibition mechanism of siolipin may reveal the role of these lipoamino acid esters.

Table XIII. Reversal Effect of Amino Acids

	Amino acids (1,000 μg/ml)	Inhibitory zone diameter (mm) SL* 500 (μg/ml)	SL*2,000 (μg/ml)
Exp. I	None (control)	22.5	35.2
	L-Isoleucine	15.3	23.2
	L-Leucine	13.7	18.7
	L-Valine	16.0	22.6
	L-Histidine	0	0
	L-Hydroxy-proline	12.7	13.1
Exp. II	None (control)	24.8	40.5
	L-Cysteine	0	0
	L-Methionine	trace	12.8
	L-Threonine	14.2	16.3
	L-Lysine	12.8	23.8
	L-Phenylalanine	12.0	24.7
	L-Alanine	12.5	20.1
	L-Arginine	15.4	29.5
	L-Aspartic acid	trace	14.9
	L-Glutamic acid	11.8	18.3
	L-Proline	12.5	23.1
	L-Tryptophan	12.5	18.1
	L-Tyrosine	11.2	15.8
	L-Ornithine	12.4	21.3
	Glycine	trace	14.1

* SL = Siolipin

Table XIV. Reversal Effect of L-Cysteine and L-Histidine

Concentration of amino acid (μg/ml)	Inhibitory zone diameter (mm)	
	L-Cysteine	L-Histidine
0	34.4	37.6
1	12.5	26.5
10	0	17.6
50	0	13.2
100	0	12.0
500	0	0
1,000	0	0

* Siolipin=2,000 μg/ml

CONCLUSION

The result of the screening test for the ninhydrin-positive lipids indicated the author that most strains contain one common spot which was later revealed to be phosphatidylethanolamine. A few strains showed two spots, whereas only Streptomyces sioyaensis had three ninhydrin-positive spots.

The author tried to study on the lipids of Streptomyces sioyaensis, which was isolated from the soil sample of Shioya district in Kobe, and named Streptomyces sioyaensis after the name of the place.

Studies on the lipid of St. sioyaensis showed that the strain contained three ninhydrin-positive lipids in addition to triglyceride, cardiolipin, and phosphatidyl-inositolmonomannoside etc.

Of the three ninhydrin-positive lipids of St. sioyaensis, two were phosphatidylethanolamines, which afforded a double spot on thin-layer chromatography, because of their different fatty acid composition. From one of the phosphatidylethanolamines was isolated α-hydroxy fatty acid (2-hydroxy-13-methyl-tetradecanoic acid), which, for the first time, suggested the possibility

of α-oxidation of the branched fatty acid in micro-organisms.

The third ninhydrin-positive substance was a new lysine-containing lipid, which was shown to be an ester of lipoamino acid and named "siolipin". Later, two kinds of siolipin were found in Streptomyces sioyaensis. One was a lysine-lipid (siolipin A) and the other was an ornithine-lipid (siolipin B). The ratio of these two siolipins varied according to culture conditions. Young cells contained more siolipin A than siolipin B, whereas aged cells synthesized much more siolipin B. Alkaline environment was favorable for the synthesis of siolipin B. Siolipin B was the same type substance as the ornithine-lipids reported in Mycobacterium,[10,11] Rhodopseudomonas spheroides,[12,14] Rhodospirillum rubrum.[13] The structure of the ornithine-lipid from Rh. spheroides was determined by Gorchein[14] independently at almost the same time with that of siolipin. From chemo-taxonomic point, it is quite interesting that fatty acid compositions of the lipoamino acids varied according to microorganisms, that is, the ornithine-lipid from Rh. spheroides (photosynthetic bacteria) contained palmitate, and stearate, which are ordinary found in higher plants, whereas siolipin from St. sioyaensis contained the branched

fatty acids, which are characteristic of the lower micro-organisms.

Siolipin showed three biological activities: 1. hemolytic action for rabbit erythrocyte, 2. accelerating effect for the fibrin clot formation, 3. antibacterial activity on Bacillus subtilis PCI-219.

Studies on lipoamino acids has just begun. Here the presence of lysine- and ornithine-lipids was shown, and similar derivatives of other amino acids might be expected to be found in microorganisms.

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