THE RELATION BETWEEN THE COMPOSITION OF FATTY ACID FROM X-RAY IRRADIATED RABBIT LIVER AND ITS BIOCHEMICAL PROPERTIES

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X線照射家兎肝より抽出せる脂肪酸分画の生化学的作用

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X線照射後，家兎肝より抽出した脂肪酸分画は癌細胞膜の nigrosin に対する透過性を高め，ミトコンドリアの膨化を促進せしめ，酸化的リン酸化反応の共軛阻害を惹起せしめると同時に latent ATPase の活性を促進せしめる．

この様な生化学的作用は非照射動物の同分画に比して強く，それは照射動物に於て不飽和脂肪酸組成が増加したためと考えられる．

Contents

I. Introduction

It is well known that the hemolysis and uncoupling of oxidative phosphorylation are caused with X-ray irradiation[1)2)3)], although the mechanisms of these phenomena are still obscure.

Surface active agent, such as fatty acid especially in *cis* form of unsaturated one, will produce lysis of the plasma membrane and uncoupling of oxidative phosphorylation in different type of cells[4)5)6)7)]. Namely the surface active agent will induce the similar results as it was observed with X-ray irradiation.

Recently the changes of lipid metabolism with X-ray irradiation have been considered with keen interest[10)11)12)13)14)15)16)] and the quantities of increased phospholipid, lysophosphatide and neutral fat and of no changes of free fatty acid were observed[10)11)]. The increased

lysophosphatide contains rather small amount of unsaturated fatty acid suggesting the liberation of unsaturated one from phospholipid[16]. Thus, the qualitative change of fatty acid in the fatty acid fraction would be expected after X-ray irradiation.

One of the authors, Inaba, has been found an uncoupling factor of oxidative phosphorylation induced with X-ray irradiation in the lipid fraction of ascites fluid of Ehrlich ascites tumor bearing mouse. Furthermore the several uncoupling factors isolated from natural materials have been shown to contain long-chain fatty acids as an active substance, i.e., extracted from rat liver microsome[22], insect sarcosome[23], tumor mitochondria and hepatome cells[7]. Since, the fatty acid plays an important role in biological effect of X-ray irradiation and of its biochemical properties of X-ray irradiated rabbit liver.

II. Materials and Methods

Mitochondria were isolated by the method of Hogeboom and Schneider[17] and 1g tissue equivalent mitochondria were suspended just before use in 1 ml of 0.25 *M* sucrose solution as the stock mitochondrial suspension. These procedures were carried out at 0-4° C.

Ehrlich ascites tumor cells were harvested 7-9 days after transplantation and were washed 3 times with physiological saline solution to remove the red blood cells.

Fatty acid fraction was extracted by the method of Seno and Yamamoto[3] with some modification from normal and X-ray irradiated rabbit liver. These fatty acids were mixed with 1/20 volume of Tween 80 and emulsified by adding physiological saline solution to 1 per cent in concentration.

ADP (adenosine diphosphate) was obtained from Shigma Chemical Co.

Effect of the fatty acid on the cell membrane was observed by the method of Hodes[9] for stainability to nigrosin: the cell suspension was diluted to 7.5×10^6 cells per ml. Five ml of the suspension of tumor cells containing 0.075 per cent nigrosin was stirred gently on ice with a magnetic stirrer. Samples were withdrawn and counts were made from each sample approximately 5 minutes after addition of fatty acid ranging 0.005-0.05 per cent at final concentration. The cells to be counted were diluted twenty times in a white cell pipette with 0.15 per cent nigrosin in isotonic saline, then counted in a blood counting chamber.

Tests of mitochondrial swelling were carried out as described in previous publication[4][5]: the incubation mixture was composed of 4 ml of 0.15 *M* KCl-0.02 *M* Tris aminomethan-HCl buffer solution (pH 7.4), 0.5 ml of 0.05 per cent fatty acid and 0.05 ml of mitochondrial suspension. The colloidal solution of fatty acid was diluted with the KCl-Tris buffer solution to 0.05 per cent. The incubation was performed at 25°C. for 30 mimutes and the mitochondrial swelling was carried out optically in Bechman spectrophotometer at 520 mμ.

Oxidative phosphorylation and physiological swelling-shrinkage of mitochondria were carried out as described in previous paper[8]: simultaneous measurement of 90° light scattering and oxygen uptake of mitochondria were carried out by the special apparatus constructed by one of the authors, K. Utsumi, in the medium consisted of 0.05 *M* sucrose, 0.02 *M* K-phosphate 0.02*M* KCl and 0.1 *mM* EDTA (pH 7.5) at 25°C. Two ml of the incubation

mixture was introduced to the sample chamber of the apparatus and 0.2 ml of stock mitochondrial suspension was added to the incubation mixture (state 1 and 2), After one minute 0.02 ml of 1 *M* sodium succinate was added (state 4) and 1 minute later 0.02 ml of 10 m*M* of ADP was again added (state 3). After reversing to state 4 (changed ADP to ATP),0.04 ml of 0.1 percent fatty acid solution was added.

Ehrlich ascites tumor cells were incubated for 30 minutes at 37°C. in the incubation mixture consisted with 10 ml of Krebs-Ringer solution containing 2 g of Ehrlich ascites tumor cells, 1 ml of 0.02 *M* K-phosphate buffer solution containing 30 μc of P^{32} and 1 ml of 0.05 per cent fatty acid diluted with physiological saline solution. The acid soluble phosphorous compound fraction of the cells was separated by the method of Terada[18] and the incorporation of P^{32} was studied.

III. Results

Effect of fatty acid fraction on the cell membrane: Most of the fresh untreated cells are unstained by nigrosin. As fatty acid is added, an increasing number of cells is stained black first in nucleus and the cytoplasm followed. The effects of fatty acid fractions extracted from X-ray irradiated and non-irradiated rabbit on the staining are shown in Fig. 1. The effect of irradiated one is more than that of non-irradiated one, and that of oleic acid is the lowest. As shown in Fig. 1, the percentage of stained cells increases with the fatty acid concentration and all the cells are stained at the concentration of 0.05 per cent of all fatty acid examined.

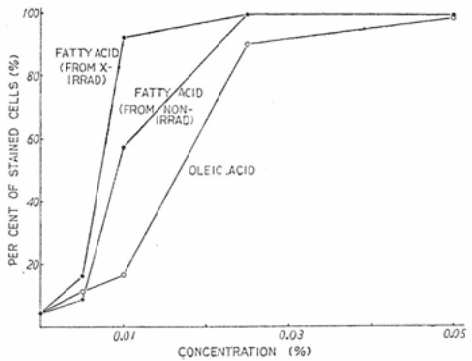

Fig. 1. Effect of oleic acid and fatty acid fraction extracted from normal and X-ray irradiated rabbit liver on the nigrosin stainability of Ehrlich ascites tumor cells.

Effect of fatty acid on the mitochondrial swelling: Oleic acid causes a great acceleration of the swelling of rat liver mitochondria suspended in a medium of 0.15 *M* KCl-0.02 *M* Tris buffer solution at pH 7.4 as shown in Fig. 2. The acceleration of the mitochondrial swelling is observed by the fatty acid fraction especially by the X-ray irradiated one with the same concentration (0.005%).

Effect of fatty acid on the physiological swelling-shrinkage and the change of respiratory

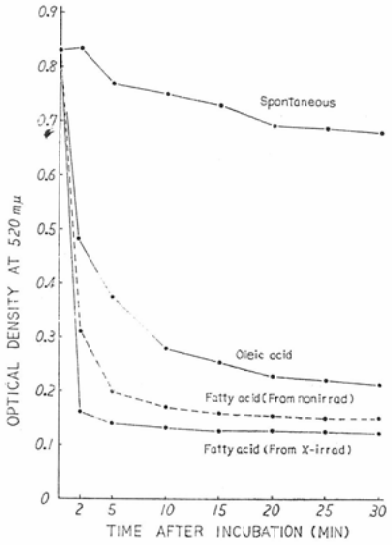

Fig. 2. Effect of fatty acid fraction extracted from normal and X-ray irradiated rabbit liver on the swelling of rat liver mitochondria. Concentration of each fatty acid is 0.005 per cent. Incubated at 25°C for 30 minutes in the 0.15 *M* KCl-0.02 *M* Tris buffer solution (pH 7.4). The effect of the fatty acids was compared with swelling action of sodium oleate at same concentration.

activity of mitochondria: The effects of fatty acid on the mitochondrial structure, i. e., swelling and shrinkage traced by 90°light scattering, and its function, i. e., respiratory activity measured by oxygen electrode are shown in Figs. 3 and 4. First of all, liver mitochondria were added to aerobic medium. Endogenous substrate is not allowed to disappear for several minutes (state 2). The addition of an oxidizable substrate, succinate, accelerated respiration 1.2 times (state 4). In this condition the mitochondrial swelling until a

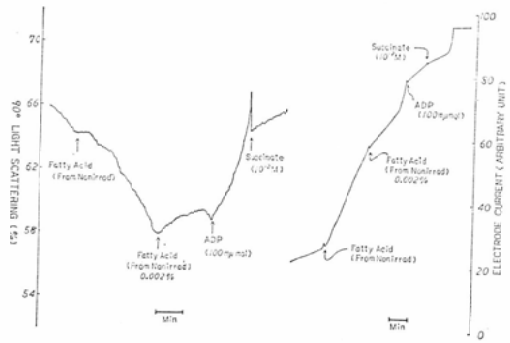

Fig. 3. Effect of fatty acid fraction extracted from normal rabbit liver on the respiration and physiological swelling of rat liver mitochondria. Respiratory release and mitochondrial shrinkage were observed by the addition of the fatty acid fraction.

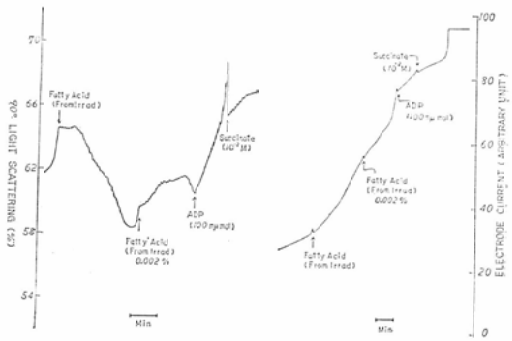

Fig. 4. Effect of fatty acid fraction extracted from X-ray irradiated rabbit liver on the respiration and physiological swelling of rat liver mitochondria. Potent respiratory release and mitochondrial shrinkage were observed by the addition of the fatty acid fraction.

lower steady state level of shrinkage is obtained (physiological swelling). If then the phosphate acceptor, ADP, is added, electron transport is increased 5 times (state 3). In this condition oxidative phosphorylation occurs, and as indicated in Figs. 3 and 4, the ADP: O ratio is 2.1 calculated by the polarographic method. The addition of ADP also causes reversal swelling (physiological shrinkage). When the small amount of added ADP is phosphorylated to ATP, respiration declines to the succinate substrate level, and swelling of mitochondria occurs again. If the fatty acid is added at this stage, electron transport is increased about 3 times and as shown in Figs. 3 and 4 the reversal swelling is induced to the level of prior to the substrate addition. In this condition oxidative phosphorylation is uncoupled and no observable changes in respiration and swelling are occured by the addition of ADP. These phenomena are found also by oleic acid[8] but more uncoupling and shrinkage are induced by the fatty acid fraction, especially by X-ray irradiated one at a concentration of 0.002 per cent. Even in the physiological condition, however, if excess amount of

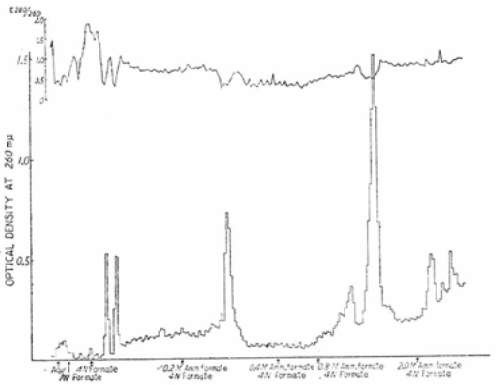

Fig. 5. A column chromatogram of acid soluble fraction of Ehrlich ascites tumor cells after incubation for 30 minutes with Krebs-Ringer phosphate solution.

fatty acid is added, the physiological shrinkage of mitochondria will be change into drastic mitochondrial swelling and into irreversible uncoupling. The effect of X-ray irradiated fatty acid on drastic swelling is more than that of non-irradiated one as shown in Fig. 2.

Effect of fatty acid fraction on the incorporation of P^{32} into acid soluble phosphorous compound: The column chromatogram of acid soluble phosphorous compound fractions from Ehrlich ascites tumor cells incubated with 0.005 per cent of fatty acid fraction extracted from X-irradiated rabbit liver exhibits the low peak of ATP and high peaks of AMP and ADP as shown in Figs. 5 and 6. In this case the incorporation of P^{32} into ATP and GTP is decreased but not into ADP. Namely, the specific activity is decreased remarkably in ATP and is relatively little decreased in ADP showing the hydrolysis of ATP to ADP and inorganic phosphate by ATPase activity which is accelerated by the fatty acid. From the data of total activity it is shown that the treatment of the fatty acid causes the uncoupling of oxidative phosphorylation and the stimulation of mitochondaial ATPase.

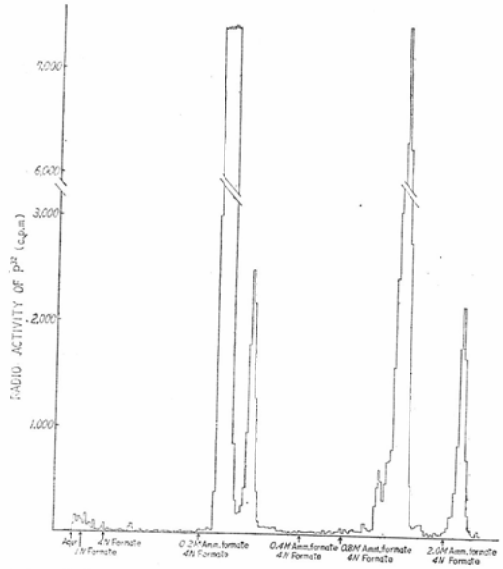

Fig. 6. Radioactivity of each fraction of Fig. 5. Active P^{32} incorporation was observed on the ATP, GTP and ADP.

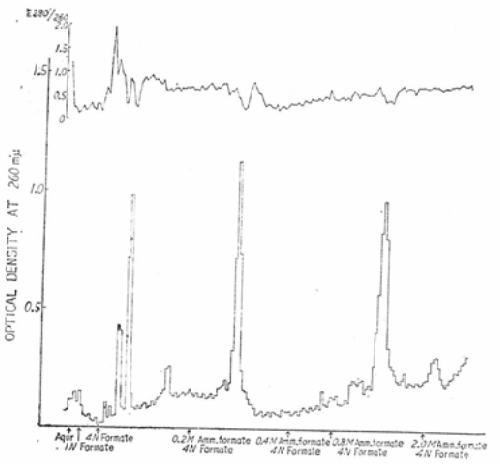

Fig. 7. A column chromatogram of acid-soluble fraction in Ehrlich ascites tumor cells after incubation for 30 minutes with Krebs-Ringer phosphate solution containing 0.005 per cent of fatty acid fraction extracted from X-ray irradiated rabbit liver. Decrement of ATP and increment of AMP and ADP were observed.

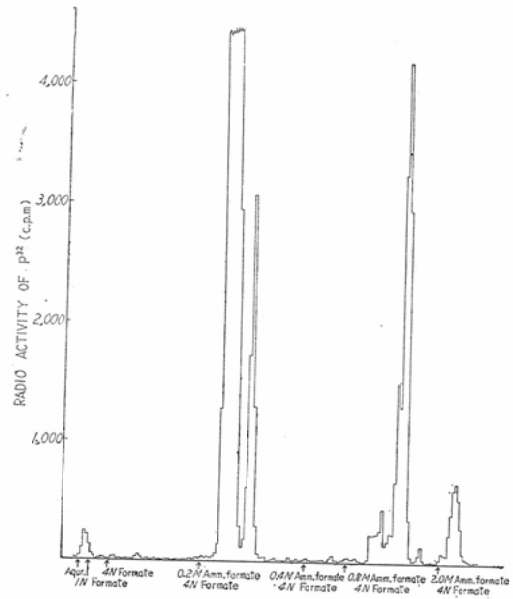

Fig. 8. Radioactivity of each fraction in Fig. 7. Showing the decrement in radioactivity of ATP and GTP.

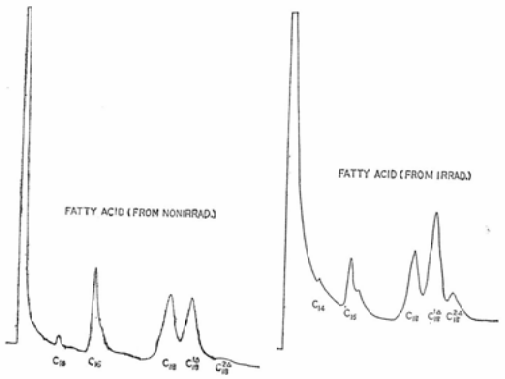

Fig. 9. Gas chromatogram of fatty acid fraction extracted from normal and X-ray irradiateds rabbit liver showing decrement of palmitic acid and increment of oleic and linoleic acids in X-ray irradiated one.

Gas chromatography of fatty acid fraction: Fatty acid composition of non-irradiated and X-ray irradiated rabbit were analysed by gas chromatography. The relative amount of palmitic, stearic, oleic and linoleic acids were compared and the following results are

obtained. Relative amount of palmitic and stearic acids are decreased but that of oleic and linoleic acids increased remarkably in X-ray irradiated one compared with non-irradiated one. And the non-identified peak, may be palmitoleic acid, is appeared following the peak of palmitic acid. Namely the increment of unsaturated fatty acid and the decrement of saturated one are observed in the fatty acid fraction of X-ray irradiated rabbit liver.

IV. Discussion

The experimental results described above demonstrate that the X-ray irradiation induces the increment of unsaturated fatty acid in the irradiated rabbit liver and this has the strong activity of lytic and uncoupling action. The lytic and uncoupling action of X-ray could be observed in the X-ray irradiated animals. Therefore, these actions of the fatty acid increased by X-ray irradiation are assumed to be related to X-ray disturbances. With respect to the changes of lipid metabolism by X-ray irradiation Elko and Luzio[10)11)] presented the elevation of phospholipid and neutral fat content and no alteration of free fatty acid in the plasma and liver of X-ray irradiated rabbit, but did not studied the component of fatty acid fraction. At the present state of research in this field nothing is yet known abont the mechanisms which may involve enzyme reactions to increase unsaturated fatty acids. The following two possibility, however, can be considered: one of them is a splitting of unsaturated fatty acid from compound lipid and the other is an unsaturation of fatty acid. The former possibility is supported by the following reason that the quantitatively increased lysoposphatide with X-ray irradiation contains the relative small amout of unsaturated fatty acid[26)]. The latter one is supported by the following reason that the ionizing radiation causes the oxidation of hydrocarbon and fatty acid *in vitro* and finally produces the double bond or the short chain fatty acids[19)20)].

The stainability of Ehrlich ascites tumor cell with nigrosin is accelerated by the treatment of fatty acid and the degree of acceleration by the fatty acid extracted from X-ray irradiated rabbit liver is larger than that of non-irradiated one. Hodes[9)] described the accelerated stainability of tumor cells to nigrosin by the teatment of surface active agents and found that the sulphonates and sulfates with C_{12} or C_{14} side chains of the anionic surfactants were most effective on the staining effect. The potentiating action of unsaturated fatty acid in uncoupling oxidative phosphorylation, especially in *cis* form, observed in the previous work[8)] using purified samples indicates that these substances play a role in damaging or deformation of membrane structure by its surface activity. With respect to the effect of fatty acid, Lardy and Pressman[5)], and Borst et al.[6)] are reported same results. Namely, the increased stainability of Ehrlich ascites tumor cell by the treatment of fatty acids may be due to their surface activity.

As the physical properties of fatty acid, the surface activity is stronger and melting point is lower in unsaturated state than that of saturated one. The action of surfactants is due to their ability to combine with lipid and or protein in membrane. The hemolysis by X-ray irradiation would not be only due to the increased unsaturated fatty acid but to unsaturation of membrane lipid because membrane permeability would be changed by the

steric hindrance and charge transfer in molecular structure of cell membrane.

Oxidative phosphorylation of rat liver mitochondria is severely uncoupled by fatty acid fraction obtained from X-ray irradiated rabbit liver. It is already known that the oxidative phosphorylation is uncoupled by X-ray irradiation but the mechanism is not clear. Lehninger[7] found the endogenous uncoupling factor in the isooctane soluble fraction from aged and swollen mitochondria. The properties of the uncoupling agent suggest that it may be long-chain fatty acid. These endogenous uncoupling factors may be formed from phospholipid of membrane by the phospholipase and the other enzyme activity may be changed by the changing of physiological state of membrane structure. Recently one of the authars, Inaba[2], found an uncoupling factor in the lipid fraction of X-ray irradiated tumor bearing mouse which contains aboundantly unsaturated fatty acid. In general, saturated and unsaturated fatty acids, especially in *cis* form of unsaturated one, act as a potent uncoupler of oxidative phosphorylation. In addition, the fatty acids have profound effect on ATPase activity of mitochondria[5,6,8].

Thus the evidences described in this paper indicate that the increased unsaturated fatty acid in rabbit liver by X-ray irradiation acts as a part of the hemolytic action and uncoupling action of oxidative phosphorylation.

V. Summary

Fatty acids from normal and X-ray irradiated rabbit liver are studied on the chemical composition and its biochemical properties and obtained following results.

1) The fatty acid acts as a damaging agent of cell membrane. Fatty acid obtained from X-ray irradiated rabbit liver accelerates more nigrosin permeability than that of non-irradiated one.

2) Rat liver mitochondria is swollen by the treatment of fatty acid. The swelling action of fatty acid extracted from X-ray irradiated rabbit liver is stronger than that of non-irradiated one.

3) Oxidative phosphorylation of rat liver mitochondria is uncoupled by the fatty acid. The uncoupling action of fatty acid extracted from X-ray irradiated rabbit liver is stronger than that of non-irradiated one.

4) Latent ATPase of rat liver mitochondria and Ehrlich ascites tumor cells are stimulated by the fatty acid extracted from X-ray irradiated rabbit liver.

5) The quantity of unsaturated fatty acid of fatty acid fraction obtained from X-ray irradiated rabbit liver is more than that of non-irradiated one.

References

1) Van Bekkum, D.W.: The effect of X-ray on the phosphorylation *in vivo*. *Biochim. Biophys. Acta*, 25, 487, 1957. — 2) Inaba, K.: The effect of X-ray on the energy metabolism of mouse Ehrlich ascites tumor cells, *Nippon Acta Radiol.* 22, 1203, 1963. — 3) Yamamoto, M.: Toxic substance found after X-ray irradiation, *Symposia Cell. Chem.*, 9, 141, 1958. — 4) Utsumi, K. and others: Mitochondtial swellig and uncoupling activity of long-chain fatty acids, *Acta Med. Okayama*, 16, 317, 1962. —5) Pressman, B. and Lardy, H.: Effect of surface active agents on the latent ATPase of mitochondria, *Biochim. Biophys. Acta.*, 21, 458, 1956. — 6) Borst, P., Loose, J.A., Christ, E.J., and Slater,

E.C.: Uncoupling activity of long chain fatty acid, *Biochim. Biophys. Acta.* 62, 609, 1962. — 7) Lehninger, A.L. and Remmert, L.F.: An endogenous uncoupling and swelling agent in liver mitochondria and its enzymic formation, *J. Biol. Chem.* 234, 2459, 1959. —8) Utsumi, K. and others: Mitochondrial swelling and oxidative phosphorylation, *Symposia Cell. Chem.*, 13, 1962. in press. — 9) Hodes, M.E., Palmer C.G. and Warren, A.: The effect of surface active agent on the permeability to dye of the plasma membrane of Ehrlich ascites tumor cells, *Exptl. Cell Res.*, 21, 164, 1960. — 10) Elko, E.E. and Luzio, N.R.: Effect of X-irradiation on plasma, liver, and bone marrow lipids of the rabbit, *Radiation Research*, 11, 1, 1959. —11) Elko, E.E. and Luzio, N.R.: Lipid metabolism in X-irradiated rabbits, *Radiation Research*, 14, 760, 1961. —12) Entenman, C., Neve, R.A., Sapplee, H. and Olmstad, C.A.: Effects of X-irradiation on lipid metabolism I. Plasma phospholipid levels in several species, *Biochim. Biophys. Acta*, 59, 97, 1955. — 13) Rosenthal, R.L.: Opalescence of serum after total body X-irradiation as a prognostic sign of death, *Science*, 110, 43, 1949. — 14) Hewitt, J.E., Hayes, T.L. Gofman, J.W., Hones, H.B. and Pierce, F.T.: Effects of total body irradiation upon lipoprotein metabolism, *Cardiologia*, 21, 353, 1952. — 15) Di Luzio, N.R. and Simon, V.A.: The effect of X-irradiation on the plasma lipid fractions of the rabbit, *Radiation Research*, 7, 79, 1957. —16) Ohara, S.: Studies on the compound lipids from X-ray irradiated animal, *Acta Med. Okayama*, 6, 333, 1962. — 17) Hogeboom, G.H.: *Method in Enzymology, Vol.* 1, 16, 1955. — 18) Terada, S.: Studies on the acid soluble phosphorus compounds in rat liver, *J. Jap. Biochem. Society*, 31, 795, 1959. — 19) Swallow, A.J.: The radiation chemistry of organic substances, *Chemical Reviews*, 56, 471, 1956. — 20) Shinozaki, Y. and Ohara, S.: On the autoxidation of unsaturated fatty acids irradiated with the X-ray in emulsion state, *The scientific report of The Faculty of Agriculture, Okayama Univ.* 20, 100, 1962. — 21) Wajtczak, L. and Lehninger, A.L.: Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria, *Biochim. Biophys. Acta*, 51, 422, 1961. — 22) Hulsman, W.C., Elliott, W.B. and Slater, E.C.: The nature and mechanism of action of uncoupling agents present in mitochrome preparation, *Biochim. Biophys. Acta*, 39, 267, 1960. — 23) Wajtczak, L. and Wajtczak, A.B.: Uncoupling oxidative phosphorylation and inhibition of ATP-Pi exchange by a substance from insect mitochondria, *Biochim. Biophys. Acta*, 39, 277, 1960.

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