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Abstract

For the purpose to reveal the mechanism of the biological indirect action of X-rays the author has conducted the analysis of the compound lipids of the organs of the rabbits irradiated with X-rays. Silicic acid column chromatography was applied for the fractionation of the compound lipids. Component of each fraction was analyzed by silicic acid-impregnated paper chromatography and infra-red spectrophotometory. The result proved that the compound lipids showed some qualitative and 10+-+ quantitative changes. The changes occurred mainly in glycerophosphatides showing the formation of high level lyso-phosphatidylcholine and lysophosphatidylethanolamine. The fatty acid compositions of the compound lipids were determined by means of gas-liquid chromatography. Further, polyunsaturated fatty acids were analyzed as fatty acid bromide. Major differences found in fatty acid patterns included: in the irradiated group the amount of C18-monoenoic, C18-dienoic, C18-trienoic, C20-tetraenoic and C22-acids were decreased and of C18- and C18-monoenoic acids were increased. It has been elucidated that lysophosphatides not only posesses a strong hemolytic power but also it has an action to induce swelling of the rat liver mitochondria. And it has been suggested that the lysophosphatides and ethanolamine will be responsible, at least partially, for the cell damage induced by X-rays.

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STUDIES ON THE COMPOUND LIPIDS FROM X-RAY IRRADIATED ANIMAL

I. CHARACTERISTICS OF COMPOUND LIPIDS FROM THE ORGANS OF X-RAY IRRADIATED RABBITS

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X-rays may damage cells or biological system by its direct and indirect actions, i. e. by both direct disintegration of the organizing substances and metabolic disturbances induced secondarily by abnormal radiation products. Therefore it is reasonably supposed that the damage of the cells in the organ may be different from those *in vitro* in their reaction modes to X-ray irradiation. Because the former will receive various inderect effcts by the radiation products which may reach them from other part of the organism, whereas the cells *in vitro* may escape from such effects. Actually, the cells in culture show almost no recognizable change by irradiating with a X-ray dose in a short period with which the living organisms or the cells in the animal body are severely damaged. This will mean that some toxic substances should be produced in animal body by irradiation and these are carried to various organs by blood or lymph stream. YAMAMOTO[1] found a strong hemolytic activity in the ethanol soluble and acetone insoluble fractions of the liver from the X-ray irradiated rabbits and this substance was supposed to be lysolecithin produced by the activation of lecithinase by X-ray irradiation. YAMAMOTO considered that this substance should be responsible for the anemia seen after irradiation. This supposition was supported by a few facts, e. g. the grade of the hemolytic activity of the acetone insoluble fraction increased paralell with the dose of X-rays, similar damage induced by X-ray irradiation could also be induced by injecting the substance in the animal, etc.[2]

On the basis of these observations the author aimed at getting the substance from various organs of the rabbit irradiated with X-rays, purifying and identifying the substance chemically.

MATERIALS AND METHODS

Normal rabbits weighing 2.5 to 3 kg served as materials. For each experiment 5 to 10 animals were used. The rabbits were irradiated with 3,000 r (in

air) on the whole body once by means of Toshiba KXC Type 18: the secondary voltage of 200 KV, 25 mA, filter Cu 0.5 mm + Al 0.5 mm, T. S. D. 40 cm, and H. V. L. Cu 1.37 mm, 116.6 r/min. Twenty-four hours after irradiation the animal were sacrificed by blood depletion, and liver, spleen, pancreas, lung, kidney were put togather and homogenized with an equal volume of ethanol. For the isolation of compound lipids from this homogenate the procedures as shown in Fig. 1 were employed. The acetone insoluble fraction containing the compound lipid is designated as CLX for short and the same fraction from the non-irradiated rabbits is designated as CL. Their yields are shown in Table 1.

Silicic acid column chromatography were applied for the fractionations of CLX and CL. The column (2.7 × 40 cm.) having glass filter (G3) and stopcock at

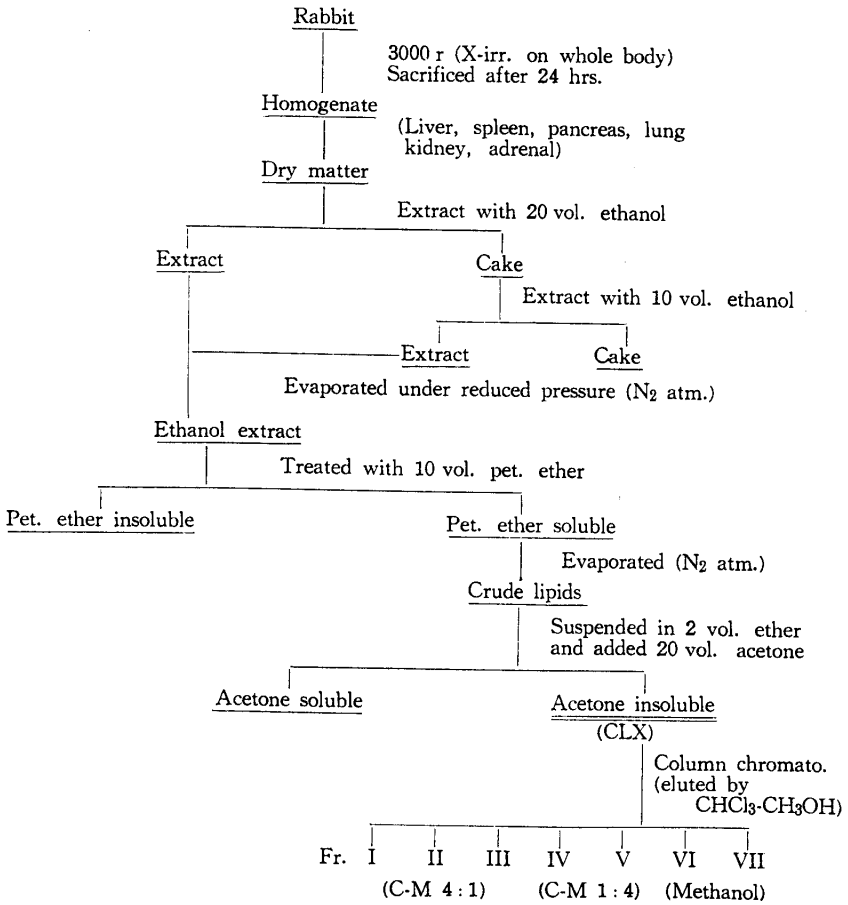

Fig. 1. Separation of Lipids from X-ray Irradiated Rabbit Organs

Table 1. Effect of 3,000 r of Whole Body X-ray Irradiation on Lipid Fractions of the Rabbit Organs

	X-ray irradiated (Mean value of 15 bodies)	Control (Mean value of 20 bodies)
Total lipid (Pet. ether soluble part)	22.0%	17.6%
Simple lipids (Acetone sol. part)	12.0%	10.8%
Unsaponifiable matter	(1.26%)	(1.19%)
Compound lipids (Acetone insol. part)	9.7%	6.6%

Yield % to dry matter

bottom was used. Before the use, the absorbent, silicic acid mixed with Hyflo-Super cel 2/1 (w/w) was dried for two hours at 120°C and cooled by leaving it in a desiccator. The 37 g of absorbent were used for one gram of the sample. Preparing the column by the routine procedures[3], the test material dissolved in chloroform-methanol 4/1 (v/v) was put in the tower. The minimum quantity of chloroform-methanol required to dissolve the material was used. Then the elution was done with chloroform-methanol of 4/1 (v/v) at first, then of 1/4, and finally with pure methanol. The flow rate was fixed at 1 ml/min. and each 20 ml of the eluate was taken separately in the calibrated flasks. The solvent was evaporated out at about 45～50°C under a stream of hydrogen at reduced pressure. The residue was further dried in desiccator. The dried substance was weighed and thus the quantity of the eluate in each tube was estimated.

Each sample was analyzed on infrared absorption spectra, Shimazu infrared spectrophotometer, AR-275 type, which had NaCl prism. The sample was measured as KBr discs or Nujol paste.

Quantitative analysis of nitrogen and phosphorus was carried out by the micro Kjeldahl method for the former and by ALLEN's method[4] for the latter.

Silicic acid-impregnated paperchrtomatography was used for the identification of phospholipids. Silicic acid-impregnated paper was made on Toyo filter paper No. 51-A by MARINETTI's method[5]. The base line was at 3 cm from the bottom edge of the paper and the sample was spotted at the interval of 2.5 cm apart. Di-isobuthyl-ketone - acetic acid - water 40/20/3 (v/v) solution was used as solvent, and development was carried for 2.5～3 hours at 22°～24°C. After drying completely, the paper was immersed in distilled water for 10 minutes and the analysis was conducted by the following methods:

a) Rhodamine 6G test for phospholipid: The dried paper is immersed in Rhodamine 6G solution (1.2 mg per cent) for one to two minutes, washed immediately with tap water for 20 minutes, check any change in the color tone under an ultraviolet lamp, and then dry for the calculation of Rf.

b) Ninhydrin test for aminophosphatide: A 0.25 per cent solution of ninhydrin in acetone-

2, 4-lutidine 9/1 (v/v) is sprayed over the dried chromatogram, and after leaving it at room temperature for five hours, amino-lipids appear as violet spots.

c) Test for choline: The dried chromatogram is immersed into one per cent phosphomolybdic acid aqueous solution for ten minutes and after washing in running tap water for 20 minutes and again immersing in one per cent $SnCl_2$-3 N HCl mixture. Choline is detected as blue spots.

d) Test for plasmalogen: The chromatogram is further washed in running tap water for 20 minutes and then immersed in 0.15 per cent 2,4-dinitrophenylhydrazine 3N HCl mixture for two minutes. Then wash again with tap water (four times for ten minutes each), and after drying plasmalogen is detected as yellowish-brown spots.

e) Test for unsaturated group:

i. Potassium permanganate test. The chromatogram washed with water is immediately immersed in one per cent $KMnO_4$ aqueous solution for one minute, washed with running tap water sufficiently and dried. Unsaturated compound is detected as brown spots.

ii. Iodine test. The dried chromatogram is suspended in a cylinder containing iodine and when the bottom of the cylinder is warmed for a moment unsaturated lysolecithin appears as bright yellow spots and saturated lysolecithin as yellowish-brown spots.

For the detection of component fatty acids of CLX and CL each fraction was saponified by refluxing with 10 volumes of N KOH methanol for 6 hours. After diluting with 2 volumes of water unsaponifiable matter was removed by extraction with ether. The resulting aqueous soap solution was acidified with 10 per cent HCl and fatty acids were extracted with ether, washed with water, and dried. Solvent was evaporated under a stream of hydrogen at reduced pressure (in this instance a white crystalline substance, which separated out at the interface, was removed by filtration) and thus mixed fatty acids were obtained. The component fatty acid isolated from CLX is designated as CLXF for short and that from CL as CLF.

Chemical properties of the fatty acids were determined by the standard oil and fat analytical method of Am. Oil Chemists' Society[6]. For the estimation of iodine value Wijs' method was used.

CLXF and CLF were esterified with diazomethane solution in ice cooled ether. The methyl esters were analyzed by gas-liquid chromatography using Shimadzu Model GC-2B. Column was $4 \times 3{,}000$ mm Cu-tube packed with 30 per cent diethylene glycol succinate polyester on 60～80 mesh fire brick. Hydrogen was used as the carrier gas. The area per cent for each component was determined by triangulation.

For the detection of polyunsaturated fatty acids bromide method was applied. CLXF and CLF were dissolved in about 40 volumes of ether, cooled at below 0°C and excess Br_2 was added. The mixture was left standing overnight at the temperature below 5°C. The precipitated bromide was harvested by filteration and washed three times with cold ether. Brom content of this bromide was measured by a slightly modified method of Boubigny-Chavaune[11].

For the detection of combined sugars the materials were hydrolyzed in 2 **N** HCl methanol solution for 24 hours by DOWSON's method[7], and then the oil components were eliminated with petroleum ether, and sugars and inositol contained in aqueous layer were identified by paper chromatography using propanol-ethanol-water 5/3/3 (v/v) solution as solvent and detected with TREVALYAN reagent[9].

Hemolytic test: CLX and CL were dissolved in physiological saline solution to a desired concentration. Each 2 ml of this solution was mixed with 2 ml of red cell suspension (which was prepared by suspending one ml of packed cell to 100 ml of physiological saline). The suspension was left standing at room temperature for 15 minutes and at 5°C for 30 minutes. Then it was centrifuged for five minutes at 1,500 r. p. m. The optical density of the supernatant was measured by Beckmann type Hitachi spectrophotometer at the wave length of 550 mμ. As blank the supernatant of the blood cell suspension similarly prepared but without CLX or CL was used. The degree of hemolysis was represented by the percentage of the optical density at complete hemolysis.

Swelling test of mitochondria: Mitochondria was isolated from the liver of albino rat by HOGEBOOM and SHNEIDER's method[9], and it was washed twice with 0.25 **M** sucrose solution. Mitochondria contained in 1 g liver tissue was suspended in 2 ml of 0.25 **M** sucrose solution. Such a stock mitochondrial suspension was freshly prepared before the use. To test the swelling of mitochondria LEHNINGER's method[10] was employed. As the suspension of mitochondria 2.7 ml of the suspension were used, which was prepared by mixing 0.25 ml of stock mitochondria with 10 ml of the mixture solution of 0.15 **M** KCl- tris-aminomethane buffer solution (pH 7.4). At observation 0.3 ml of the sample (or 0.15 **M** KCl- tris-aminomethane buffer as a control) was added to the mitochondrial suspension, and the change in the optical density was recorded from 0 to 30 min. at the wave length of 520 mμ by Beckmann type Hitachi spectrophotometer.

RESULTS

The column chromatographic fractionation of the compound lipids, CLX and CL, have been carried out under exactly the same conditions. Peaks were identified by the paper chromatography and infra-red spectroscopy to demonstrate whether each eluate of the peak in column chromatography of CLX corresponded to respective peak of ones in CL. The result coincided with some difference in detail. Seven peaks in CLX and 5 peaks in CL appeared as illustrated in Fig.s 2A & B. Five experiments repeated showed the same results.

To investigate the biological properties of each fraction, the effects of hemolysis and the mitochondrial swelling have been observed. Observations have revealed that the hemolitic activity of CLX is considerably stronger than

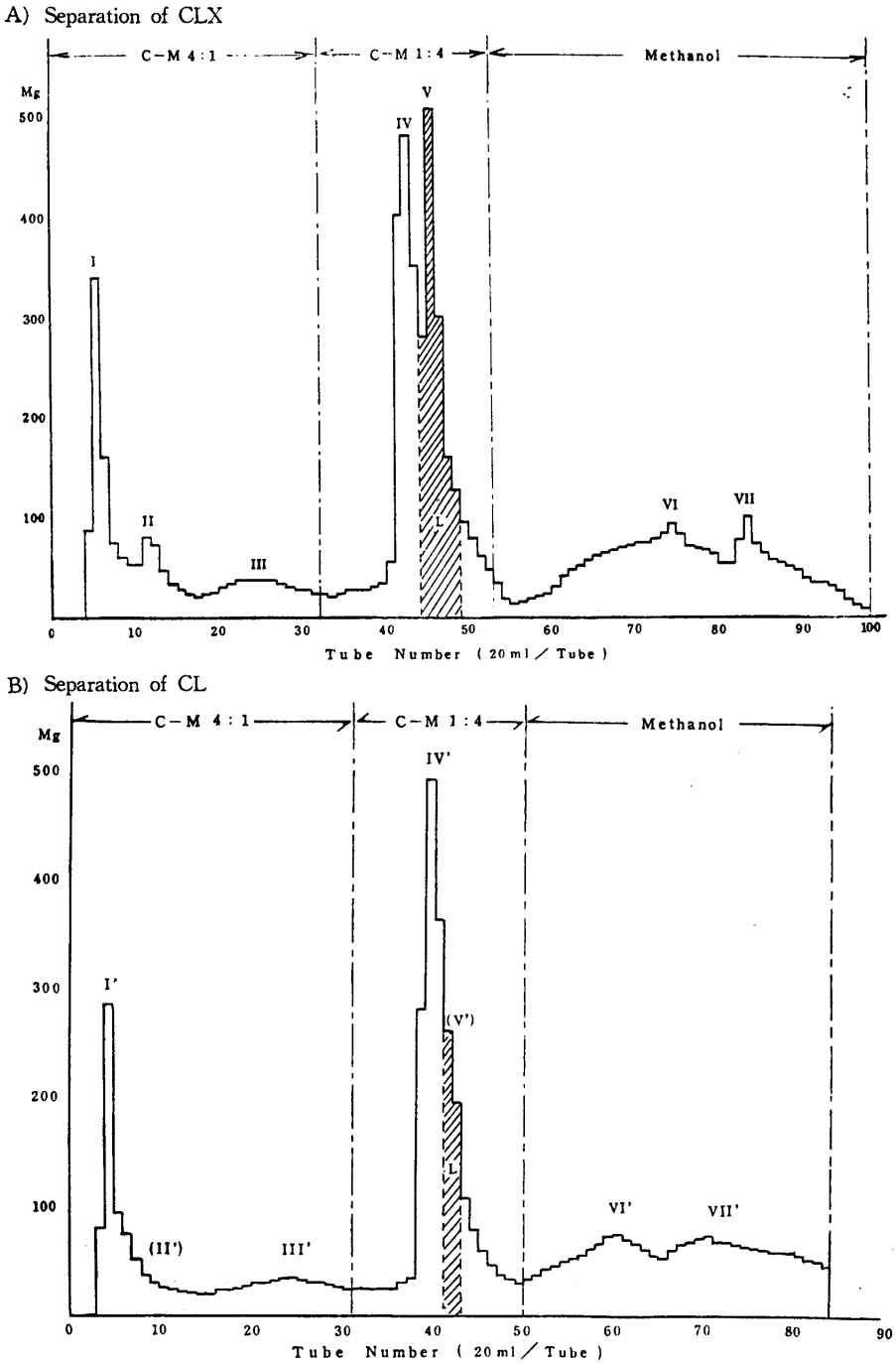

Fig. 2. Chromatograms showing the separations of compound lipids from X-ray irradiated (CLX) and non-irradiated rabbit organs (CL) by stepwise elution from silicic acid with varying concentration of $CHCl_3$ (C)-CH_3OH (M). Note peaks V and II in CLX which are lacking in CL. Sign of L indicates the strongly positive for a test of lysophosphatidylcholine.

CL, i. e. CLX was about 3 times high in activity as CL in the range of 2^{-2} to 2^{-3} per cent, and 10 to 20 times high in 2^{-4} to 2^{-5} per cent. In the dilution of 2^{-6} per cent CLX showed a retained activity but CL showed no activity (Table 2). The marked mitochondrial swelling of albino rat liver and hemolysis were demonstrated by adding fraction V, which is found only in CLX as shown in Fig. 4. (see Table 3A)

The characteristics of eluate in the fraction corresponding to the peaks are shown in Table 3A and B Further, phospholipids contained in each fraction

Table 2. Comparison of Haemolytic Activities between CLX and CL

	Concetration (%)					
	2^{-2}	2^{-3}	2^{-4}	2^{-5}	2^{-6}	2^{-7}
CLX	100	65.5	27.4	9.45	0.56	0
CL	36.6	13.4	1.83	0.15	0	0

Table 3. Characteristics of Eluate by Silicic Acid Chromatography

A) CLX

Fraction		I	II	III	IV	V	VI	VII
Tube Number		6	12	25	43	46	75	84
nitrogen (N) %		1.09	1.42	3.38	2.73	3.49	2.44	2.78
Phosphorus (P) %		2.82	3.40	5.04	4.90	3.02	3.29	4.05
Ratio N/P		0.87	1.00	1.49	1.24	2.56	1.64	1.52
Qualitative test	Molish	−	−	+	+	+	+	+
	Ninhydrine	−	−	−	+	+++	+	+
	Liebermann-Burchard	+	−	−	−	−	−	−
Hemolysis		−	−	±	−	+++	−	−
Mitochondrial Swelling (·△ 520 mμ, after 10 min.)		−	15.7	13.0	12.2	48.3	10.6	11.5

B) CL

Fraction		I′	(II′)	III′	IV′	(V′)	VI′	VII′
Tube Number		5	11	25	40	42	61	71
Qualitative test	Molish	−		+	+		+	+
	Ninhydrine	−		±	+		+	+
	Lieberman-Burchard	±		−	−		−	−
Hemolysis		−		−	+		−	−
Mitochondrial Swelling (·△ 520 mμ, after 10 min)		5.9		9.9	15.5		5.3	8.8

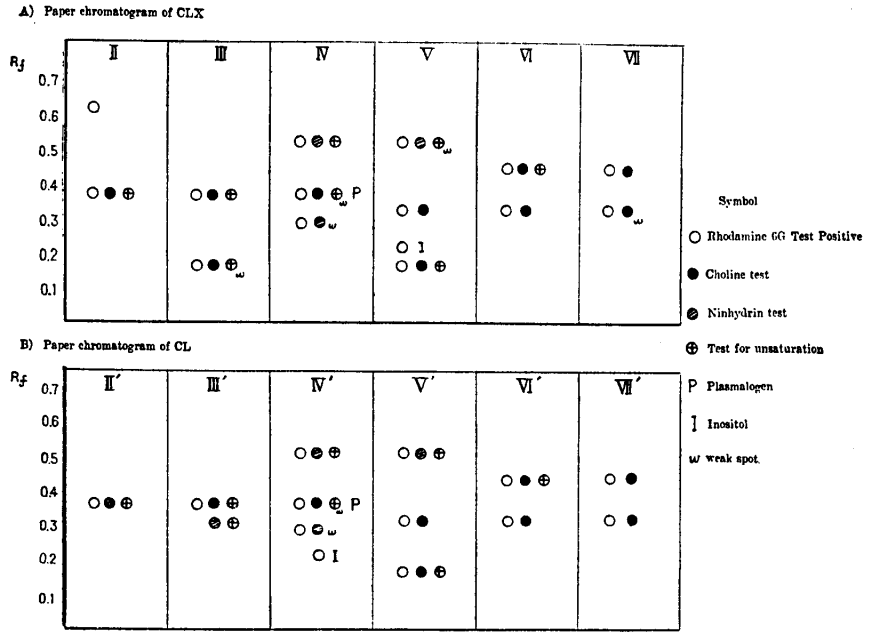

Fig. 3. Silicic acid impregnated paper chromatogram of the main fractions of CLX and CL isolated by silicic acid column chromatography.

were identified by silicic acid impregnated paper-chromatography. Fig. 3 illustrates the results obtained by such tests conducted for each fraction as Rhodamine 6G test, choline test, ninhydrin test, and tests for unsaturation on the silicic acid impregnated paper chromatogram.

Fraction I of CLX, which was obtained as white powder, presented a strong Liebermann-Burchard reaction, and showed the infrared absorption at 3,600—3,400 cm^{-1} corresponding to OH band. While fraction I' of CL gave only a weak Liebermann-Burchard reaction and showed absorption bands at 1,750 cm^{-1}, 1,170 cm^{-1}, which were specific to cholesteryl ester. The results suggested that the fraction I contains mainly free-type cholesterol while the fraction I' ester-type sterol. The silicic acid impregnated paper chromatography indicated the presence of phosphatidylcholine in the fraction I' but not in I. Further, the presence of cholesterol was also identified as follows. The fractions were alkaline hydrolyzed and the unsaponifiable matter recrystallized with ethylacetate, and the white powder of m. p. 147.8°C. was obtained from I' as well as from I. The melting point was not depressed by mixing with pure cholesterol. The absorption spectra coincided with that of cholesterol.

Fraction II was demonstrated by the silicic acid impregnated paper chromatography. In fraction II, there appears the spots of Rf 0.37 as revealed by

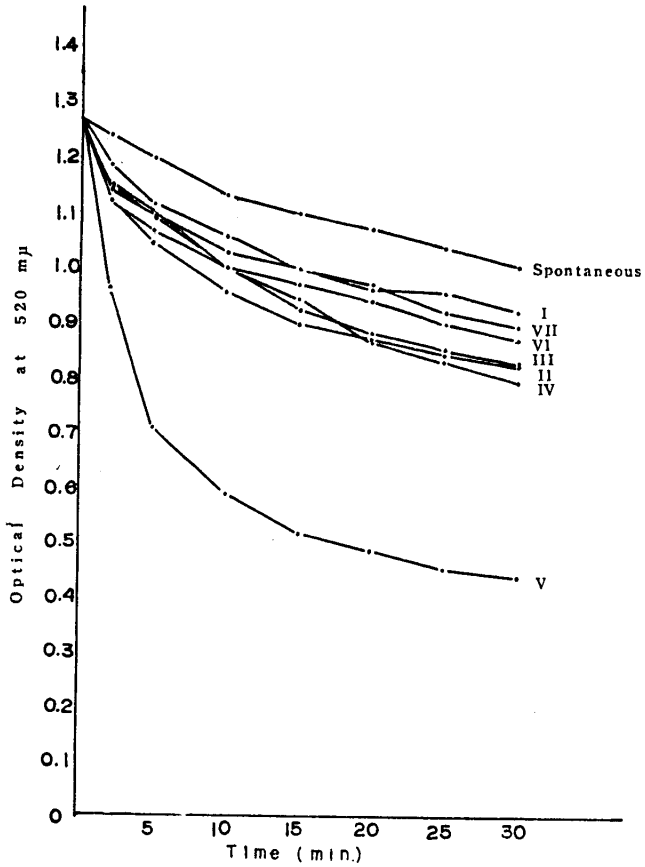

Fig. 4. Normal Rat Liver Mitochondrial Swelling by Each Fraction of CLX Isolated by Silicic Acid Column Chromatography. (Final concentration 0.01 %)

the Rh. 6G test. The spot gave positive reaction of the tests for choline and unsaturation, being identified to be unsaturated phosphatidylcholine. Besides on the fraction II, there appeared a spot of Rf 0.62 which showed blue coloration by Rh. 6G test but gave no other reactions suggestive of cardiolipin according to the Table of Marinetti[5].

In both of the fractions III and III' were contained unsaturated phosphatidylcholine. Besides in III there appeared a spot of Rf 0.17, which did not appear in III'. It gave the same reactions as those of phosphatidylcholine, showing to be lysolecithine. Fraction III' gave another spot of Rf 0.32, which was positive to ninhydrine test. The paper chromatography of the HCl-hydrolysate of III' revealed galactose but the substance has been left to be identified.

In fraction IV there appeared three spots of Rf 0.51, 0.37 to 0.39 and 0.28

by Rh. 6G test. The spot of Rf 0.51 was confirmed to be unsaturated phosphatidylethanolamine by ninhydrin test and $KMnO_4$ test. The spot of Rf 0.28 was to be lysophosphatidylethanolamine by ninhydrin test and iodine test. The spot of Rf 0.37 ~ 0.39 was thought to contain aldehydic phospholipid as it gave positive choline reaction, weakly positive $KMnO_4$ reaction and positive reaction for plasmalogen. The fraction IV' which occupied the main portion of CL (see Table 3), contained the same substances as IV, giving the spots of Rf 0.51, 0.37 and 0.28. Besides these in IV' was contained inositide, showing Rf 0.22 not demonstrated in the fraction IV. The presence of inositide in IV' was further confirmed by the detection of the spot of inositol in the paper chromatography of sugar after HCl-hydrolysis.

The fraction V, which showed strong hemolytic activity (77.4 per cent at the concentration of 0.0025 per cent), was found only in CLX. The silicic acid impregnated paper chromatography of V gave positive spots for four substances, phosphatidylethanolamine (Rf 0.51), inositide (Rf 0.21), lysophosphatidylcholine (Rf 0.162~0.18) and sphingomyelin (Rf 0.32).

All other four fractions VI, VI', VII and VII' proved to contain monomethylphosphatidylcholine and sphingolipid as revealed by silicic acid impregnated paper chromatography. HCl-hydrolysate of these fractions liberated galactose. Infrared absorption spectrum of these fractions showed the absorption bands of amide at 1,550 cm^{-1} and 1,650 cm^{-1} and the specific band of trans-double bond at 970 cm^{-1}. These results indicate the existance of sphingolipid.

Analysis of fatty acids composing of the total compound lipids proved the reduction of the unsaturated fatty acids in quantity in CLX comparing to that of CL. The chemical and physical properties of the fatty acids obtained by hydrolysis (CLXF and CLF) are shown in Table 4.

Table 4. Characteristics of Fatty Acids of Compound Lipids

		X-ray irradiated CLXF	Control CLF
Mixed F. A.	Acid V.	196.7	196.6
	Sap. V.	197.0	197.5
	Iodine V.	79.0	89.8
Liquid F. A.	%	44.1	45.0
	N_D^{25}	1.4711	1.4720
	Sap. V.	189.6	195.5
	Iodine V.	168.0	187.0
	Conj. diene %	2.05	1.39
	Conj. triene %	0.09	0.03
	Conj. tetraene %	0.07	—

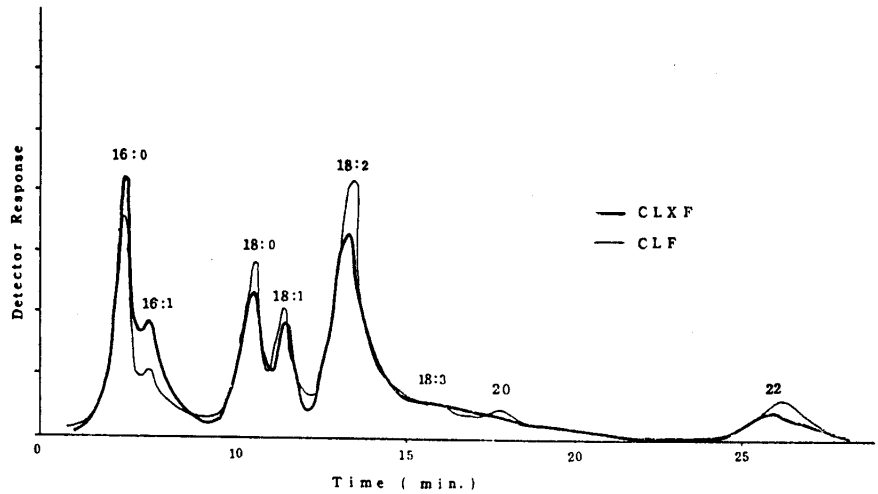

Fig. 5. Gas-liquid Chromatograms of Methyl Esters of CLXF and CLF. Temperature, 200 °C; Flow rate, 52 ml/min.; Bridge current, 140 mA; Chart speed, 10 mm/min.

In a comparison of fatty acid composition in CLXF and CLF, the kinds of fatty acids were quite similar, as shown in Fig. 5. But in the former C_{18} stearic, C_{18}- monoenoic, C_{18} dienoic, C_{18}- trienoic, C_{20}- and C_{22}- acids were decreased in quantities and then, C_{16} palmitic and C_{16}- monoenoic acid were increased.

Further, the quantities of cold ether insoluble bromide were 8.06 per cent in CLF and 6.57 per cent in CLXF, suggesting that a considerable amount of highly unsaturated fatty acid was diminished in compound lipids by irradiation. The brom percentage in CLXF-bromide was 66.4 per cent and 67.4 per cent in CLF-bromide (67.75 per cent calulated as hexabromo-eicosanoic acid, $C_{20}H_{32}O_2Br_8$) as shown in Table 5.

Table 5. Characteristics of CLXF- and CLF-Bromide

	X-ray irradiated CLXF-Bromide	Control CLF-Bromide
Yield %	6.57	8.06
M. P. (°C)	218.0	223.0
Br. %	66·4	67.4

DISCUSSION

As it has been introduced briefly, the biological action of X-rays has not yet fully been clarified. Especially, the so-called "indirect action" of X-rays, which will be the most impotant one, is left obscure, though there may be some hypothetical considerations. The chemical analysis, both quantitative and quali-

tative, revealed remarkable changes occurring on the lipids of the organs of irradiated animals. Total amount of the lipid fraction from the rabbit organs markedly increased by the whole body irradiation. This increase was mainly prominent in compound lipid fractions (phospholipids, glycolipids). Beside these, cholesterol found in the unsaponifiable matter was mainly of free-type in the irradiated animal, whereas the ester-type was predominant in non-irradiated animal. These findings substantiate the result reported by ELKO *et al.*[12,13].

Biological test, the hemolytic test and the effects of each fraction on the swelling of isolated rat liver mitochondria, suggested that the qualitative changes would also be occurring on glycerophosphatides by whole body irradiation. Then, the author applied a silicic acid column chromatography for the separation of biological active component and obtained the active fraction V, which contained four substances lysophosphatidylcholine, inositide, sphingomyelin and phosphatidylethanolamine. Therefore, the same biological experiments as done on fraction V were carried out on soya lecithin, egg-yolk lecithin containing lysolecithin, inositol, shingomyelin, ethanolamine and choline. As the results it was demonstrated that the substance containing lysolecithin or ethanolamine had solely the action to induce the mitochondrial swelling and hemolysis. Lysophosphatidylcholine was recognized in the range of tubes 45～49 in the column chromatogram of CLX, while only in 42 and 43 of CL. (see Fig. 2A and B)

These increases of lysophosphatide will mean concurrent release of the fatty acids from phosphatides by irradiation. The gas-liquid chromatography on the mixed fatty acids from the compound lipids proved a considerable amount of higher unsaturated fatty acids, arachidonic-, linolenic- and linoleic acids. But, quantitative analysis revealed a marked decrease in these fatty acids longer than C_{18}, in the fractions from the irradiated animal. This elucidates that lecithin is the main source for the lysophosphatide produced by irradiation. Another noticeable change appearing on each component is the increase of palmitoleic and palmitic acids. It seems to be one results from whether the synthesis of the long-chain fatty acids is inhibited or the β-oxidation is promoted in the oleic, linoleic and linolenic acids, though it cannot be decided, at present, which one is the course.

This experiment proved that X-ray irradiation of animal body results in a release of unsaturated fatty acids from phospholipids yielding the substance having a powerful hemolytic activity. The release of such hemolytic substances will be of the important part of the indirect action of X-rays. Detailed biological observations related with these substances are to be reported in the next paper.

SUMMARY

For the purpose to reveal the mechanism of the biological indirect action of X-rays the author has conducted the analysis of the compound lipids of the organs of the rabbits irradiated with X-rays. Silicic acid column chromatography was applied for the fractionation of the compound lipids. Component of each fraction was analyzed by silicic acid-impregnated paper chromatography and infra-red spectrophotometory. The result proved that the compound lipids showed some qualitative and quantitative changes. The changes occurred mainly in glycerophosphatides showing the formation of high level lyso-phosphatidylcholine and lysophosphatidylethanolamine.

The fatty acid compositions of the compound lipids were determined by means of gas-liquid chromatography. Further, polyunsaturated fatty acids were analyzed as fatty acid bromide. Major differences found in fatty acid patterns included: in the irradiated group the amount of C_{18}-monoenoic, C_{18}-dienoic, C_{18}-trienoic, C_{20}-tetraenoic and C_{22}-acids were decreased and of C_{16}- and C_{16}-monoenoic acids were increased.

It has been elucidated that lysophosphatides not only posesses a strong hemolytic power but also it has an action to induce swelling of the rat liver mitochondria. And it has been suggested that the lysophosphatides and ethanolamine will be responsible, at least partially, for the cell damage induced by X-rays.

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