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Abstract

Chemical and biological characteristics of the unsaturated fatty acids from the liver of irradiated and non-irradiated animals and some unsaturated fatty acids in sale have been described. The unsaturated fatty acid fractions from the rabbit liver taken after irradiating animal with x-ray show hardly any difference from those of non-irradiated animal in each component. But the former were distinguished from the latter in the increased rate of velocity of autoxidation. Similar characteristics were observed on the unsaturated fatty acids irradiated in vitro. They developed less labile free radicals with the shift of the double bonds to the carboxylic group and the conjugated double bonds, dienoic and trienoic acids. Biologically, the fatty acids from the irradiated animal suppressed the growth of bacteria requiring unsaturated fatty acid. And they are slightly stronger in the activity of uncoupling effect for the oxidative phosphorylation and the swelling of mitochondria comparing to those of general fatty acids, oleic and linoleic acids. They showed a strong lytic activity on the cell membrane as in the case of general fatty acids, linoleic, oleic, and some long chain unsaturated fatty acids. Tumor cells surviving through the treatment with unsaturated fatty acids changed the cell characteristics temporarily, with a slow-down of the ascites development and the cell growth.

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**CHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES
OF FATTY ACIDS FROM THE LIVER OF X-RAY
IRRADIATED RABBIT, THE ANTITUMOR
AGENT SO-CALLED OX***

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The antitumor activity of the unsaturated fatty acid fraction from the liver of x-ray-irradiated rabbit has been found through the experiments on the Brown-Pearce sarcoma of rabbit¹, Ehrlich ascites tumor in solid form², and several human carcinomas³. In several cases of human tumor treated with the fatty acids from the irradiated rabbit the tumors disappeared completely or extremely reduced in size by local application. The striking effects were observed on some of the squamous cell carcinomas of the skin and on some of rectum adenocarcinomas³, to which the fatty acids could effectively come in contact at high concentration. Ehrlich ascites tumor moderately developed in subcutaneous tissue became necrotic and disappeared after the injections of the fatty acids into the surrounding tissues⁴. Since then chemical analysis and biological properties of the fatty acids have been exhaustively studied by us with the collaboration of colleagues for the purpose to reveal the mechanism of the antitumor activity. In this paper the chemical, biological and morphological findings obtained through our experiments for the last two years are summarized.

CHEMICAL ANALYSIS

Unsaturated fatty acid fraction was obtained from the liver of rabbit which had received a whole body irradiation with x-ray, 3,000r at one time, 24 hours before the liver was harvested. Fatty acids were obtained with fresh liver homogenate or dried liver powder by the method of FOLCH *et al*^b, and unsaturated fatty acid fraction was obtained by urea abduct method. Chemical analysis proved that the refractory index n_D^{20} was 1.4715, acid value 25.4, saponification value 179.1, iodine number 90 to 150, peroxide value 30.7, unsaponifiable substance 17.5%, conjugated dienoic acid 1.279%, conjugated trienoic acid 0.033%, negative Ninhydrin reaction, negative biuret reaction, negative Molish reaction and positive Liebermann reaction⁶.

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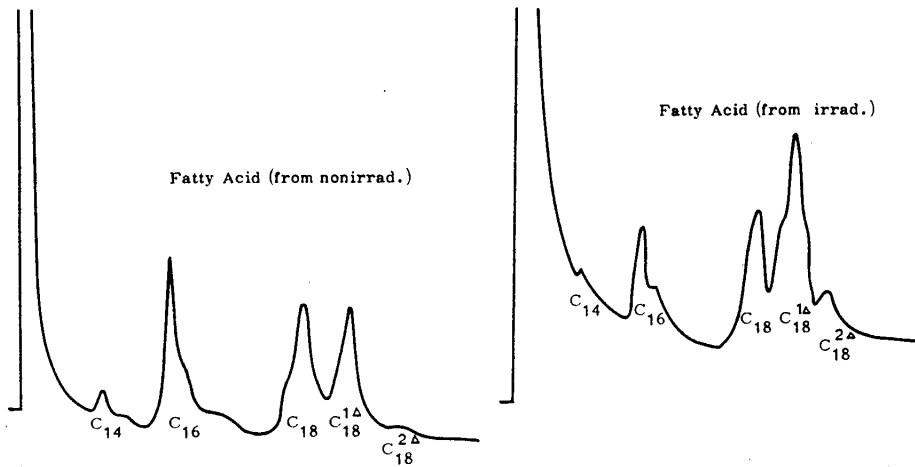


Fig. 1 Gas chromatogram of fatty acid fraction extracted from normal and x-ray-irradiated rabbit liver, showing decrement of palmitic acid and increment of oleic and linoleic acids in that from x-ray-irradiated rabbit.

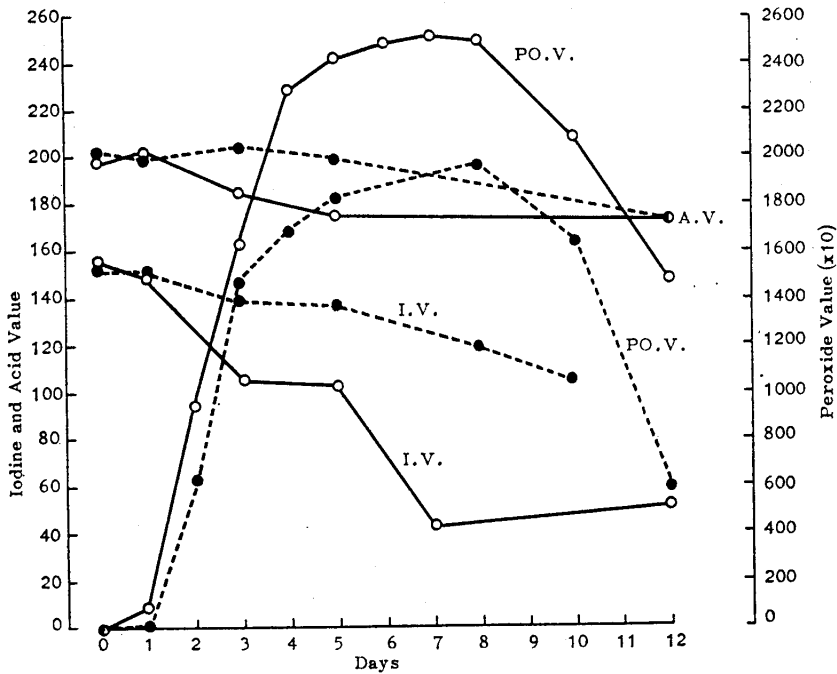


Fig. 2 Changes of chemical properties of fatty acid fractions from irradiated (Solid lines) and non-irradiated (broken lines) animals during autoxidation
I. V. : Iodine value, A. V. : Acid value, PO. V. : Peroxide value.

Gas chromatographic analysis revealed that the main constituents were oleic and linoleic acids with some stearic, palmitic and palmitoleic acids (Fig. 1), though the percentage of these acids varied according to the samples tested. These fatty acids are distinguished from general oleic, linoleic and other unsaturated fatty acids or from those obtained with non-irradiated animal at the rate of autoxidation velocity, i. e. they are rather rapidly oxidized in the air environment (Fig. 2)⁷. Very similar characteristics were observed on the fatty acids irradiated *in vitro*⁸ (Fig. 3). Infrared spectrography also showed an accelerated

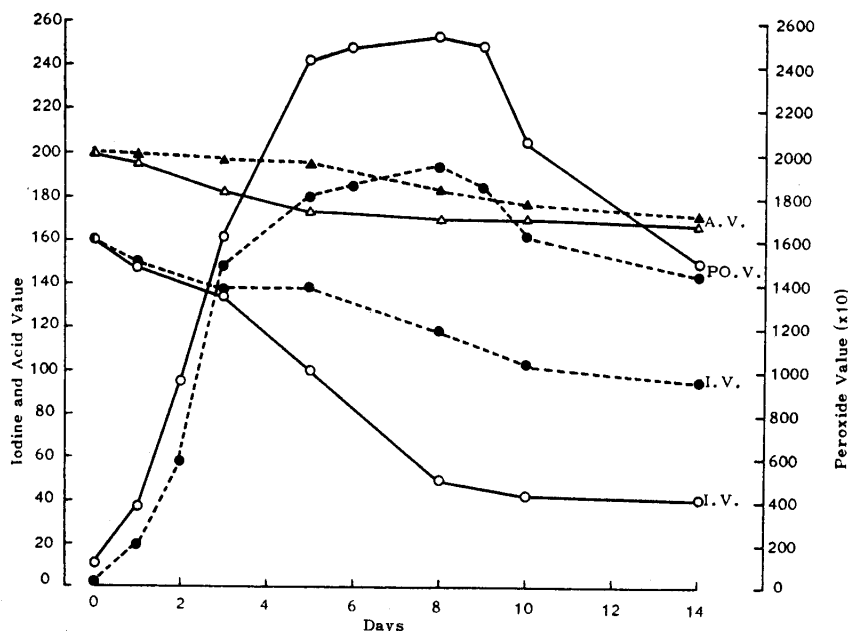


Fig. 3 Changes of chemical properties of x-ray-irradiated linoleic acid (solid lines) during autoxidation
I. V.: Iodine value, A. V.: Acid value, PO. V.: Peroxide value, Broken lines: control.

increase of the OH group in the fatty acids irradiated *in vitro* (Figs. 4, 5)⁸. The results suggested the formation of free radicals less labile which might be correlated to the conjugated double bonds newly formed. By the gas chromatographic analysis after oxidizing with permanganate and esterification a considerable amount of the positional isomers have been detected, which have the double bonds in the position shifted toward the carboxylic group (Table 1)⁹. And by observing the electron spin resonance the free radicals were detected¹⁰.

From these results it is suggested that the fatty acids separated from the x-ray-irradiated rabbit undergo similar changes in chemical structure as those

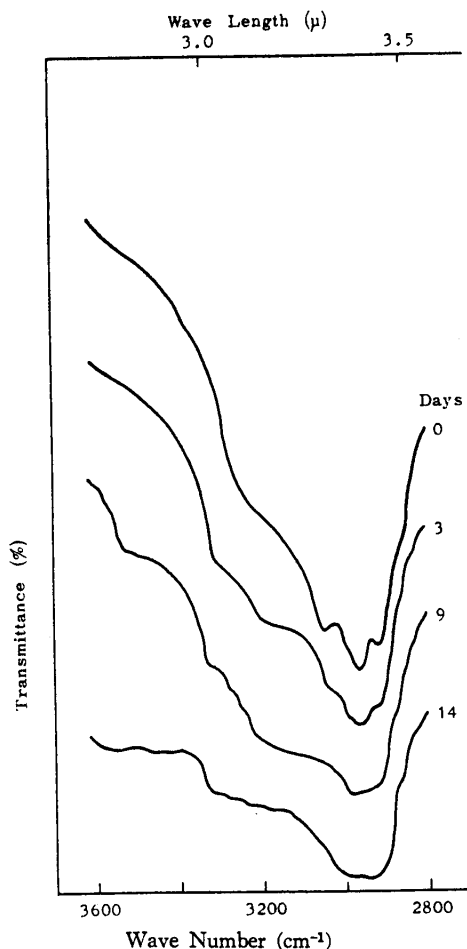


Fig. 4 Changes of infrared spectra of x-ray irradiated linoleic acid during autoxidation.

irradiated *in vitro*. They have some double bonds in the shifted position toward the carboxylic group and free radicals probably associated with conjugated double bonds, which have been newly formed by the irradiation.

BIOLOGICAL ACTIVITIES

The rabbit received the injection of the fatty acids from irradiated animal showed a severe damage in the spermatogenesis, abnormal mitosis of spermatogonias, degeneration and finally disappearance of the cells¹¹. From these results the anti-tumor activity of the fatty acids from the irradiated animal has simply been attributed to the direct arrest of mitosis. But through the studies on bacteria it has been revealed that they should act also as the metabolic antagonist for fatty acids. Observations on *Saccharomyces carlsbergensis* growing on the Sabouraud's medium with glucose, which requires unsaturated fatty acids under anaerobic condition, revealed that the cell proliferation had been extremely

suppressed in the presence of the fatty acids from irradiated animal, while the proliferation had been greatly enhanced with linoleic acid, cholesterol or lecithin under anaerobic condition (Table 2)^{12,13}. Under aerobic condition the fatty acids from irradiated animal suppressed the respiration of the bacteria (Table 3)¹³. These experiments have revealed that the fatty acids are a metabolic antagonist against general fatty acids or lipids, and it is probable that the anti-mitotic activity of the fatty acids on germ cells may be the result of the metabolic disturbance, because mitosis of the bone marrow cells is not so severely damaged by the fatty acids. Besides these, as it suppressed the respiration of the bacteria, it was expected that the energy producing system of mammalian cells

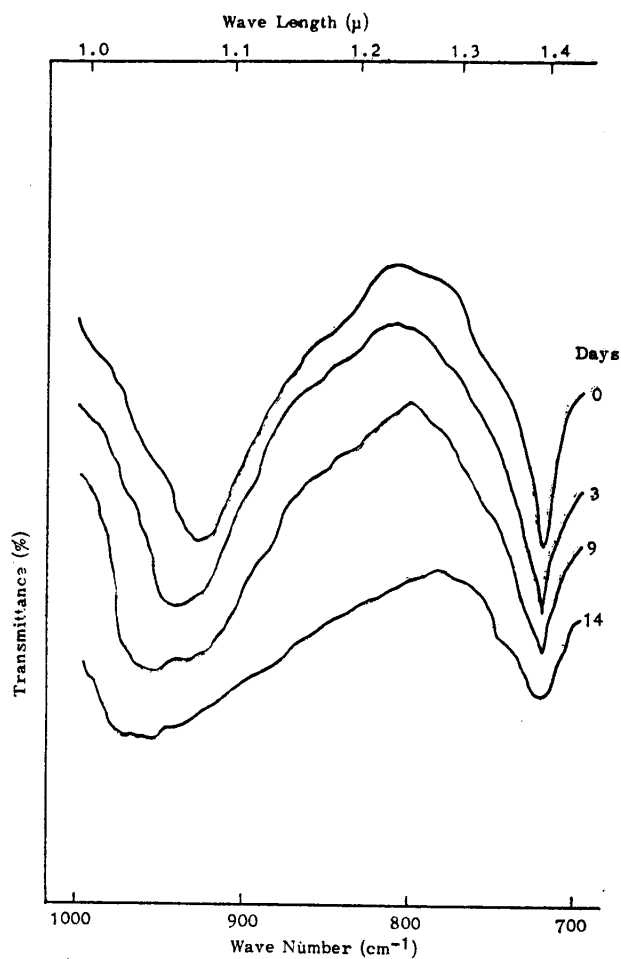


Fig. 5 Changes of infrared spectra of x-ray-irradiated linoleic acid during autoxidation.

Table 1 Main dicarboxylic acid fragments by oxidation

	x-ray-irradiated abbit	Non-x-ray- irradiated rabbit	Plant oil
Hexadecenoic	C ₅ C ₁₀	—	C ₁₀
Octadecenoic	C ₃ C ₄ C ₈ C ₉	C ₉	C ₉
Octadecadienoic	C ₃ C ₅ C ₆ C ₇ C ₉	C ₃ C ₉	C ₃ C ₉
Octadecatrienoic	C ₃ C ₈ C ₉	C ₃ C ₉	C ₃ C ₉

Table 2 The effect of the unsaturated fatty acid fraction from x-ray-irradiated rabbit (OX) on the proliferation of *Sacharomyces carlsbergensis* under an anaerobic condition in the presence of glucose. The concentration of glucose in the media was 2%, and those of lecithin, linoleic acid, and OX were 0.05% in final, respectively. After one week of incubation at 37°C optical density was measured.

Substrate	Optical density at 490 m μ	Rate of proliferation
Glucose	0.071	100
Glucose + lecithin	0.194	290
Glucose + linoleic acid	0.167	250
Glucose + OX	0.108	160
Glucose + Licithin + Linoleic acid	0.208	310
Glucose + Leithin +CX	0.167	250

Table 3 The effect of the fatty acid from irradiated animal on the oxygen uptake of *Saccharomyces carlsbergensis* in the presence of glucose and lecithin as substrate. Concentrations of glucose and lecithin are both at 0.05% and incubation time 60 minutes.

Substrate	Oxygen Uptake (μ l)
None	17.1
Lecithin	23.2
Lecithin + 0.005% fatty acids from irradiated animal	32.0
Lecithin + 0.01% "	33.2
Lecithin + 0.05% "	26.9
Lecithin + 0.1% "	18.2
Glucose	95.2
Glucose + lecithin + 0.005% fatty acids from irradiated animal	112.0
Glucose + lecithin + 0.01% "	113.8
Glucose + lecithin + 0.05% "	70.9
Glucose + lecithin + 0.1% "	66.3

might be arrested by the fatty acids. It has been well established that long chain unsaturated fatty acids have a strong activity as the uncoupling agent for oxidative phosphorylation^{14,15}.

Thus the activity as an uncoupling agent for oxidative phosphorylation has been tested by using the mitochondria of rat liver. By means of a modified method of LEHNINGER¹⁶ for the measurement of the swelling and the contraction of mitochondria, the apparatus attached with oxymeter, the oxygen consumption and the swelling-contraction of mitochondria have been observed concomitantly by adding the fatty acids to the suspension medium¹⁷. The experiments have revealed that the fatty acids from the irradiated animal has a strong uncoupling

effect on oxidative phosphorylation being accompanied by contraction of mitochondria and a marked swelling is induced with the increased amount of fatty acids (Fig. 6). A similar effect has been observed on the fatty acids from non-

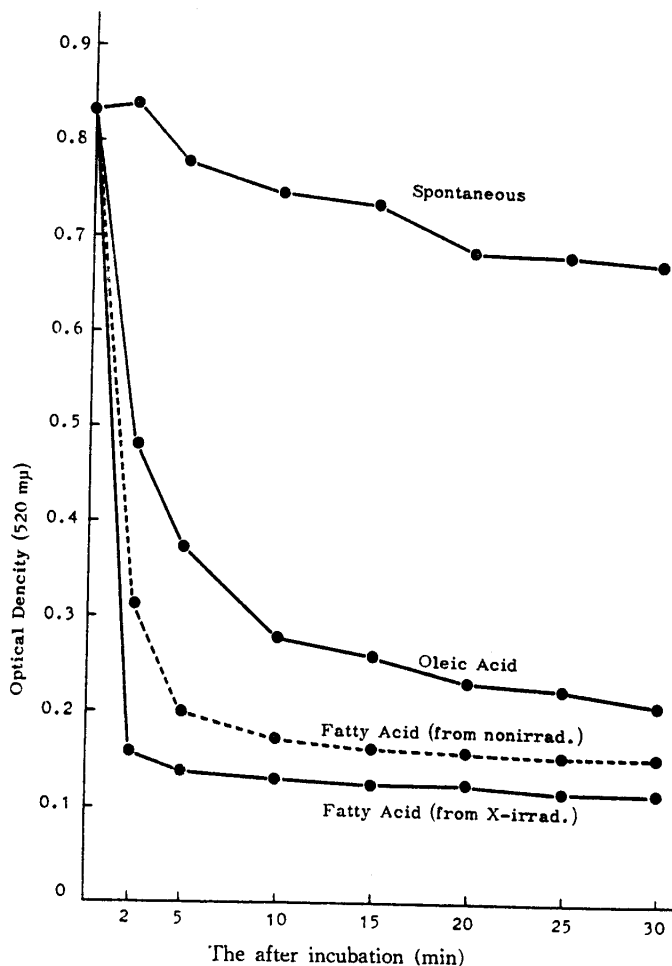


Fig. 6. Effect of fatty acid fraction extracted from normal and X-ray irradiated rabbit liver on the swelling of rat liver mitochondria. Concentration of each fatty acid is 0.005 per cent. Incubated at 25°C for 30 minutes in the 0.15 M KCl-0.02 M Tris buffer solution (pH 7.4). The effect of the fatty acids was compared with swelling action of sodium oleate at the same concentration.

irradiated animal and also in oleic acid but the activity is rather low compared with those from the irradiated animal¹⁸. Thus it has been demonstrated that the fatty acids from the irradiated animal is rather stronger in the uncoupling activity for oxidative phosphorylation and the swelling activity of the mitochondria

of mammalian cells as compared to those of the fatty acids from non-irradiated animal or oleic acid⁸.

The electron microscope observations on the mitochondria swollen by the action of oleic acid have shown destruction of the cristae and the inner limiting



Fig. 7 The picture of mitochondria swollen with 0.1 mM oleic acid. Note the vesicular decomposition of cristae and inner limiting membrane.

membrane into vesicular form (Fig. 7). Differing from normal mitochondria these swollen ones are easily invaded by ATP added to suspending medium resulting in the shrinkage of the vesiculated cristae and the inner membrane



Fig. 8 The picture of mitochondria which were thought to be contracted from the increased light scattering. They were treated with 0.1 mM oleic acid, forming dense masses but the outer limiting membrane remaining almost unchanged.

excepting the outer membrane (Fig. 8)¹⁹. The change induced by the fatty acids from irradiated animal are essentially the same as that induced by oleic acid. The electron microscope findings seem to suggest that the uncoupling activity of unsaturated fatty acids for oxidative phosphorylation may account for the loosening of the mitochondrial membrane.

Hemolytic activity was tested with rabbit erythrocytes suspended in physiologic saline solution, then a marked hemolytic activity was observed with the fatty acids from the irradiated animal as well as with those from the non-irradiated animal and with oleic acid, but the former was slightly stronger in the activity²⁰. Permeability test for the cell membrane with Nigrosin was also observed on Ehrlich ascites tumor cells. In these observations, too, the cell stainability increased by the addition of fatty acids and the strongest effect was observed in the case of the fatty acids from irradiated animal (Fig. 9)¹⁸.

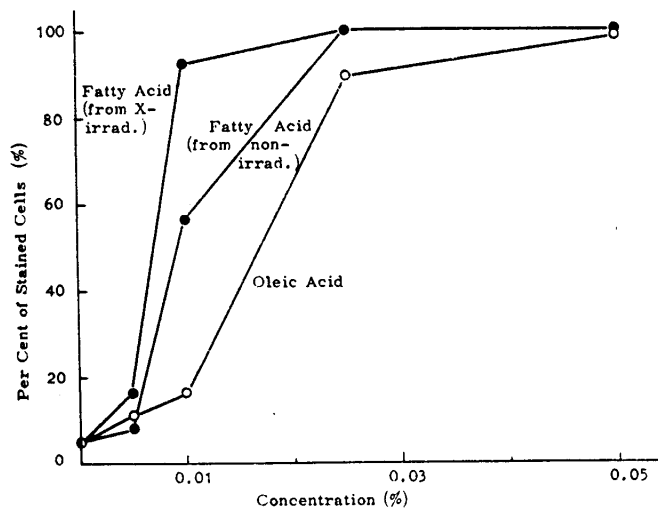


Fig. 9 Effect of oleic acid and fatty acid fraction extracted from normal and x-ray-irradiated rabbit liver on the Nigrosin stainability of Ehrlich ascites tumor cells.

The Ehrlich ascites tumor cells affected by the fatty acids from irradiated animal, oleic and linoleic acids, were observed under light microscope. The changes induced with these fatty acids were essentially the same. After a short period of incubation with the bovine serum containing 1% fatty acid, the cells lost their basophilicity with adsorption of fatty acid droplets all over the cell surface, showing the specific affinity of fatty acid to the cell membrane (Fig. 10, inset)²¹. Electron microscope observation revealed the loosening of the cell membrane with the leak-out of basophilia, ribosomes, but endoplasmic reticulum, mitochon-



Fig. 10 Electron microscope picture of an Ehrlich ascites tumor cell incubated with oleic acid bovine serum mixture at 37°C for 10 minutes. Inset: Light microscope picture of the tumor cells treated similarly, smeared and stained with Nile blue.

dria and other organelles were found in the original cytoplasm without leaking out (Fig. 10)²¹. When the cells exposed to these acids for a short period of time were transplanted into the peritoneal cavity of mice, some of them survived through this treatment but they seemed to change their cell characteristics temporarily.

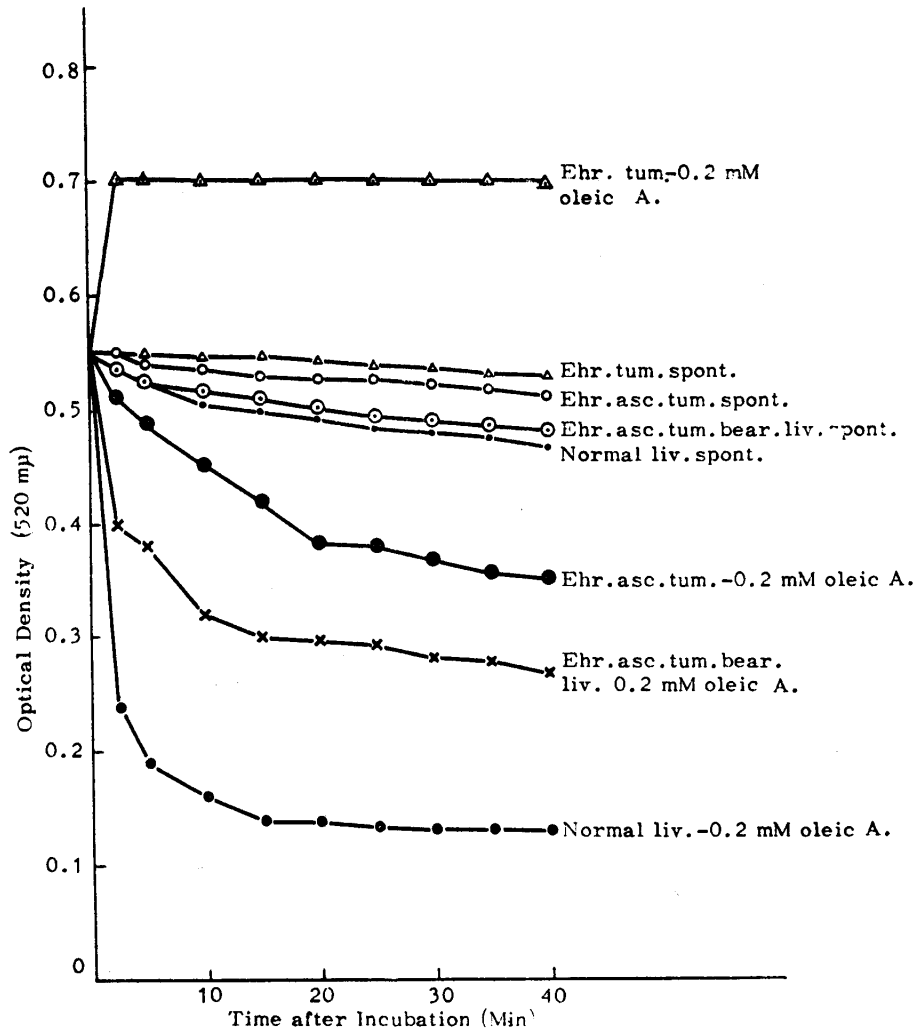


Fig. 11 Effect of sodium oleate (0.2 mM) on the mitochondrial swelling of mouse liver (50 mg), Ehrlich tumor bearing mouse liver (50 mg) and Ehrlich ascites tumor cells (250 mg) (solid and ascites form). The medium consisted of 5.0 ml of 0.15 M KCl-0.02 M Tris buffer, pH 7.4. The amount of oleate shown was added, and optical absorbancy changes were measured at 520 μ at 20°C after the addition of washed rat liver mitochondria derived from 100 mg fresh liver. Spont.: Spontaneous swelling

Newly growing cells in the peritoneal cavity were less basophilic in the cytoplasm and had dense nucleus with ambiguous nucleoli. The growing rate was rather low initially but later restored to the original characteristics, strong basophilicity of the cytoplasm and active mitosis²¹. The mitochondria of Ehrlich ascites tumor cells showed a marked resistance against the swelling action of fatty acids (Fig. 11)¹⁷. The mechanism is unknown but this might be correlated to the survival of the tumor cells through the treatment with fatty acids. This last finding that the tumor cell survives through the treatment with the fatty acids and changes in characteristics even temporarily may be important biologically, because it seems to suggest a possibility that tumor cell may change its characteristics to more mature type receiving some substance from the environment or losing their microsomes through the loosened cell membrane.

SUMMARY

Chemical and biological characteristics of the unsaturated fatty acids from the liver of irradiated and non-irradiated animals and some unsaturated fatty acids in sale have been described. The unsaturated fatty acid fractions from the rabbit liver taken after irradiating animal with x-ray show hardly any difference from those of non-irradiated animal in each component. But the former were distinguished from the latter in the increased rate of velocity of autoxidation. Similar characteristics were observed on the unsaturated fatty acids irradiated *in vitro*. They developed less labile free radicals with the shift of the double bonds to the carboxylic group and the conjugated double bonds, dienoic and trienoic acids.

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Tumor cells surviving through the treatment with unsaturated fatty acids changed the cell characteristics temporarily, with a slow-down of the ascites development and the cell growth.

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