TOXIC LIPIDS IN THE ROE OF A BLENNY, STICHAEUS GRIGORJEWI HERZENSTEIN

TOXIC LIPIDS IN THE ROE OF A BLENNY, STICHAEUS GRIGORJEWI HERZENSTEIN*

By

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Introduction

In the preceding paper (1), the authors reported on the toxicities of lipoprotein and lipid fractions in the roe of a blenny fish, Stichaeus grigorjewi Herzenstein. The investigators of the Hokkaido University claimed that the food poisoning of this fish is exclusively due to a "phospholipid" type complex lipid in the roe (2). Thus, it is the purpose of the present investigation to develop a better purification method and to clarify so far as possible the chemical, physical and pharmacological properties of the lipid fractions.

Experimental

1. Comparison of the extraction methods of the toxic roe lipids.

In search of a more efficient extraction method, the author tried various ways of extraction and compared the yield and toxicity of the lipid fractions obtained. Those procedures are outlined as follows;

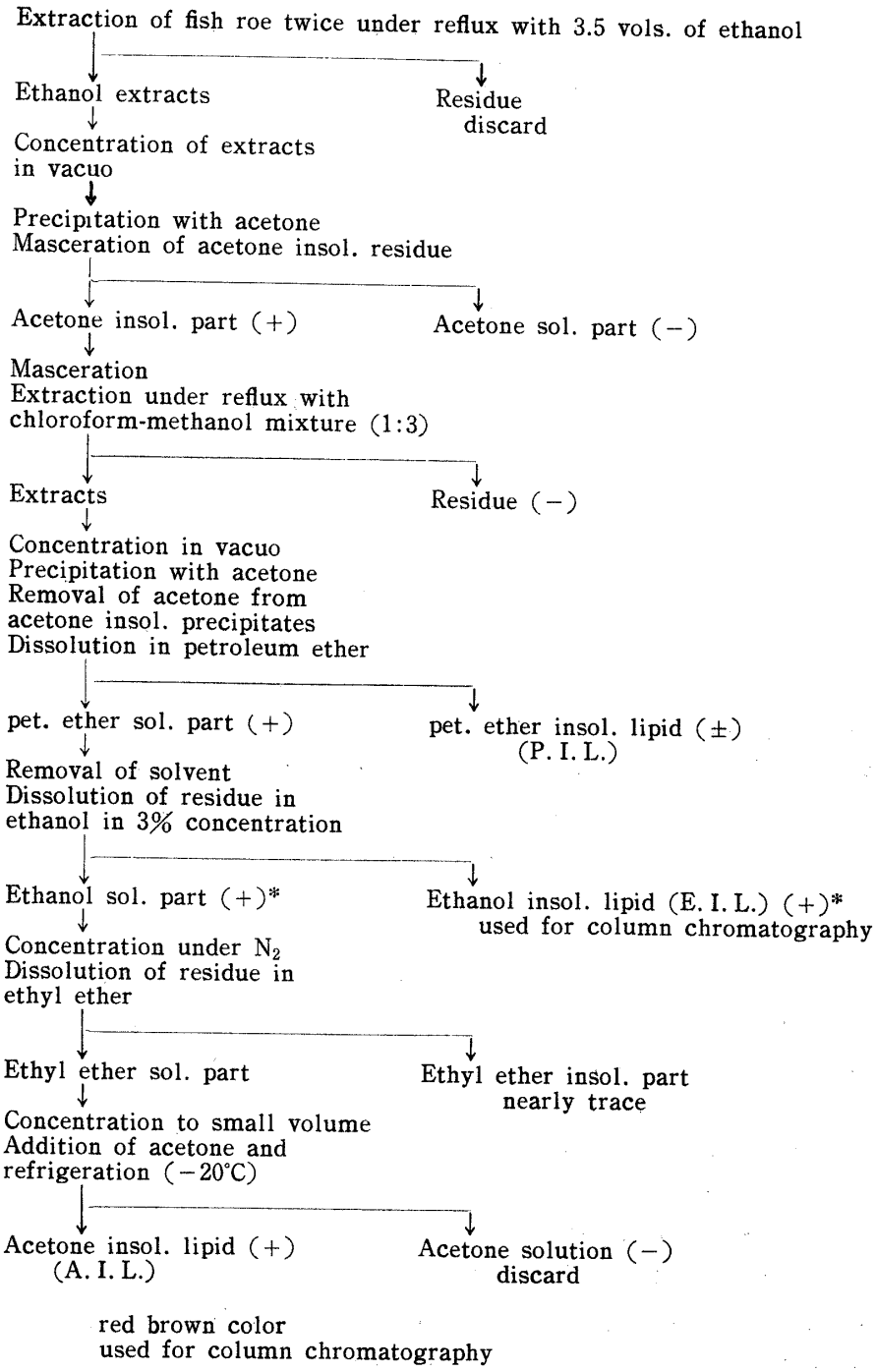
(A) Thiele's method (3).

The flow sheet of the method is given in Fig. 1.

(B) Sakai's method (2).

Extraction of the fish roe once with anhyd. acetone (3 volumes) and three times with aqueous acetone (3 volumes, 1:3), concentration of the pooled acetone extracts to the volume of about one fifteenth, extraction of this concentrates with an equal volume of ethyl ether five times, removal of the solvent from the extracts, extraction of the residue with 20 volumes of acetone four times and leaving to stand at −15°C for 24 hours, fractionation of acetone soluble- and

* This was lectured at the annual meeting of the Japanese Society of Scientific Fisheries held at Tokyo in April 4, 1964.
According to the opinion of Dr. Tokiharu Abe, Tokai Regional Fisheries Research Laboratory, the scientific name of this blenny fish "Stichaeus" is better than "Dinogunellus," therefore, the former was adopted.



* Procedure below ethanol fractionation was added by the author to the original method of Thiele.
(+) toxic (−) nontoxic (±) sporadically toxic

Fig. 1. Flow sheet of Thiele's method.

insoluble fractions. Besides the dominant toxic fraction (acetone insoluble fraction), Sakai found toxicities in both ethanol insoluble- and ethyl ether insoluble fractions.

(C) The author's method using dry fish roe powder or wet intact roe.

The samples used were dry fish roe powder which was prepared by drying at 70°-80°C and pulverization, or wet intact roe.

This method involves the following steps. Extraction of the sample with ethanol under reflux, removal of the solvent under nitrogen, shaking with ether, concentration of ether solution, fractionation of the residue with acetone. The acetone insoluble fraction was toxic. The results of the comparison of these extraction methods are shown in Table 1.

Table 1. Comparison of different methods for the extraction of toxic roe lipids.

Method	Starting material	Toxic fraction	Yield of toxic fraction	Toxicity*
Thiele	1040 g(wet roe)	Ethanol sol. lipid (=Acetone insol. lipid A. I. L.)	47.5g (4.56%)	3/3
		Ethanol insol. lipid (E. I. L.)	3.5g (0.33%)	3/3**
Sakai	1000 g (wet roe)	A. I. L.	38.5g (3.85%)	3/3
		E. I. L.	1.0g (0.10%)	3/3**
Author's	390 g (Dry powder from 1010 g wet roe)	A. I. L.	34.0g (3.36%)	2/3
	1000 g (wet roe)	A. I. L.	28.5g (2.85%)	3/3

Remarks: * Numerator means the number of killed mice (dd strain). Denominator means the number of injected mice.

** Although this fraction was less in yield, the death time was shorter than that of A. I. L. fraction.

2. Silicic acid column chromatography

(A) Acetone insoluble lipid (abbreviated as A. I. L.)

Sample was A. I. L. obtained by the Thiele's method as described in the former section. The procedure for column chromatography was based principally on Hanahan's method (4). The silicic acid used was Mallinckrodt's analytical reagent, 100 mesh. As a typical run, 60 grams of the silicic acid were activated for 8 hours at 110°C. As an aid in the flow of the solvent through the column, Celite 545 or diatomaceous earth (Kieselguhr nach Stahl, Merck) was mixed with silicic acid (2 parts silicic acid to 1 part Celite). Preparation of the column was the same as in the report of Hanahan. The silicic acid and Celite were mixed and suspended in the initial solvent system (i. e., chloroform-methanol, 4:1 v/v). After washing with the same solvent mixture, the suspension was poured into the column. (32 × 400 mm). A. I. L., weighing 1507 mg, was dissolved in a small quantity of the initial solvent system and applied onto the column. The size of the fractions collected was about 10 ml. The solvent system was the mixture of chloroform and methanol consecutively applied in the following proportions and

quantities: (1) 4:1 v/v, 400 ml, (2) 3:2 v/v, 800 ml, (3) 1:4 v/v, 400 ml. The progress of the elution was followed by phosphorus determination on every two other tubes, namely No. 1 tube and next No. 4, No.7 tubes and so on. This was depicted as in Fig. 2.

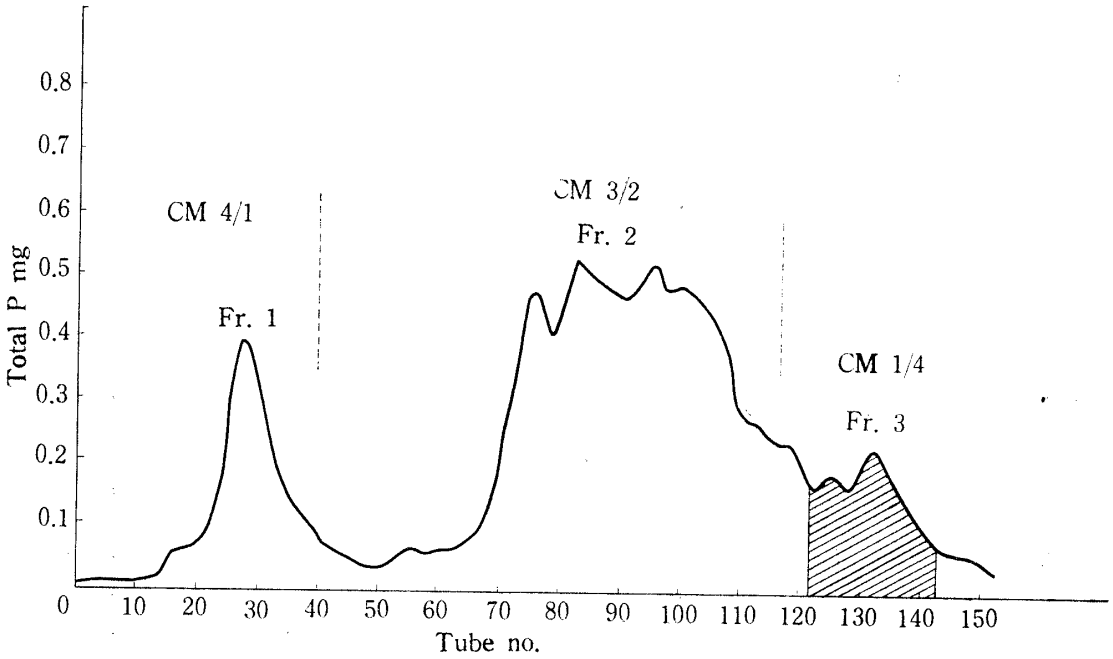


Fig. 2. Chromatogram of A.I.L on a 60 g silicic acid-30 g celite 545 column.

Toxicity test was also carried out on each fractions as indicated in Table 2. The results showed that toxic lipid occurs exclusively in fraction 3. (shaded area in the Figure. Designated as A. I. L. Fract. 3)

Table 2. Toxicity test of each fractions obtained by silicic acid chromatography using A. I. L. as a sample.

Fraction No.	Solvent system*	Body weight & sex of mice	Volume & weight of injected lipid**			Death or Survival Death time
1	CM 4/1 400 ml	20.6 g ♂	1 ml	26.3 mg	1.27 mg/g	survived
		21.6 ♂	〃	〃	1.21	〃
		23.4 ♂	〃	〃	1.12	〃
2	CM 3/2 800 ml	20.6 ♀	1 ml	27.8	1.34	survived
		20.9 ♀	〃	〃	1.33	〃
		20.1 ♀	〃	〃	1.38	〃
3	CM 1/4 400 ml	23.1 ♂	1m l	22.15	0.95	died 29h
		21.9 ♂	〃	〃	1.00	〃 56h
4***		21.9 ♂	1m l	23.6	1.07	survived
		21.2 ♂	〃	〃	1.11	〃

Remarks: * CM 4/1 means chloroform-methanol mixture (4:1, v/v).
** Nearly equal amount of Tween 60 (Polyoxyethylene sorbitan monostearate) was added to lipid specimen as a surfactant. It was emulsified and injected intraperitoneally. (ip)
*** Residual part remaining in the column was squeezed and concentrated.

For further purification of A. I. L. Fract. 3, it was subjected to the second chromatography using the solvent system of chloroform-methanol mixture in a ratio (CM 9:1, 1:1, 1:9, v/v) respectively. The results are given in Table 3. The authors found toxicity exclusively in the last fraction. (designated as A. I. L. Fract. 3-3).

Table 3. Toxicity test of each fractions obtained by silicic acid chromatography using A. I. L. fract. 3 as a sample.

Fraction No.	Solvent system	Body weight & sex of mice	Volume & weight of injected lipid*			Death or survival Death time
Fr3—1**	CM 9/1 500 ml	20.0 g ♀	1 ml	27.1 mg	1.35 mg/g	survived
Fr 3—2	CM 1/1 1000 ml	21.9 ♀	1 ml	33.1	1.56	survived
		20.9 ♂	〃	〃	1.58	〃
		20.6 ♀	〃	〃	1.60	〃
Fr 3—3	CM 1/9 500 ml	20.9 ♂	1 ml	33.1	1.58	died 18h
		21.1 ♂	〃	〃	1.56	〃
		21.2 ♂	〃	〃	1.56	〃

Remarks: * Surfactant Tween 60. ip. injection.
** Owing to the shortage of the sample, only one mouse was injected.

(B) Ethanol insoluble lipid (E. I. L)

Although the yield of E. I. L. was much lower than that of A. I. L., its toxicity surpassed the latter. This lipid was also obtained by the Thiele's method and subjected to the silicic acid chromatography. The progress of elution was followed by phsophorus determination similarly as before (Fig. 3). The results of the toxicity test are shown in Table 4.

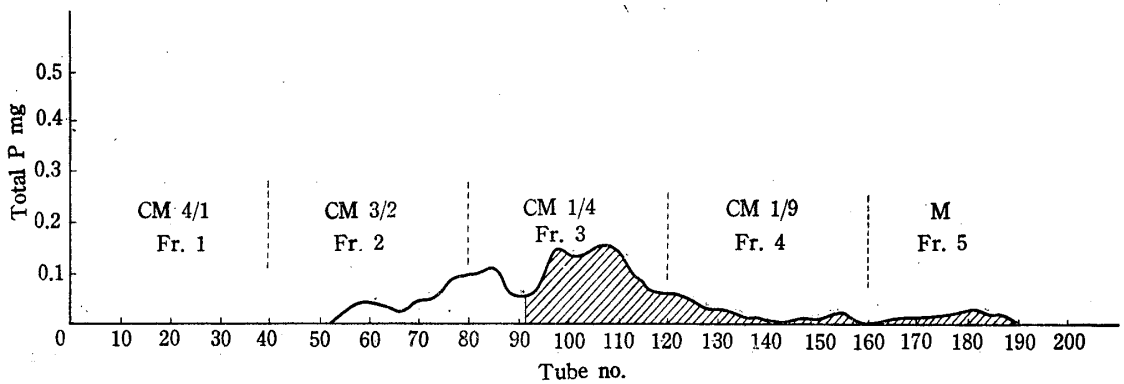


Fig. 3. Chromatogram of E. I. L on a 60 g silicic acid-30 g celite 545 column.

Contrary to the A. I. L., it was a characteristic of this lipid that residual toxicity was observed in methanol washings. Toxicity was observed in the shaded area.

Table 4. Toxicity test of each fractions obtained by silicic acid chromatography using E. I. L. as a sample.

Fraction No.	Solvent system	Body weight & sex of mice	Volume & weight of injected lipid*			Death or survival Death time
1	CM 4/1	22.7 g ♀	1 ml	40.1 mg	1.76 mg/g	Survived
		24.6 ♀	〃	〃	1.63	〃
		26.1 ♀	〃	〃	1.53	〃
2	CM 3/2	22.4 ♀	1 ml	30.1	1.34	Survived
		25.2 ♀	〃	〃	1.19	〃
		26.2 ♀	〃	〃	1.14	〃
3	CM 1/4	23.3 ♀	1 ml	30.3	1.30	Died 22h
		23.0 ♀	〃	〃	1.31	〃
		22.2 ♂	〃	〃	1.36	〃
4	CM 1/9	24.7 ♀	1 ml	32.1	1.29	Died 70h
		22.1 ♀	〃	〃	1.45	〃 22h
		21.2 ♀	〃	〃	1.51	〃 〃
5	M**	25.0 ♂	1 ml	30.7	1.22	Died 22h
		24.0 ♂	〃	〃	1.27	〃
		22.0 ♀	〃	〃	1.39	〃

Remarks: * Surfactant Tween 60. ip. injection.
** Residual part was washed out with methanol.

(C) Petroleum ether insoluble lipid (P. I. L.)

Sakai reported on the occurrenec of toxicity in ethyl ether insoluble fraction. The authors found sporadic toxicity in petroleum ether insoluble fraction, however, owing to the smallest amount of the fraction, whether this is really a toxic entity or contaminated by other toxic fraction, is still obscure.

3. Trials of purification of A. I. L. by the Pangborn method (5).

The author attempted to examine the efficacy for purifying the A. I. L. by the Pangborn method. The materials used were A. I. L. from the fish roe and the beef heart. The purification procedure involves the following steps; In the case of beef heart, the material was treated with acetone five times and dehydrated to dry powder. In the case of A. I. L., this step was omitted. Then the material was extracted with 95 per cent ethanol by shaking for half an hour on a mechanical shaker. The alcoholic extract was filtered by suction and the filter cake discarded. The alcoholic extract was precipitated with a slight excess of 50 per cent $CdCL_2$ solution and stood in a refrigerator. The lecithin-$CdCL_2$ precipitated was washed twice with acetone, then dissolved in chloroform. The chloroform solution was poured with constant mixing into seven fold volume of ethanol to which a small amount of 50 per cent $CdCL_2$ solution was added. After the mixture had stood about 10 minutes at room temperature with frequent shaking, the precipitate flocculated, leaving a clear supernatant; the mixture was filtered by suction. The precipitate was redissolved in chloroform and the solution poured into alcohol containing $CdCL_2$ exactly as before. This treatment

was repeated. After three chloroform-alcohol precipitations, the cadmium salt was suspended in petroleum ether to which was added petroleum ether saturated 80 per cent ethanol containing 0.1 per cent $CdCL_2$ (this solution is expressed for convenience as the 80 per cent alcohol mixture). The suspension was shaken in a separatory funnel until solution was complete; The petroleum ether layer was reextracted twice more with the 80 per cent alcohol mixture. The combined extracts were concentrated in vacuo to approximately two-thirds of the original volume and refrigerated at −5℃ overnight. The precipitated lecithin-$CdCL_2$ was dissolved in chloroform to which was added an equal volume of 30 per cent aqueous alcohol and shaken. The upper alcoholic layer was tested to check cadmium-free with silver nitrate solution. This was repeated several times until completely free from cadmium. Then the cadmium-free chloroform solution was evaporated to dryness, washed with acetone and dissolved in anhyd. ether. To ether solution was added 1/5 volume of acetone and stood overnight in a refrigerator. A finely flocculent, dense precipitate was removed and the supernatant was evaporated to dryness in vacuo and the residue was dissolved in absolute ethanol (Step A). At this step, a part of the residue was taken and its toxicity was checked up. (cf, Table 5, test No.2).

Table 5. Toxicity test of the purified fraction from A. I. L. and beef heart lecithins by Pangborn's method.

Test No.	Starting material	Body weight & sex of mice	Volume & weight of injected lipid	Death or survival Death time	Remarks
1	A.I.L. from fish roe (toxic)	18.8g ♀	1 ml 5.4 mg 0.28 mg/g	survived	completely purified product.
		18.7 ♀	〃 〃 0.28	〃	
		18.0 ♀	〃 〃 0.30	〃	
		21.6 ♀	〃 10.84 0.50	〃	
		19.2 ♀	〃 〃 0.56	〃	nontoxic
		19.7 ♀	〃 〃 0.55	〃	
		22.3 ♀	〃 22.7 1.01	〃	
		20.5 ♀	〃 〃 1.10	〃	
		19.4 ♀	〃 〃 1.17	〃	
		20.3 ♀	〃 31.3 1.54	〃	
		19.7 ♀	〃 〃 1.58	〃	
		21.0 ♀	〃 〃 1.49	〃	
		20.6 ♀	〃 38.9 1.88	〃	
		20.5 ♀	〃 〃 1.89	〃	
		22.4 ♀	〃 〃 1.73	〃	
2	A.I.L. from fish roe (toxic)	23.3 ♀	1 ml 31.3 1.34	died 116h	half-purified product at step (A) in the text. retains toxicity
		21.4 ♀	〃 〃 1.46	〃 18h	
3	Beef heart lecithins	21.8 ♀	1 ml 31.6 1.44	survived	Mixture of phosphatidal- and phosphatidyl cholines.
		25.1 ♂	〃 〃 1.25	〃	
		22.1 ♀	〃 〃 1.42	〃	
		20.4 ♀	〃 34.7 1.66	〃	
		21.8 ♀	〃 〃 1.59	〃	
		20.1 ♀	〃 〃 1.72	〃	nontoxic

The alcoholic solution was made alkaline to phenolphthalein with saturated baryta solution. The mixture was neutralized with CO_2 and a small amount of saturated NaCL solution to flocculate the precipitate. Precipitated acidic phospholipids were removed and the filtrate was reprecipitated with $CdCL_2$. The cadmium salt was twice precipitated by pouring the chloroform solution into alcohol and was fractionated once by the petroleum ether-80 per cent alcohol method as before. Cadmium was removed and the residue (cadmium-free lecithin) was dissolved in ether, precipitated with acetone and refrigerated at −5°C overnight. The toxicities of the purified fractions from both A. I. L. and the beef heart were tested (Table 5).

4. Characterization of toxic lipid fractions.

(A) Chemical analysis of the components of toxic lipid fractions.

Nitrogen determination was made by micro-kjeldahl method and phosphorus by the Allen's method (6). Choline liberated in the hydrolysates of lipid was estimated by the Kushner's method (7) and by the Glick's method (8). Higher fatty aldehyde determination was made by p-nitrophenylhydrazine method of Wittenberg (9) and by the modification of Schiff reaction by Rapport (10). Iodine value was estimated by the Wijs' method (11). The results obtained by these methods are summarized in Table 6.

Table 6. Chemical analysis of the components of toxic lipid fractions.

Fraction	P%	N%	N/P	Choline%	Aldehyde%	Iodine value	Toxicity*
A. I. L.	3.54	2.72	1.70	13.11(K)** 11.88(G)	11.86(W)**	97	LD_{100} 15 mg LD_{50} 10 mg
A. I. L. Fract. 3	3.62	2.08	1.27	13.98(K) 15.79(G)	10.93(W)	108	
A. I. L. Fract. 3-3	2.63	5.54	4.66	12.41(K)	10.75(W) 11.88(S)	108	LD_{100} 10 mg LD_{50} 7 mg
E. I. L. Fract. 3	2.07	7.48	8.02	trace	8.34(W) 8.00(S)	107	LD_{100} 5 mg
P. I. L.	0.27	8.23	67.4	trace	9.75(W)	26	
Purified fraction from A. I. L. by Pangborn method	3.99	1.50	0.83	14.27(G)		152	nontoxic by i. p.
Beef heart sample by Pangborn method◎	3.75+	1.60#	0.94	12.89		109+	nontoxic by i. p.

Remarks: * LD_{50} 50% lethal dose (20g mouse) LD_{100} 100% lethal dose (20g mouse)
** (K) Kushner method (G) Glick method (W) Wittenberg method (S) Schiff's modification method
◎ Mixture of phosphatidal- and phosphatidylcholines prepared by the Pangborn method.
+ Figures reported by Rapport. # Figures obtained by the author.

(B) Thin layer chromatography

Thin layer chromatography of toxic lipid fractions using silica gel (Kiesel gel nach Stahl, Merck) was carried out. Samples examined were A. I. L. Fract. 3-3 and E. I. L. Fract. 3 (Table 4) from the fish roe. Authentic phosphatidyl serine and phosphatidyl ethanolamine were also used as reference substances. After development with the solvent mixture of chloroform-methanol-water (65 : 25 : 4, v/v), the following reagents were sprayed on the chromatograms; Aqueous H_2SO_4 (1 : 1), 1 per cent iodine in methanol, 1 per cent potassium permanganate, 0.3 per cent ninhydrin in water-saturated n-butanol, rhodamine B, 0.1 per cent bromthymolblue in 95 per cent ethanol, Dragendorff reagent, 0.2 per cent 2,4-dinitrophenylhydrazine in 3N HCL, aniline hydrogen phthalate, acid ammonium molybdate reagent for phosphorus, Chargaff reagent, hydroxylamine-ferric chloride reagent and Schiff reagent.

Both A. I. L. Fract. 3-3 and E. I. L. Fract. 3 are not single substances and still contain certain impurities. The principal spot of A. I. L. Fract. 3-3 was located at Rf 0.28-0.40 and showed positive reactions to the following reagents; Phosphorus (+), Schiff (卅), Choline (卅), acetal lipid (卅), Dragendorff (+), unsaturation (+). The principal spot of E. I. L. Fract. 3 which was located at Rf 0-0.20 showed positive to the following reagents: Phosphorus(+), Schiff(+), acetal lipid(sl.+), unsaturation(+). Both lipid samples showed ninhydrin positive at origin. Chromatograms are illustrated in Fig. 4.

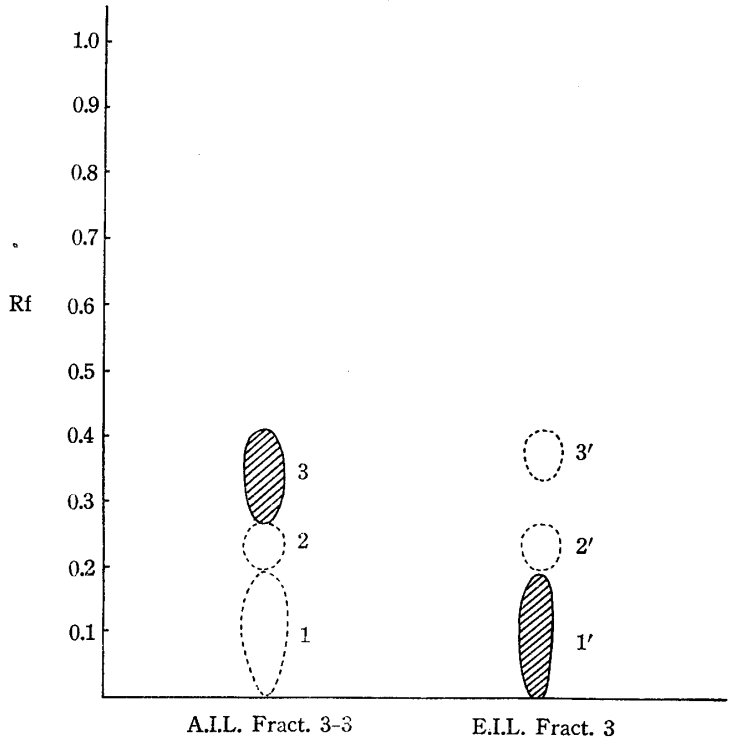
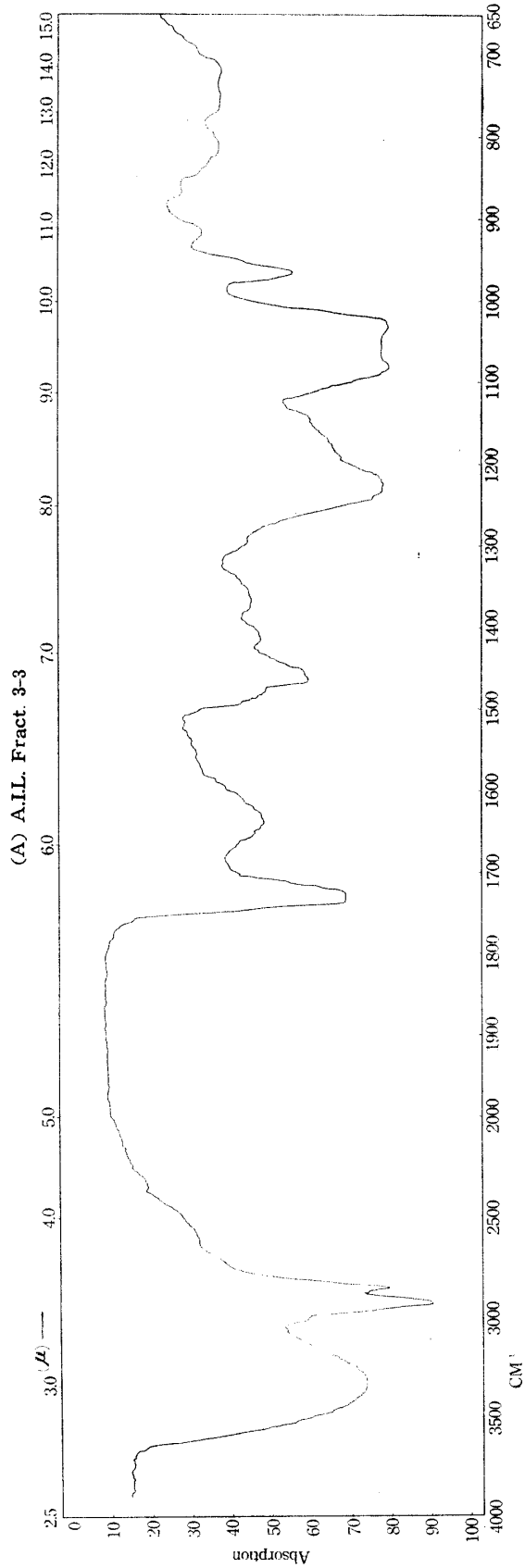


Fig. 4. Thin layer chromatography of toxic lipid fractions, Solvent system: chloroform-methanol-water (65 : 25 : 4, v/v)


(A) A.I.L. Fract. 3-3
Absorption
CM⁻¹
(μ)

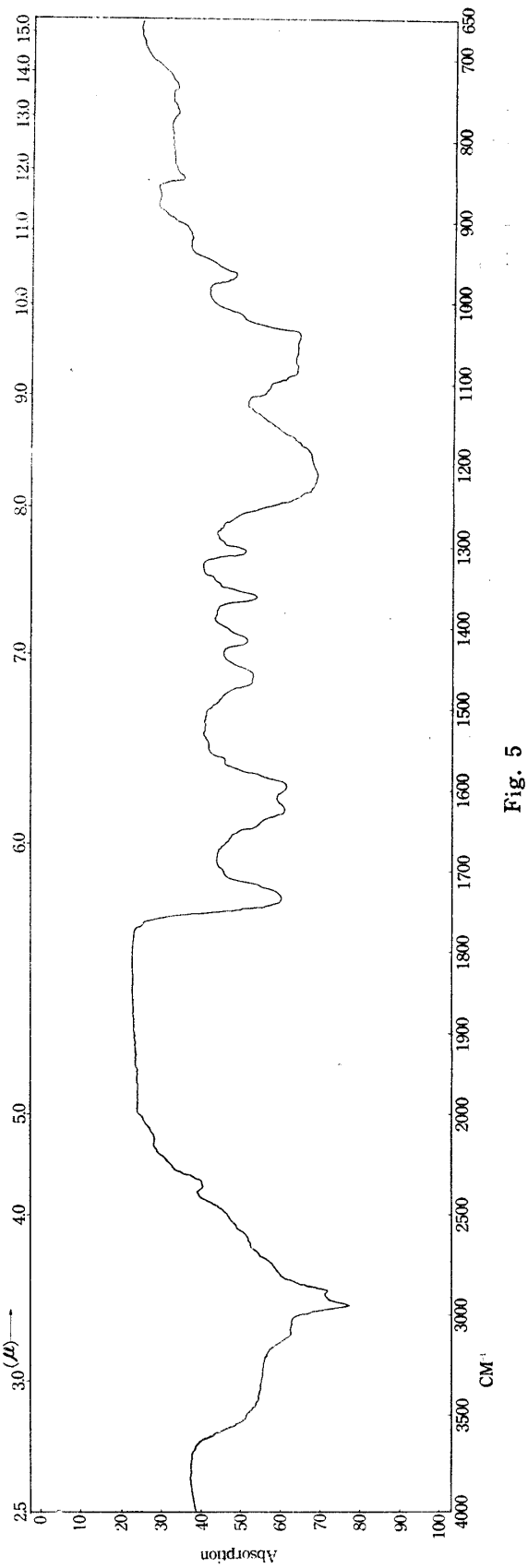

(A') A.I.L. Fract. 3-3 Dried sample.
Absorption
CM⁻¹
(μ)

Fig. 5

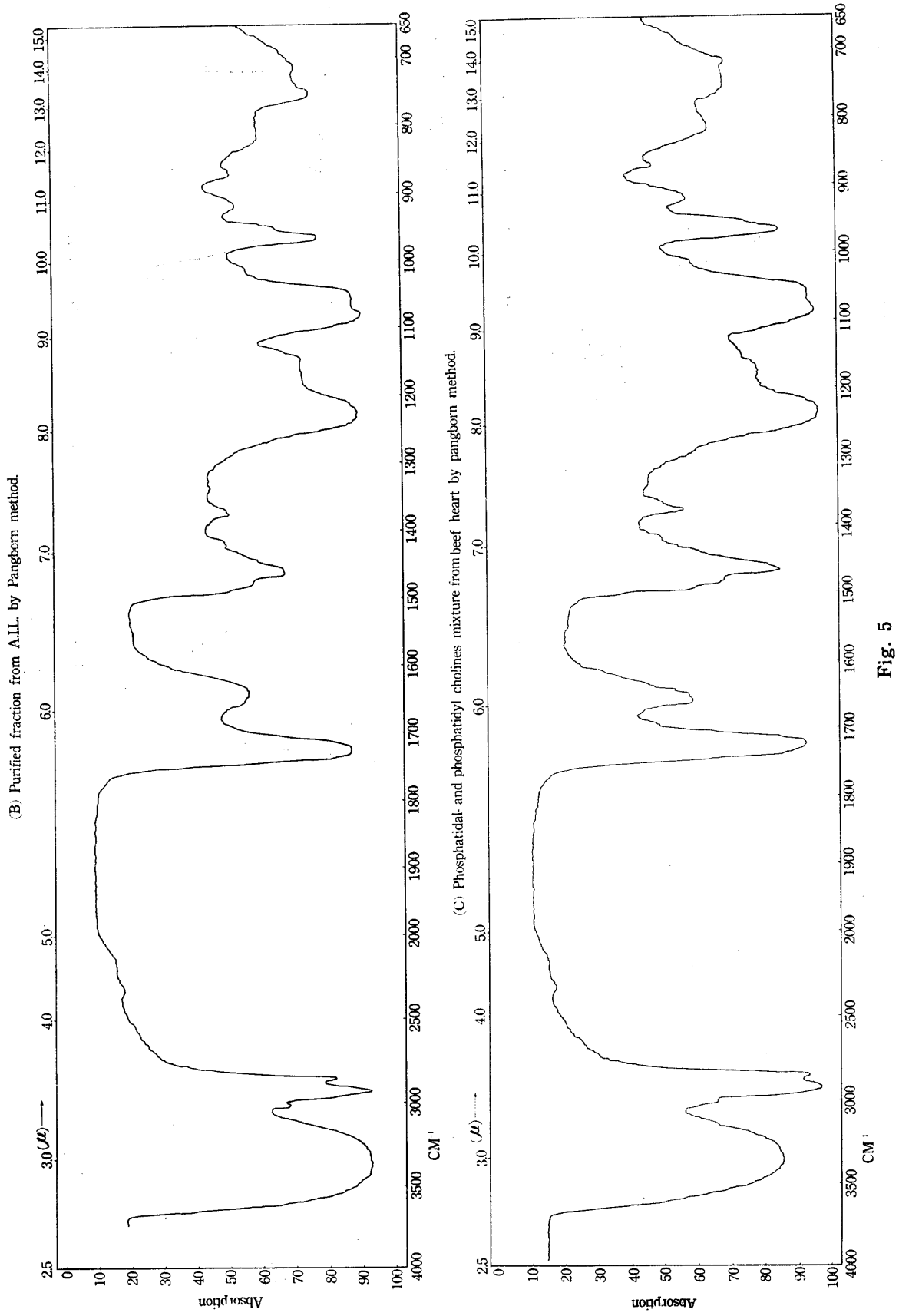

(B) Purified fraction from A.LL. by Pangborn method.
(C) Phosphatidal- and phosphatidyl cholines mixture from beef heart by pangborn method.
Absorption
CM⁻¹
(μ)

Fig. 5

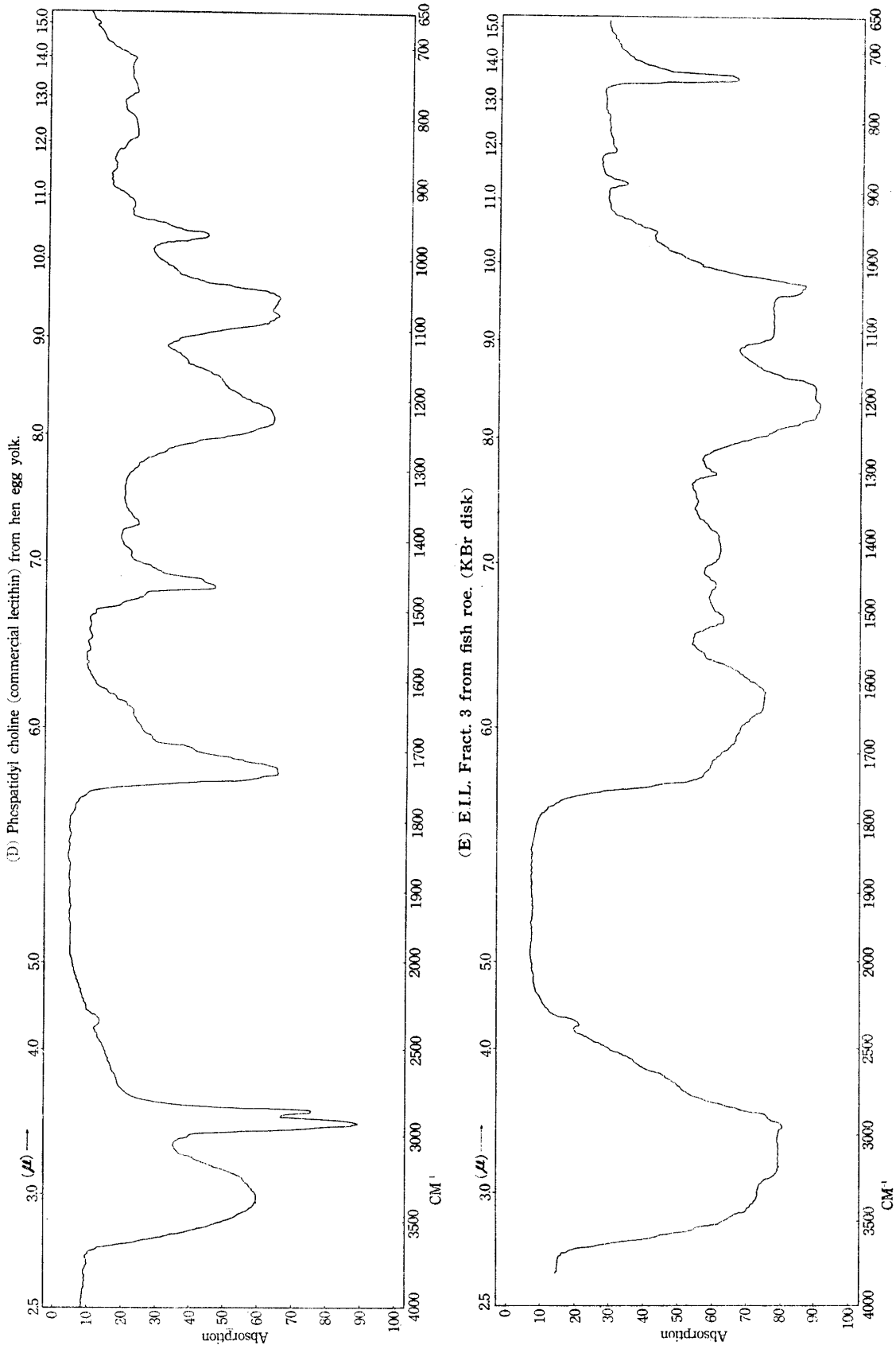

(D) Phospatidyl choline (commercial lecithin) from hen egg yolk.
(E) E.I.L. Fract. 3 from fish roe. (KBr disk)
Absorption
CM⁻¹
(μ)

Fig. 5

(C) Infrared absorption spectra

Samples measured were A. I. L. Fract. 3-3, E. I. L. Fract. 3 (these are derived from the fish roe), commercial lecithin from egg yolk (Merck), and phosphatidal- and phosphatidylcholines mixture from the beef heart prepared by the Pangborn method. The spectra were measured by the so-called "sandwitch method" except

Table 7A. The probable assignment of infrared absorption bands.*

Wave number (cm^{-1})	Probable assignment	Remarks
3340—3360	νO-H (Polymeric, intermolecular)	broad, strong
2925—2930	ν_{as}C-H(CH_2, CH_3)	sharp, strong
2850	νC-H(CH_2, CH_3)	strong
1730	νC=O(Carbonyl)	strong
1655	νC=C (α, β unsaturated ether)	medium, present in beef heart preparation. absent in egg lecithin.
1640	νC=C(shift) or δN-H(Deformation of NH)?	medium or weak present only in fish samples
1460—1465	δC-H(CH_2, CH_3)	medium
1415	δC-H(=CH_2)	weak
1375	δC-H(CH_3)	weak
1235	νP=O and ν_{as}C-O-C	strong, broad overlapped stretchings
1085	νP=O-C	strong, broad
1050	νP-O-C	strong
965	δC-H	strong-medium

* These bands were commonly observed in the spectra of A. I. L. Fract. 3—3, purified fraction by the Pangborn method, beef heart sample and egg lecithin sample, except specified bands in remarks.
These spectra were measured with specimens prepared by the sandwitch method.

Table 7B. The probable assignment of infrared absorption bands.*

Wave number (cm^{-1})	Probable assignment	Remarks
2960—3460	νO-H(Polymeric) νN-H νC-H(CH_2, CH_3)	Composite band very broad and strong
1570—1715	νC=O(Carbonyl) CO Absorption(Amide 1) δN-H(Amide 11)?	Composite band very strong and broad
1510	δN-H(Amide 11)	weak
1460	δC-H(CH_2, CH_3)	weak
1400—1410	δN-H	weak, broad
1300	δN-H(Amide 111)	weak
1205—1210	νP=O and νC-O-C	overlapped stretching strong, broad
1040	νP-O-C	strong, sharp
735—740	Skeletal vibration -$(CH_2)_4$-	strong, sharp

* These bands were observed in the spectrum of E. I. L. Fract. 3 prepared by KBr disk method.

for E. I. L. Fract. 3 by KBr disk method, using Perkin-Elmer model 21 infrared spectrophotometer. The spectra are illustrated in Figures 5 A-E and the probable assignment of the absorption bands are given in Tables 7A-B.

5. Pharmacological aspects of toxic lipid fractions.

Previously the authors reported that both lipoprotein- and lipid injections into rats caused the symptom of a fatty liver due to the accumulation of fat in the liver (1). The author now tried to examine whether toxic lipid injection into mice also induce the similar symptom. Thus the curde fat contents of toxic lipid-injected mice liver were determined. The results are indicated in Table 8.

Table 8. Crude fat contents of livers of mice killed by injection of various lipid fractions.*

Treatment	Crude fat contents on wet basis	Remarks
Intact, non-injected	3.71%	Control, sacrificed
Injected with A. I. L. Fract. 3	9.00	
Injected with A. I. L. Fract. 3—3	6.89	
Injected with E. I. L.	5.42	
Injected with beef heart lecithins	4.88	survived after injection, therefore, sacrificed and analysed

* Surfactant Tween 60. ip. injection.

The crude fat contents in mice liver increased by the injection of toxic lipid as well as those in rats. Next, the author suspected that this "fatty liver" should be regarded as a real fatty liver or a kind of fat infiltration. Therefore, antagonism between A. I. L. Fract3-3 and some chemical agents was tested as in Table 9.

It is very likely that the toxic principle might be lost from A. I. L. during the process of purification. If this assumption is true, the toxic principle should be acidic phospholipid which was removed during the process. It is a problem to be examined further.

As shown in Table 6, phosphorus and nitrogen occur in these toxic lipids, indicating that these belong to "phospholipid" categories. The reversal of the order of both elements was observed, namely, for phosphorus, A. I. L., E. I. L., and P. I. L., and for nitrogen, P. I. L, E. I. L. and A. I. L. in decreasing order. The N/P ratio of A. I. L. Fract. 3-3 increased remarkably when compared to those of A. I. L. and A. I. L. Fract 3, showing a wide deviation from unity. The ratio increased much more in E. I. L. and P. I. L. Hatano(14) informed that he also observed the rise of N/P ratio (4.1) in A. I. L.. On the contrary, the ratio approached to unity in case of the purified fraction from A. I. L. and the beef heart specimen, both prepared by the Pangborn method.

Table 9. Antagonism between A. I. L. Fract. 3—3 and some chemical agents.

Fraction No.	Contents of injectant	Body weight & sex of mice	Volume & weight of injectant	Survival or death Death time
1(control)	A.I.L. Fract. 3-3* 159mg, Tween 60** 150.8 mg were emulsified in 5 ml of water	21.7 g ♀	1 ml 31.8mg 1.46mg/g	died 17 hours
		23.5 ♀	〃 〃 1.35	〃 41 〃
		25.2 ♀	〃 〃 1.26	〃 41 〃
2	A.I.L. Fract. 3-3 178.6 mg, L-methionine 150.1 mg, Tween 60 155.1 mg were emulsified in 5ml of water	21.5 ♀	〃 35.7 1.66	died 41 〃
		23.5 ♀	〃 〃 1.51	〃 41 〃
		21.2 ♀	〃 〃 1.68	〃 41 〃
3	A.I.L. Fract. 3-3 152.5 mg, egg yolk lecithin*** 158.3 mg, Tween 60 151.7 mg were emulsified in 5ml of water	23.7 ♀	〃 30.5 1.28	died 22 〃
		23.0 ♀	〃 〃 1.32	〃 22 〃
		22.4 ♀	〃 〃 1.36	〃 41 〃

Remarks: * Toxic fraction from A.I.L. of fish roe, purified by column chromatography.
** Surfactant.
*** Commercial product, Merck.

It is a significant feature that a considerable amount of choline exist in A. I. L. but not in E. I. L. and P. I. L..

Rapport (15) described recently that the substantial part of marine animal lipids are aldehydogenic and almost all plasmalogens in marine invertebrates have α,β-unsaturated ether structure. The author found that these toxic lipids are also aldehydogenic. Concerning the toxicity of lipid fractions, the toxicity of E. I. L. surpassed that of A. I. L., although the yield of the former was much lower than that of the latter. When compared the lethal doses of A. I. L. and A. I. L. Fract. 3-3, the dose of the latter should decrease about 1/25 theoretically, since the yield of toxic fraction after a run of column chromatography was roughly 20 per cent ($1/5 \times 1/5 = 1/25$). However, in fact, the toxicity rose up slightly, the reason being uncertain.

Thin layer chromatography of A. I. L. Fract. 3-3 and E. I. L. Fract. 3 showed that both fractions still contain trace contaminant in spite of their purification, however, it is evident that both are different entities.

Discussion

To characterize and identify the toxic lipid components may be a good approach to the elucidation of the poisonous principles in fish roe, consequently to the final solution of this food poisoning problem. Putting aside the detailed

examination of lipoprotein, the author attempted to characterize the toxic lipid components as far as possible in the present report. In search of a more efficient method, the comparison of different extraction methods was made. Among them, the Thiele's method originally designed for the extraction of choline plasmalogen, showed the highest yield, therefore, this method with additional modification was used for the routine preparation of toxic lipids afterward. (Table 1). For the purification of toxic lipid fractions, silicic acid column chromatography was employed throughout the experiment. As seen in Table 1, A. I. L. predominates in amount, followed by E. I. L. and then P. I. L.. Owing to the lowest yield and sporadic toxicity of P. I. L., it was left untouched in this report. Although E. I. L. is much lower in yield, its toxicity surpasses that of A. I. L. (Table 6), therefore, the author examined them separately. At first, from the data of chemical analysis, he suspected whether A. I. L. is identical or closely related substance to phosphatidalcholine named by Rapport (13). It is well known that the beef heart contains a large amount of phosphatidalcholine (about 60 per cent) together with common phosphatidylcholine. Recently Rapport (12) was able to prepare a pure native phosphatidalcholine from the beef heart by using the selective hydrolysis of the phosphatidylcholine component by snake venom (*Crotalus atrox* venom). Although the author did not perform enzymatic hydrolysis, he prepared the phosphatidal and phosphatidylcholines mixture from the beef heart by the Pangborn method. Parallel with it, he purified the fish roe lipid by the same method, using A. I. L. as a starting material. Thus, the beef heart specimen, the completely purified product of fish roe lipid and the partly purified roe lipid until step (A) were prepared and their toxicities were checked (Table 5). It was a very interesting finding that the beef heart specimen and the completely purified roe lipid were proved to be nontoxic, while the partly purified roe product still retains toxicity.

The infrared absorption spectrum of E. I. L. Fract. 3 differs appreciably from the other spectra, although the former only was measured by the KBr disk method because of its stickiness whereas the other spectra by the "sandwitch method". The most characteristic absorption distinguishing these samples are the band at 1650~1655 cm^{-1} which is attributable to "C=C" stretching in case of the beef heart sample, the band at 1640 cm^{-1} in case of A. I. L. Fract. 3-3 and the purified sample from A. I. L. by the Pangborn method, and very broad composite band at 1615~1630 cm^{-1} in case of E. I. L. Fract. 3. The workers of the Hokkaido University (13) assumed that the band at 1640 cm^{-1} is attributable to amide linkage and referred to its possible relationship to toxicity. However the author found that the purified fraction from A. I. L. by the Pangborn method retains the band at 1640 cm^{-1} but lost its original toxicity (Table 5). Since the band at 1640 cm^{-1} is lacking in the spectrum of the usual lecithin, it certainly represents one of the significant bands for characterizing the structure of toxic

lipid molecule. Very broad band near 3300~3400 cm^{-1} indicates the presence of intermolecular polymeric hydrogen bond. Especially in case of E. I. L. Fract. 3, two very broad composite bands were observed, one ranging 2960~3460cm^{-1} and the other at 1570~1715 cm^{-1} (peak 1615~1630 cm^{-1}). Although it is very difficult to assign these composite bands, the author assumed that the former includes N-H stretching and the latter includes amide deformation in addition to the corresponding absorption bands as shown in Table 7A. The absorption near 1000~1250 cm^{-1} found in all spectra showed that all of them belong to phospholipid category. According to Rapport (12), α,β unsaturated (vinyl) ethers should show a characterisric strong band at 1200 cm^{-1} attributable to C-O-C deforming, however, this can not be observed in the spectrum of pure phosphatidalcholine probably due to the masking by P-O absorption at 1200~1250 cm^{-1}. The author observed the identical band at 1235 cm^{-1} in four samples and at 1205~1210 cm^{-1} in E. I. L. Fract. 3. Another characteristic band in E. I. L. sample is a strong sharp band at 735~740 cm^{-1} which may be attributable to skeletal vibration.

Intraperitoneal injection of toxic lipid into mice caused the similar symptom as previously reported in case of rats. However, the simultaneous injection of toxic lipid with the lipotropic substances such as L-methionine proved to be unefficient for preventing death.

Therefore, it is better to regard the symptom as "fat infiltration" caused by the decrease of liver function rather than as the "fatty liver" caused by the deficiency or unbalance of nutrients. The exact nature of the accumulated fat in the livers of dead animals, the mechanism of causing fat infiltration should be clarified in future. At the same time, the decision of toxic lipids being artifact or inherent in its origin, the confirmation of toxicity by oral administration of toxic lipoprotein and lipids are the problems to be solved. Anyway, the occurrence of these toxic "glycerophospholipid" (glycerol occurs in A. I.L. Fract. 3-3 and E. I. L. Fract. 3) which have such high N/P ratios, in a blenny fish roe is very interesting from not only food sanitary aspects but also from comparative biochemical points of view, as well as Ciguatera type toxin in reef and inshore fishes in the Central Pacific, although the latter belongs to acetone soluble lipid(s).

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Summary

1. Among the various methods, Thiele's method for the extraction of choline plasmalogen, showed the highest yield, therefore, it was employed afterward for the extraction of toxic lipids from the fish roe.

2. Acetone insoluble lipid (A. I. L. Fract. 3-3), the most predominant toxic fraction, is a complex lipid containing phosphorus, nitrogen, choline, aldehyde and glycerol as the components of molecule. It is not identical with phosphatidal-choline.

3. Ethanol insoluble lipid (E. I. L. Fract. 3), occurs much less in amount, however, it surpasses A. I. L. in toxicity. E. I. L. is also a complex lipid containing phosphorus, nitrogen, aldehyde, and glycerol as its components. Higher N/P ratio, none or trace in choline are the characteristics of this lipid.

4. From the chromatographic behavior, solubility, chemical data, and infrared spectra, the author concluded that toxic lipids in the fish roe consist of at least two different complex lipids described above.

5. The Pangborn method used for the purification of lecithin, is not applicable for the purification of A. I. L., since its toxicity is lost during the process of purification.

6. Intrapritoneal injection of toxic lipids into mice caused the increase of crude fat contents in animal livers as well as in rats. Simultaneous injection of toxic lipid with lipotropic substance proved to be unefficient for preventing death, therefore, this fat accumulation in livers should be regarded as fat infiltration rather than fatty liver of dietary origin.

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