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Abstract

For the purpose to clarify the causes of X-ray disturbances a series of experiments have been conducted on biological and biochemical properties of compound lipids extracted from normal and X-ray irradiated rabbit organs with a special reference to the P^{32}-labeled compound lipids uptake, inhibitory action to L cell proliferation and uncoupling of oxidative phosphorylation, and the following results have been obtained. The compound lipids (lysophosphatide rich fraction) isolated from the X-ray irradiated rabbit organ have been found to possess a strong hemolytic action and also an action to inhibit the cell proliferation as well as to accelerate the respiration of the mitochondria in the rabbit liver and spleen. It has also been proven that they act as to induce a marked swelling of mitochondria, to impede the formation of high energy phosphate as well as to act as an uncoupler of oxidative phosphorylation in vivo. In the test to see the uptake of P^{32}-labeled compound lipids by various organs, a marked uptake has been observed in spleen, bone marrow, and liver of both irradiated and non-irradiated groups. Further, the uptake of P^{32}-labeled compound lipids in the rabbits given intravenous injections of compound lipid fraction for 30 consecutive days previously has been found to be greatest in pancreas followed by bone marrow, spleen, liver in the order mentioned in male group, whereas it is greatest in spleen, followed by liver and bone marrow in the female group. With these results the discussion was conducted concerning the relation between the lipid metabolism and X-ray disturbances.

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STUDIES ON THE COMPOUND LIPIDS FROM X-RAY IRRADIATED ANIMAL

II. BIOLOGICAL AND BIOCHEMICAL PROPERTIES OF THE COMPOUND LIPIDS IN THE X-RAY IRRADIATED RABBIT ORGANS

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In the previous paper[1] the author reported that by irradiating rabbits with X-ray lysophospholipids and fatty acids are liberated in the living organisms, as demonstrated in the alcohol extract from the liver of the animals received a large dose of X-ray. These two substances, both of lysophospholipids and free fatty acids, have a strong hemolytic activity as is well known in the biological action of snake venom which has a strong activity of lecithinase A. Consequently, it is reasonably supposed that these hemolytic substances will be responsible for the hemolysis appearing at a certain period after X-ray irradiation. Of course, these substances will have the cytolytic activity affecting the cell membrane of somatic cells as well as red cells. And this activity may be largely concerned with the cell lesion by X-ray irradiation. Besides the action on cell membrane, the fatty acids are the typical uncoupling agents for the oxidative phosphorylation, as has been revealed by UTSUMI and others[33] by using rat liver mitochondria. Namely, these substances can be considered to act as an inhibitor on the energy metabolism of cell and mitochondria. Thus it is supposed that the tissue damage seen after X-ray irradiation will greatly be dependent upon the biological activities of these substances. In this paper, the observation on the biological activities of the hemolitic substances produced by X-ray irradiation, the distribution in various organs of rabbits after the injection inhibitory activity for cell growth, and the uncoupling activity for the oxidative phosphorylation, are reported. The purpose of this study is to clarify the mechanism of the indirect tissue damage by X-ray irradiation.

MATERIALS AND METHODS

L cell strain (from the Institute for Infectious Deseases, Tokyo University, Tokyo) was used for the tissue culture experiment. Hybrid rabbits were used for obtaining the compound

lipids and for testing the uptake of P^{32}-labeled compound lipids. Mitochondria were isolated from liver and spleen of rabbit and rat liver by the method of HOGEBOOM-SCHNEIDER[3] and served for the experiments of the mitochondrial swelling and the oxidative phosphorylation, compound lipids used as test materials were CLX (compound lipids from the organs of X-ray irradiated rabbits), CL (compound lipids from the organs of the non-irradiated normal rabbits) and CLX-fr. V (lysophospholipid-rich fraction isolated by silicic acid column chromatography from CLX) which were prepared as reported in the previous paper[1].

The morphological changes of L cells were observed by the following methods: L cells were cultured in advance for 24 hours at 37°C in TD-15 tubes containing YLE-bovine serum (9 : 1) medium and then CLX, CL and CLX-fr. V diluted with the culture medium were added to make the final concentrations of 0.1, 0.05 and 0.025 per cent. The morphological changes were observed at the intervals of 1, 3, 6, 9, 12, 24 and 48 hours after addition of test materials under Olympus inverted phase contrast microscope. For some series of experiments on L cells cover-slip culture was done placing cover-slip on the bottom of each TD tube. After 24 hours cultivation of L cells lipid samples to be tested were added to the tubes, and further cultivation was made for another 24 hours. The L cells growing on the cover-slips were taken out and stained with Giemsa solution for morphologic examination.

Effects of the compound lipids on the proliferation of L cells were estimated in culture medium. An aliquot number of L cells were transplanted into culture tubes containing CL, CLX, and CLX-fr. V diluted in the serial concentrations described above. The numbers of cells were counted at the end of 2, 4 and 6 days of cultivation. Growth curves were obtained from the mean values of 5 culture tubes in each step of cultivation.

For the preparation of P^{32}-labeled compound lipids, normal rabbits were irradiated with 3,000 r and 18 hours later phosphate-glucose solution containing 0.4 mc P^{32} was injected into the aural vein. Six hours after the injection the liver, spleen, pancreas, kidney, lung, adrenal and heart were taken out with aseptic precaution and immediately homogenized with a small amount of ethanol. To the homogenate of the organs (154 g. total wet weight) from two rabbits 1 liter of absolute ethanol was added and extracted for 24 hours at 50°C and filtered. The residue was extracted twice with 500 ml. of absolute ethanol and filtrates were combined. Ethanol was evaporated under a stream of hydrogen gas at reduced pressure. These extracts were treated in the same way as described in the previous paper[1] and the P^{32}-labeled compound lipid fraction (designated as P^{32}-CLX) was obtained. The same fraction similarly obtained from non-irradiated rabbit group was designated as P^{32}-CL.

The uptake of P^{32}-labeled compound lipids in the organs of rabbit was determined as follows. The P^{32}-CLX and P^{32}-CL fractions were diluted to 5 per cent with physiological saline solution and each of these solution for 30 consecutive days. After a given time the animals were bled by severing the carotid artery and the organs were excised and weighed aseptically and ashed by sulfuric acid-nitric acid method. The radio-activity was measured in a G-M counter according to the ammonium-magnesium phosphate method[2].

For the study of the swelling of mitochondria, the fraction of mitochondria prepared from the liver and spleen of rabbits as well as from the liver of hybrid rats as described were washed twice with 0.25 M sucrose solution, and measured the absorbancy at 520 mμ with the swelling test system as described in previous paper[1].

The respiration of mitochondria was measured by the routine method with WARBURG's apparatus using following media: 1.92 ml of stock mitochondrial suspension (1 g tissue

equivalent mitochondria suspended in 10 ml of 0.25 *M* sucrose solution) and 0.3 ml of Krebs-Ringer phosphate (KRP) solution (pH 7.4) in main chamber, 0.2 ml of 20 per cent KOH in center well, 0.3 ml of 0.05 *M* sodium succinate in one side arm and 0.28 ml of 0.25 per cent CLX or CL physiological saline solution (0.9 per cent NaCl for control) in another side arm. The incubation was carried out under air phase at 38°C.

In the other experiment, parallel observation was made on the effects of compound lipids on the respiration and phosphorylation of mitochondria by means of the oxygraphy. Incubation mixture consisted with 0.05 *M* sucrose, 0.02 *M* KCl, 0.02 *M* K-phosphate buffer (pH7.5) and 0.02 *M* EDTA. The incubation was carried out under the 2.5 ml semiclosed reaction cell at 25°C. 0.2 ml sucrose suspension of rat liver mitochondria (0.1 g tissue equivalent) was added (states I and II) and after 1~2 minutes 0.025 ml. of 1 *M* sodium succinate was added (state IV) and 1 minute later 0.02 ml of 10 m*M* of ADP was added (state III). After reversing to state IV 0.025 ml of 2 per cent compound lipid (CLX or CL) was added and after lapse of 1 minutes 0.02 ml of 10 m*M* of ADP was again added. Then the changes of oxygen consumption and oxidative phosphorylation were estimated by the ratio of succinate substrate respiration and by the ratio of succinate substrate level oxidative phospholylation, both before and after the treatment of compound lipids.

For the estimation of the P^{32} incorporation into organic phosphate compound (Δ 10 P fraction) the following incubation system was used: 2 ml of the stock mitochondria (1 g tissue equivalent fresh rat liver mitochondria suspended in 1 ml of 0.25 *M* sucrose solution), 0.5 ml of KRP solution containing 9 μc of P^{32}, 2.3 ml of 0.15 *M* KCl-0.02 *M* Tris buffer solution and 0.6 ml of 0.25 per cent CLX or CL. Incubation was conducted at 30°C for 30 minutes and immediately cooled to 0 °C and centrifuged at 14,000 × g for 10 minutes, and then washed once with 0.25 *M* sucrose solution. To the precipitated mitochondria 3.0 ml of 5 per cent trichloroacetic acid (TCA) was added and let it stand at 0°C for 30 minutes. This was again centrifuged at 700 × g, and with 1 ml of this supernatant the quantitative analysis of phosphorus (Pi) was conducted by TAKAHASHI's method[4], and estimated the radio activity of P^{32} by using G-M counter (G-M-2504-A, Nihon Musen TDC-1 type). Further, to 1 ml of the same supernatant 1 ml of 1.5 *N* H_2SO_4 was added and treated at 100°C for 10 minutes, Pi and the activity of P^{32} were estimated as above mentioned and the values were taken as these of Δ 10 P.

RESULTS

Distribution of P^{32}-labeled compound lipids in various organs—The uptake of P^{32}-CLX and P^{32}-CL in various organs have been studied. As the result it has been proven that the uptake of these fraction differs according to different organs, namely, it was greatest in the spleen followed by that in the liver, bone marrow, pancreas, gonad, kidney, and lung in the order mentioned, as illustrated in Fig. 1. As for P^{32}-CLX, lipids isolated from the rabbit organs after X-ray irradiation, its uptake was most marked in the spleen and bone marrow followed by the liver, lung, pancreas, kidney and gonad decreasing gradually in that order. In contrast to these, the uptake of P^{32}-CL in the male group of the rabbits, given the intravenous injection of CL for 30 consecu-

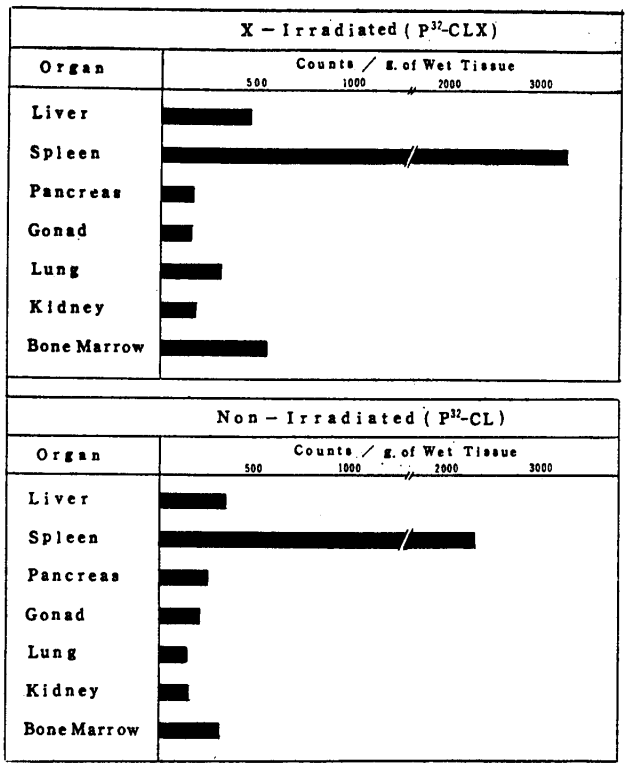


Fig. 1. Specific activity of P^{32} in various organs after the injection of P^{32}-labeled compound lipids (P^{32}-CL or P^{32}-CLX).

tive days previously, was greatest in the pancreas, followed by the bone mar- of P^{32}-CLX uptake row, spleen, and liver. The same trend was also observed in the group given the prior intravenous injections of CLX (Fig. 2A). On the other hand, in the female group of rabbits given the prior intravenous injections of CL or CLX for 30 consecutive days the uptake of P^{32}-CL and P^{32}-CLX was greatest in the spleen (Fig. 2B). In either case, moreover, the increase in the uptake of P^{32}-labeled compound lipids was greatest in the irradiated group.

Furthermore, in the observations conducted with lapse of time on the uptake of P^{32}-CLX and P^{32}-CL, no change in the order of uptake amounts can be recognized (Table 1).

This means, at least, that phosphatides are taken up most markedly by the spleen and bone marrow, indicating that there are no great metabolic transfer among the organs and phosphatides metabolism was quite active in the spleen and bone marrow. In other words, this suggests that the metabolism of phosphatides is a factor to govern the susceptibility to X-rays, hence affecting the extent of X-ray damage.

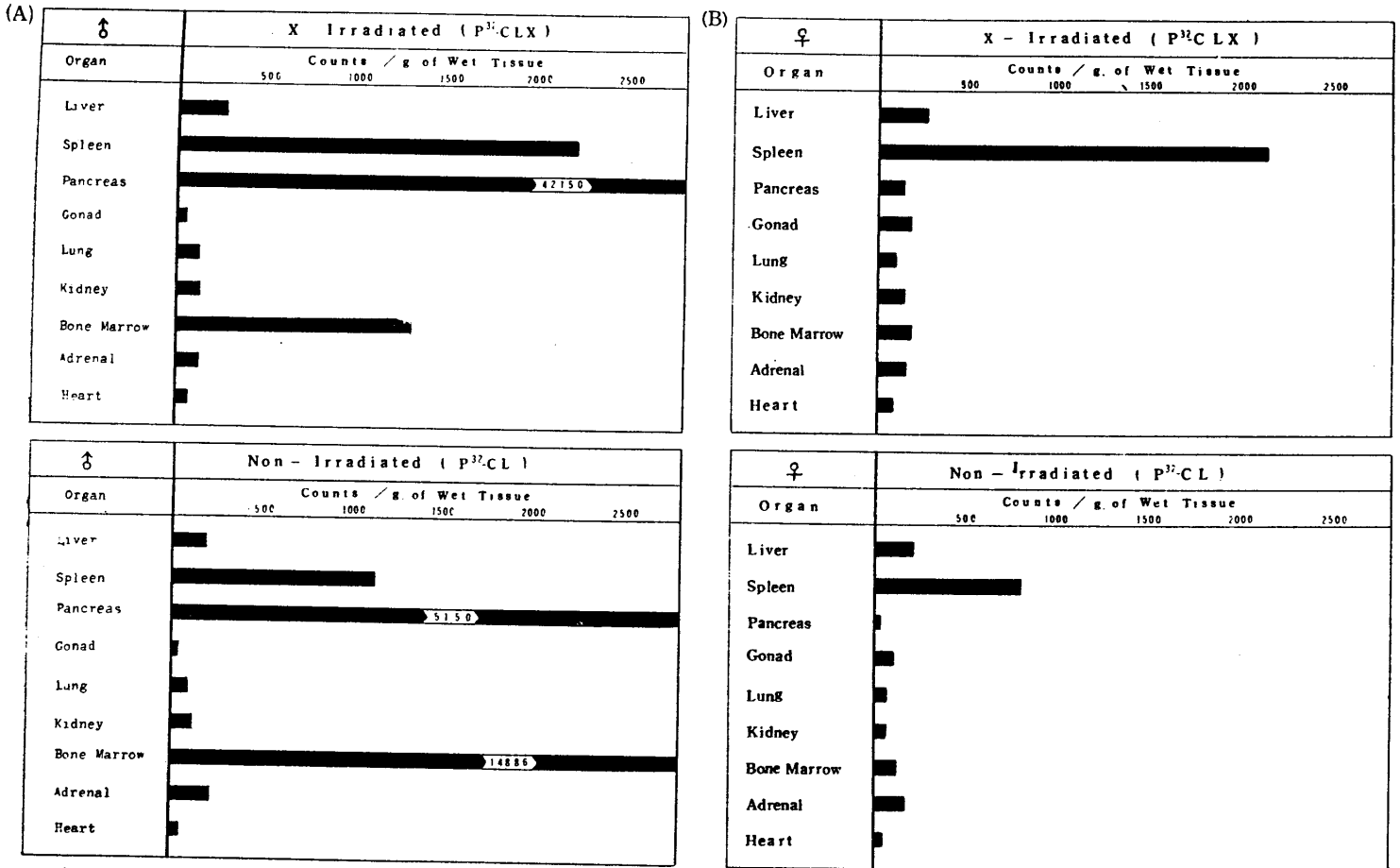
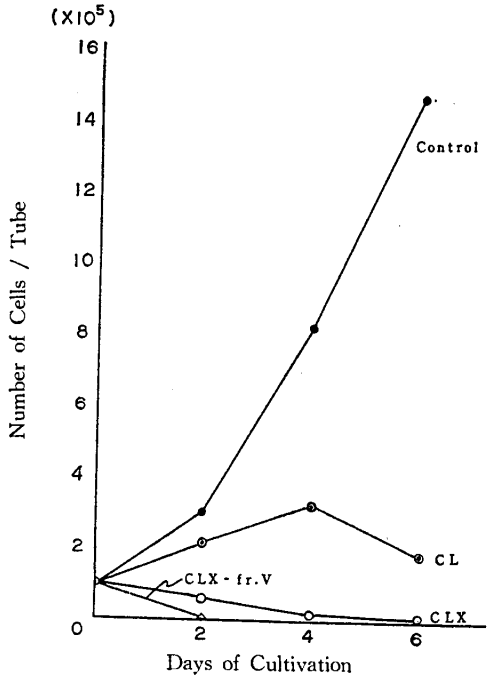


Fig. 2. Specific activity of P^{32} in various organs after the injection of P^{32}-labeled compound lipid (P^{32}-CL or P^{32}-CLX) in rabbits given the prior intravenous injections of CL (or CLX) for 30 consecutive days. (A) The male group (B) The female group

Table 1. Incorporation of P^{32}-CLX and P^{32}-CL into various organs
Figures indicate counts/g. wet weight.

Organ	Time after injection of P^{32}-CLX (hr.)					
	3.5	4	24.5	25	48	48.5
Liver	653	683	507	381	331	276
Spleen	5451	3991	3754	2617	2164	1330
Pancreas	248	188	234	103	125	119
Gonad ♀	223	248	340	—	—	—
Gonad ♂	—	—	—	46	60	45
Lung	878	475	177	141	195	104
Kidney	223	105	239	160	223	140
Bone Marrow	1150	994	600	213	191	163

Organ	Time after injection of P^{32}-CL (hr.)					
	3.5	4	24.5	25	48	48.5
Liver	333	418	550	220	160	360
Spleen	3186	3163	2645	2461	900	1407
Pancreas	177	250	52	96	833	111
Gonad ♀	933	0	0	—	—	—
Gonad ♂	—	—	—	175	63	85
Lung	174	71	180	65	130	220
Kidney	94	109	185	180	145	150
Bone Marrow	331	380	302	461	183	220



Effect of lysophosphatide-rich compound lipids on the proliferation of L cells—The hemolytic effect of lysophosphatides is one of the causes of the decrease[5] in red blood cells accompanying X-ray irradiation and there is a possibility of these substances inhibiting the cell proliferation. Therefore, the effect of these lysophosphatides on the proliferation of L cells in tissue culture has been studied. As shown in Fig.3,

Fig. 3. Effect of the compound lipids extracted from X-irradiated and non-irradiated rabbits on the proliferation of L-cells. (Final concentration of lipids is 0.05 per cent)

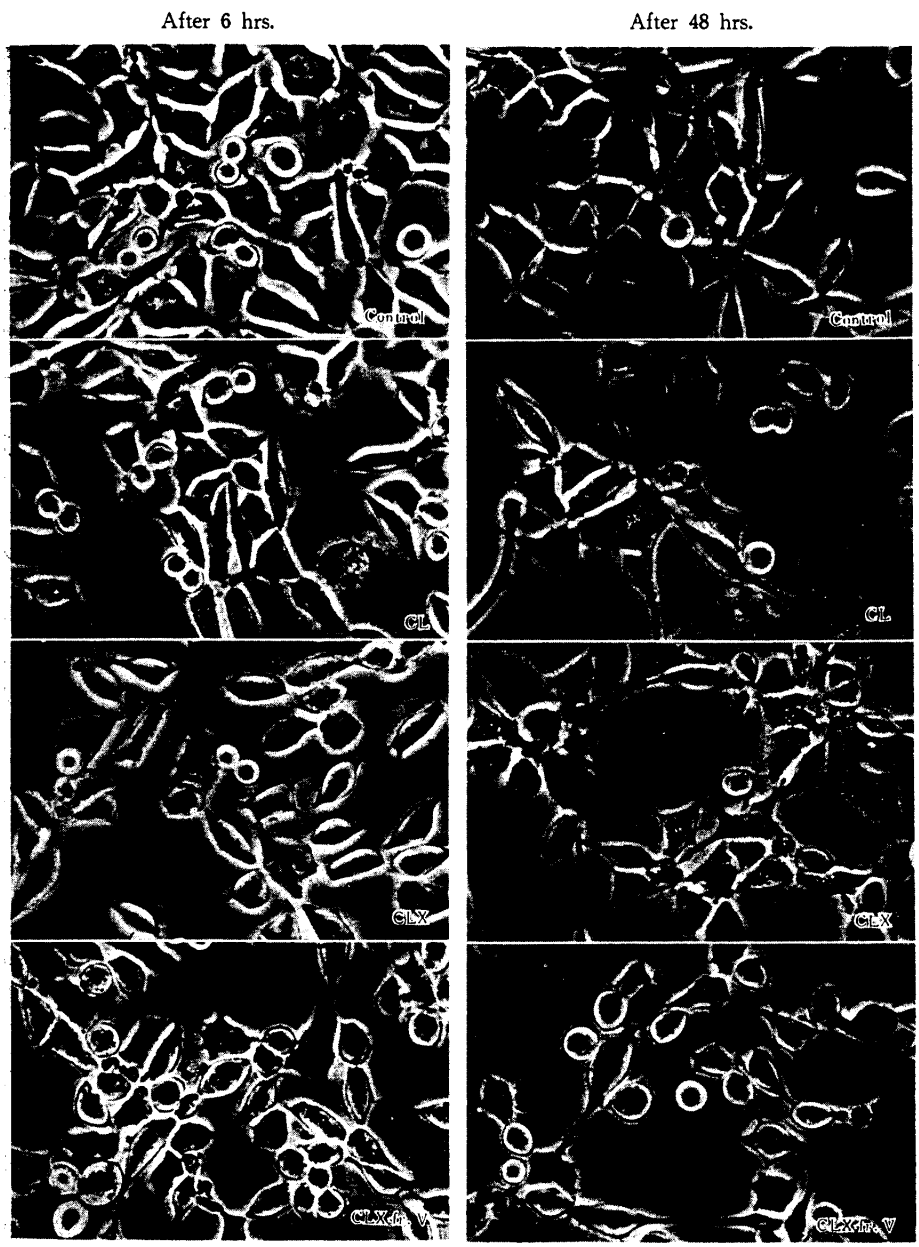


Fig. 4. Morphological changes of L cell challenged by compound lipids fractions extracted from normal rabbit organs (CL) and X-irradiated rabbit organs (CLX), and lysophospholipid rich fraction of CLX (CLX-fr. V).

After 6 hrs.: Treated 6 hours at 37°C.
After 48 hrs.: Treated 48 hours at 37°C.

addition of 0.05 per cent CL, CLX, and CLX-fr. V, all proves to inhibit the proliferation of L cells but the inhibition was especially marked with CLX-fr. V. When these cells were observed under phase contrast microscope either after the addition of CL or CLX fraction, in each case many lipid granules appeared in their cytoplasm as compared with untreated control. And such an appearance is particularly marked in the case of adding CLX-fr. V (Fig. 4). Also in the observation of those L cells stained with Giemsa, the cells similarly showed many vacuoles due to the appearance of intracellular granules and no other morphological change can be recognized with the CL-treated group of the cells, but in the CLX-treated group, especially in the CLX-fr. V-treated group, the cells have become somewhat spherical with little mitotic picture, and often nucleoles tend to be ill-defined, some cells being disintegrated.

Biochemical properties of compound lipid fractions extracted from X-irradiated rabbit organs—From the data that CLX has a strong hemolytic effect and inhibits cell proliferation, CLX can be assumed to act as an inhibitor on energy metabolism of cell and mitochondria, and hence it might induce structural changes in mitochondria and inhibit oxidative phosphorylation. From this viewpoint, morphological changes in the rabbit liver and spleen mitochondria affected by compound lipids have been observed, and the results are as shown in Figs. 5, 6. Namely, by CL both the liver and spleen mitochondria were hardly

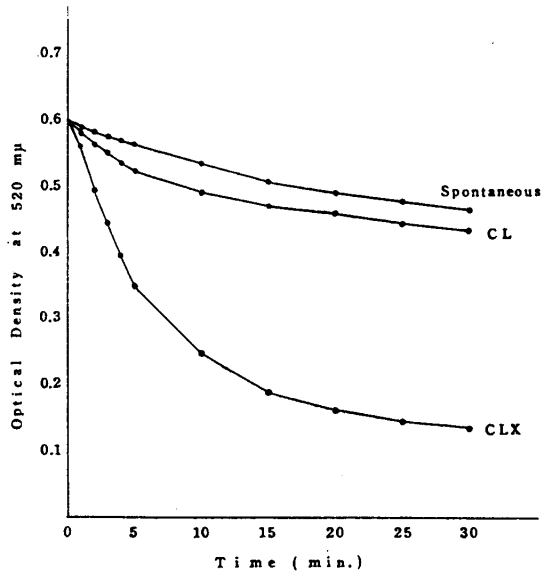


Fig. 5. Effect of CL and CLX (final concentration 0.01%) on the rabbit liver mitochondrial swelling in the medium of 0.15 *M* KCl-Tris buffer (pH 7.4).

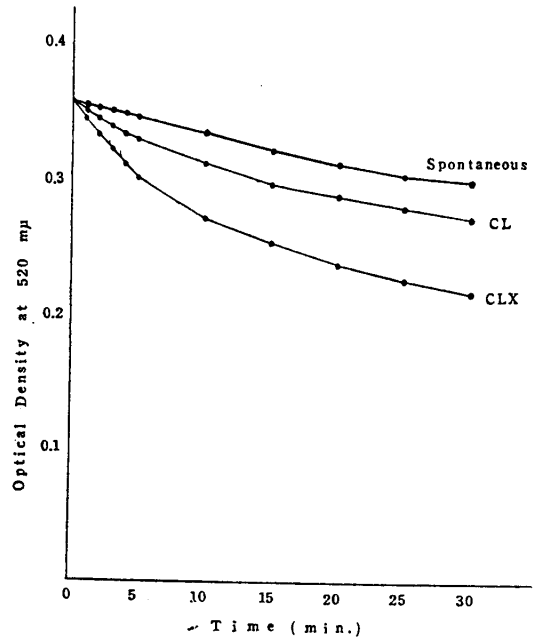


Fig. 6. Effect of CL and CLX (final concentration 0.01%) on the rabbit spleen mitochondrial swelling in the medium of 0.15 *M* KCl-Tris buffer (pH 7.4).

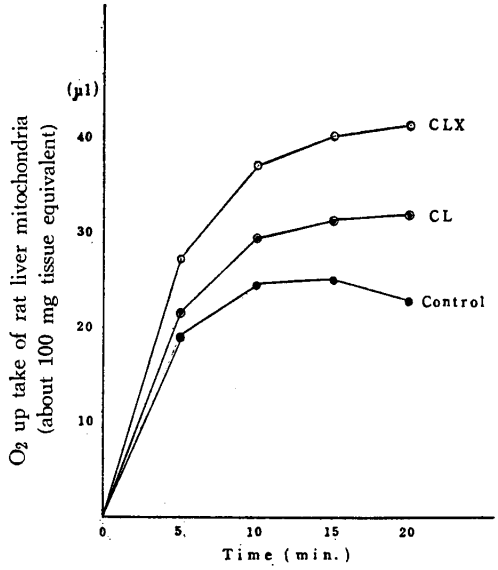
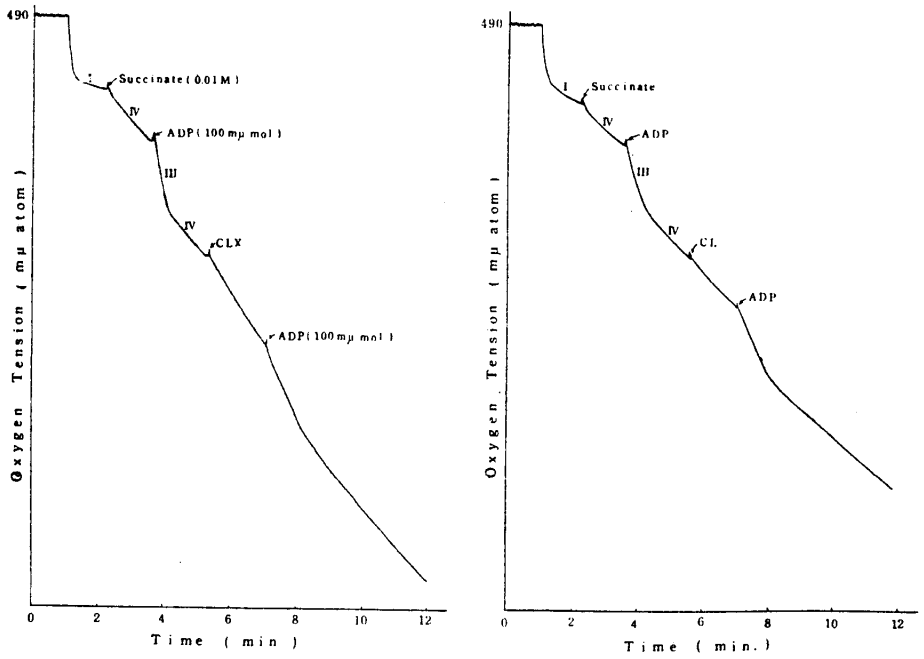


Fig. 7. Effect of compound lipids extracted from X-irradiated and non-irradiated rabbits on the respiration of rat liver mitochondria. CLX: Compound lipids extracted from organs of irradiated rabbit (final concentration 0.025%). CL: Compound lipids extracted from the organs of normal rabbit (final concentration 0.025%).

affected, but CLX induced marked mitochondrial swelling being particularly marked in the liver. From this, it is assumed that CLX, like its hemolytic action, induces a marked change in the cellular membraneous structure, and caused

Table 2. Effect of compound lipids extracted from X-irradiated and non-irradiated rabbits on the incorporation of P^{32} into rat liver mitochondria.

	Ratio of RA of Pi	Ratio of RA of Δ 10 P
Control	100	100
CLX	45.5	3.2
CL	52.5	31.0



	Before treatment		After treatment		Ratio (%)	
	Respiration (mμ atoms oxygen)	P/O	Respiration (mμ atoms oxygen)	P/O	Respiration	P/O
CLX	32.5	1.74	41.0	1.21	133	68.0
CL	27.5	1.74	30.0	1.54	110	80.8

Fig. 8. Effect of compound lipid on succinate oxidation and oxidative phospholylation of rat liver mitochondria. The respiratory release and uncoupling of oxidative phosphorylation of mitochondria are marked in the case of CLX.

uncoupling of oxidative phosphorylation. In the further observations on the influence of CL and CLX on the O_2-uptake of mitochondria of rat liver both substances have been found to increase the oxygen uptake of mitochondria as illustrated in Fig. 7, especially such an increase by CLX was marked. In the simultaneous estimation of the incorporation of P^{32} into labile phosphate of rat liver mitochondria both CL and CLX inhibit the incorporation of P^{32} into Δ10P, as demonstrated in Table 2, and such an inhibition by CLX was especially marked, being almost completely inhibited.

The uncoupling oxidative phosphorylation of mitochondria by compound lipids was also observed by the oxygraphic method (Fig. 8). Namely, succinate substrate respiration of mitochondria was stimulated and the oxidative phospholylation was uncoupled by compound lipids, especially markedly in the case of CLX, and parallel relationships could be observed in the extent uncoupling action and the swelling action. In other words, it has been clarified that CLX acts as a typical uncoupler of oxidative phosphorylation *in vivo*.

DISCUSSION

Despite numerous works on the damages caused by X-rays, it appears that studies on its initial effect is quite difficult, and at present the studies[6] by means of electron spin resonance in the field of biochemistry seem to have opened up a way leading to its resolution. For the elucidation of this problem a back-door approach seems to be more fruitful, and in this paper are presented the results of just such a study. In the previous paper[1] it was thought that the increase of lysophosphatides in the organs after X-ray irradiation was a cause of hemolysis accompanying the irradiation. In the present report it has been elucidated that compound lipid fractions including lysophosphatides enhance the respiration of cells or mitochondria, inducing the swelling of mitochondria and the inhibition of formation of high energy phosphate compound. According to the recent work on the mitochondrial swelling by LEHNINGER, and others,[7-12,32,33] these compound lipid fractions have been proven to be a typical uncoupler of oxidative phosphorylation *in vivo*. Therefore, the formation of these lysophosphatides is considered to be one of the causes of the disturbance in energy metabolism due to X-ray irradiation[13-17]. The fact that in the swelling test of mitochondria in the rabbit liver and spleen the swelling of the liver mitochondria is much more marked than that of the spleen mitochndoria can be understood from the data of the more active lipid metabolism in the spleen as demonstrated by the P^{32}-CL uptake-test of various organs.

In addition from the data of inhibitory action of CLX on the cell proliferation and of relatively active CLX-uptake in bone marrow, the CLX seems to

become a factor in the decrease of erythrocytes (hypoerythrocytosis).

Such a phenomenon of the increase in lysophosphatides accompanying X-ray irradiation suggests a simultaneous release of fatty acids. It is well known that unsaturated fatty acid has a strong hemolytic action and also an action to induce the swelling of mitochondria[33]. Namely, the increase of lysophophatides by X-ray irradiation may be one of the causes of the uncoupling of oxidative phosphorylation in the case of X-ray irradiation. It is also reported that lipid peroxide is formed *in vivo* by X-rays[5] and this lipid peroxide inhibits the cell division, induces depolymerization of DNA, and reveals various secondary effects[15,18,19,20]. In the previous experiments of the author[21], when normal fatty acid is exposed to X-rays, it has been clarified that autoxidation is quite rapid and the formation of lipid peroxide is accelerated. Thus, it is doubtlessly clear that lysophosphatides and fatty acids formed by irradiation constitute fairly important factors in the X-ray disturbances. Though the mechanism of the changes of phospholipids by X-ray irradiation is not clear, there are many reports about these phenomena by various authors. Namely, these author noticed an accelerated lipid metabolism[29], an increased fatty acid in the blood and an induced hyperlipemia[30]. Moreover, according to ALEXANDER[6] intracellular membrane phospholipid is the site of most sensitive to X-ray. On the other hand, according to the report of CRIDDLE *et al.*[22], NYGAARD *et al.*[23,24], FLEISCHER *et al.*[25] the important component of membrane structure is the phospholipid and this substance is concerned with the permeability or active transport of membrane as pointed out by HOKIN & HOKIN[26], and YOSHIDA[27]. Furthermore, UNGAR[28] holds the opinion that the formation of adrenal steroids is impeded by X-rays and thereby induces alteration in the fatty acid metabolism. Namely, the hormonal unbalance caused by X-ray irradiation will cause the changes in the lipid metabolism accompanying the damage of various important biological structures. WILLIAMS *et al.*[31] reported the distribution of phospholipid is about in the same amount in the heart, kidney, lung, testicles and liver but the above mentioned data show the large amount of compound lipid uptake in spleen and bone marrow. Such discrepancies may signify that the susceptibility to X-rays is partly related to the phospholipid metabolism of spleen and bone marrow.

SUMMARY

For the purpose to clarify the causes of X-ray disturbances a series of experiments have been conducted on biological and biochemical properties of compound lipids extracted from normal and X-ray irradiated rabbit organs with a special reference to the P^{32}-labeled compound lipids uptake, inhibitory action to L cell proliferation and uncoupling of oxidative phosphorylation, and the following results have been obtained.

The compound lipids (lysophosphatide rich fraction) isolated from the X-ray irradiated rabbit organ have been found to possess a strong hemolytic action and also an action to inhibit the cell proliferation as well as to accelerate the respiration of the mitochondria in the rabbit liver and spleen. It has also been proven that they act as to induce a marked swelling of mitochondria, to impede the formation of high energy phosphate as well as to act as an uncoupler of oxidative phosphorylation *in vivo*. In the test to see the uptake of I^{32}-labeled compound lipids by various organs, a marked uptake has been observed in spleen, bone marrow, and liver of both irradiated and non-irradiated groups. Further, the uptake of P^{32}-labeled compound lipids in the rabbits given intravenous injections of compound lipid fraction for 30 consecutive days previously has been found to be greatest in pancreas followed by bone marrow, spleen, liver in the order mentioned in male group, whereas it is greatest in spleen, followed by liver and bone marrow in the female group.

With these results the discussion was conducted concerning the relation between the lipid metabolism and X-ray disturbances.

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