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Biochemical Properties of OX substance, Fatty Acid Fraction from X-irradiated Rabbit, on Cancer Cells

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癌細胞に対するOX物質の生化学的性質

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X線照射家兎より抽出した高級不飽和脂肪酸分
画であるOX物質は癌細胞の増殖を阻害する。癌
細胞に対するかかるOX物質の作用について生化学的に研究した結果、OX物質は癌細胞の酸化
リン酸化を阻害することが明らかとなつたので報告する。

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I. Introduction

The anti-cancerous properties of OX substance which is a higher unsaturated fatty acid fraction extracted from X-irradiated rabbit liver has already been reported by M. Yamamoto and his coworkers¹⁾²⁾. The mechanism of anti-cancerous action of OX substance has been inferred from morphological observation as a factor which directly destroys the structure of cell membrane or works as a sort of metabolic antagonist in the cells.

The present experiment has been conducted to clarified the mechanism of chemical action of OX substance on cancer cells.

II. Materials and Methods

1. Experiment using HeLa cells

HeLa cells (given by Dr. H. Katsuta, the Institute for Infectious Diseases, University of Tokyo, Tokyo, Japan) were used for tissue culture experiments. HeLa cells

were cultured in advance for 24 hours at 38°C in T-D No. 15 tubes containing YLE-bovine serum medium (80:20) and then OX diluted with culture medium was added in the final concentration of 0.1, 0.05, 0.02, 0.01 and 0.001 per cent. The morphological changes were observed by an inverted phase contrast microscope.

Cover slip culture of HeLa cells was done for the same series placing cover slip in the bottom of each T-D tube. Treating with OX for 24 hours the HeLa cells growing on the cover slips were taken out and stained with Giemsa solution. These preparation were used for counting the numbers of cell divisions. In the same series the rest of the HeLa cells growing in each tube was taken out with culture medium from the T-D tube and collected by centrifugation, and washed slightly with YLE culture solution. To the cells were added sodium succinate (0.2 M) 0.2 ml, neotetrazorium solution (0.2%) 0.2 ml, and phosphate buffer solution (pH 7.6) 0.2 ml. After incubation for an hour in 37°C the activity of succinic acid dehydrogenase of the cells was measured by the method of Oda³⁾.

One hundred thousand of HeLa cells suspended in the culture medium were transplanted into each culture tube and were cultured in YLE-bovine serum medium for 48 hours. Then they were cultured for a week in YLE-bovine serum medium containing OX in the concentration described above. The numbers of cells were counted after 2, 4, 6 and 7 day cultivation, and growth curve was obtained from the mean value of 5 culture tubes in each step of cultivation. The pH of each medium was also measured at the same time by glass-electrode pH meter.

The cells after being cultured for 2 days at the above series were collected, and DNA and RNA were separated by the method of Schmidt and Thannhauser⁴⁾. Colorimetric determinations of DNA and RNA were performed by indol reaction⁵⁾ and orcin reaction⁶⁾, respectively.

2. Experiment using Ehrlich ascites tumor cells:

Transplanting 0.2 ml of Ehrlich ascites tumor cells suspension containing about 5,000,000 of the cells into the peritoneal cavity of C₃H mouse, the cells usually were taken out after 9 days. The tumor cells were washed three times with Krebs-Ringer solution by centrifugation and a fixed volume of tumor cells were suspended into Krebs-Ringer solution (pH 7.4).

i) The method of measuring oxygen consumption

Oxygen consumption of the cells was determined by way of Warburg manometric measurement. The composition of reaction mixture and condition are as follows:

main chamber: The suspension of tumor cells (average number of cells, 50,000,000) suspended in Krebs-Ringer phosphatesolution, pH 7.4), 2.2 ml.

center well: 20% KOH, 0.2 ml.

side arm a: 0.1% OX (or 0.9% NaCl solution), 0.3 ml.

side arm b: 0.18 M glucose (or 0.9% NaCl solution), 0.3 ml.

The total volume of reaction mixture was 3.0 ml. Gas layer was air and reaction

was conducted for an hour in 38°C. For the determination of the volume of carbone dioxide produced in aerobic condition 0.2 ml of distilled water and 0.3 ml of 5N H₂SO₄ was placed in the center well and the side arm b in stead of KOH and glucose (or NaCl solution), respectively.

ii) The method of measuring anaerobic glycolysis

The volume of carbon dioxide produced in anaerobic condition was determined by Warburg manometric measurement of the following condition :

main chamber : the suspension of tumor cells (the average number of cells, 50,000,000 suspended in Krebs-Ringer bicarbonate solution, pH 7.4), 2.2 ml.

center well : distilled water, 0.2. ml.

side arm a : 0.1% OX (or 0.9% NaCl solution), 0.3 ml.

side arm b : 0.18 M glucose, 0.3 ml.

The total volume was 3.0 ml. Gas layer was CO₂-N₂ gas (5:95) and the reaction mixtures were incubated for an hour in 38°C.

iii) The method of lactic acid determination

After the Warburg manometric measurement of oxygen consumption and carbon dioxide production the reaction was stopped by the addition of trichloroacetic acid (TCA) solution to the mixture in a final concentration of 5%. Leaving 30 minutes in room temperature, it was centrifuged at 2,000×G. The supernatant fluid was used to determine lactic acid by the method of Barker and Summerson⁷⁾.

iv) The method of measuring the incorporation of P³² into Δ 10 P

By the following reaction mixture and the condition using Warburg manometric apparatus, oxygen consumption and the incorporation of P³² into Δ 10 P were determined

main chamber : the suspension of tumor cells (the average number of cells, 50,000,000), 1.7 ml.

center well : 20% KOH, 0.2 ml.

side arm a : Krebs-Ringer phosphate solution containing 20 μ C P³², 0.5 ml.

side arm b : 0.18 M glucose (or 0.9% NaCl solution), 0.3 ml and 0.1% OX (or 0.9% NaCl solution), 0.3 ml.

The total volume of reaction mixture was 3.0 ml and the mixture was incubated for 30 minutes in 38°C. After the reaction the reaction mixture was centrifuged at 2,000×G in 4°C. The cells were washed with cold Krebs-Ringer solution. To these cells 5 ml of cold 8% TCA solution was added. After leaving for 30 minutes they were centrifuged and the supernatant was used for the determination of Δ 10 P. To 1 ml of this supernatant 1 ml of 1.5 N sulfuric acid was added, and then it was heated for 10 minutes in 100°C.

Takahashi's method³⁾ was used to isolate and determine inorganic phosphate. The value of Δ 10 P was calculated from the defference between the value of the phosphate in the supernatant to which 1.5 N sulfuric acid was added and heated 10 minutes in 100°C and the value of inorganic phosphate in the supernatant before

hydrolysis with sulfuric acid. By taking 1 ml of the above samples on a stainless disk and after drying the radioactivity of P^{32} was determined on the infinite thinness by G-M counter (manufactured at the Kobe Industrial Co. 131 type).

v) Fractionation of acid soluble phosphate compounds

After 9 days of transplanting Ehrlich ascites tumor cells into the peritoneal cavity of mouse, 0.3 ml of 2% OX emulsion was injected. Three hours later the ascites tumor cells were taken out, collected by centrifugation, and washed with 0.9% NaCl solution. Acid soluble phosphate compounds in 2 g of the tumor cells were extracted with 5% TCA solution and fractionated by Terada's method⁹⁾ and which is a modification in a microscale of ion exchange column chromatography of Potter's method¹⁰⁾.

The OX used in this experiment was prepared as follows: OX (the original oil given by the Toshiba Pharmaceutical Ind. Co.) was homogenized by a Waring Blender with 1/20 (W/W) of tween 80 and 0.9% sodium chloride solution and 1% OX emulsion thus prepared was used as a standard OX in this experiment.

III. Results

The morphological changes of HeLa cells with the advance of time after the treatment with OX are as shown in Picture 1. The HeLa cells treated with 0.02% OX form blister and the treatment of 0.05% OX the cancer cells are almost destroyed.

Table 1. Effect of ox on the derobic and anderobic glycolysis and respiration of Enrlich ascites tumor cells

System	Lactate formation (μ mole/mg cell/hr)	Qo ₂	Qco ₂	RQ	WQ	QL ^{air}	QL ^{N₂}
Control		6.35	5.70	0.89			
+ OX (0.01%)		3.27	3.41	1.04			
glucose	0.482	3.50	3.62	1.03	3.5	12.3	13.4
glucose + OX (0.01%)	0.666	2.63	2.95	1.12	6.6	17.0	29.2

The growth curve of Fig. 1 shows that the growth of HeLa cells as inhibited about 50% by the treatment of 0.02% OX and shows little growth by 0.05% OX treatment.

The effect of OX on the mitosis of HeLa cells is as shown in Fig. 2. At 24 hours after treatment the mitosis of HeLa cells is inhibited considerably by 0.01% OX and inhibited completely by 0.02% OX.

The effect of OX on the amounts of DNA and RNA is as shown in Fig. 3. The amounts of DNA and RNA of HeLa cells per tube cultured for 2 days increase by the treatment of 0.001% OX, but decrease when treated with more than 0.01% OX.

The effect of OX on the succinic dehydrogenase activity of HeLa cells is as shown in Fig. 4. It increases when treated with less than 0.01% OX but decreases rapidly when treated with more than 0.02% OX.

Fig. 1 Effect of OX on the growth of HeLa cells

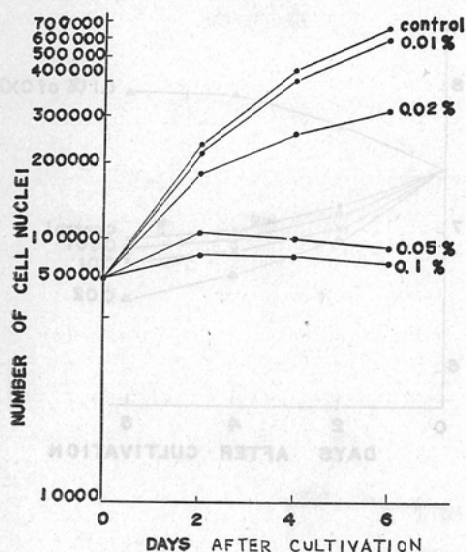


Fig. 2 Effects of OX on the mitosis of HeLa cells (24hr. treatment of OX)

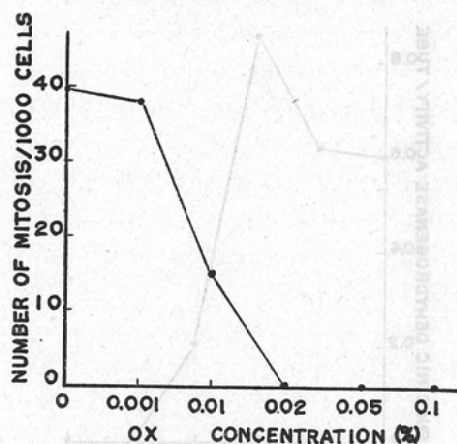
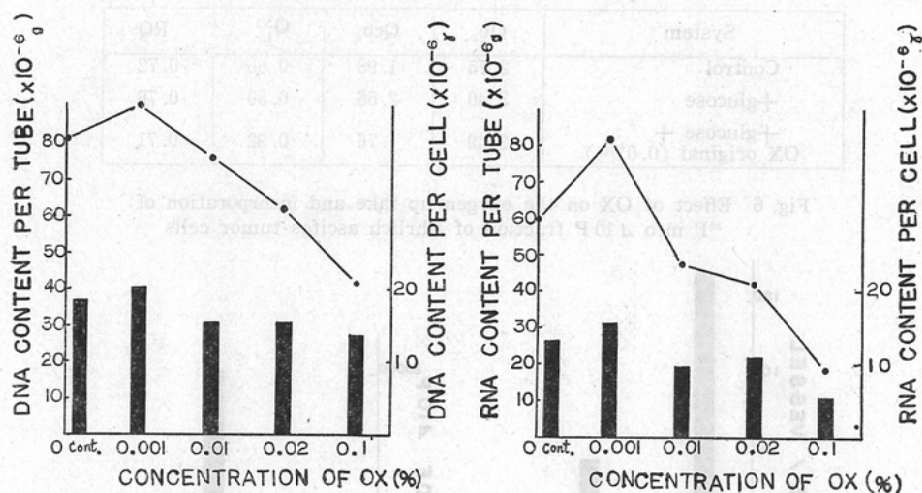


Fig. 3 Effect of OX on the DNA and RNA contents of HeLa cells



The changes of pH of the medium during the culture of HeLa cells are shown in Fig. 5. It decreases when treated with less than 0.02% OX. The reason of this decrease is concerned to be due to the fact that lactic acid increases by the treatment of OX.

The effects of 0.01% OX treatment on Q_{O_2} , Q_{CO_2} , Q_L^{air} , $Q_L^{N_2}$, RQ and WQ are shown in Table 1. Q_{O_2} and Q_{CO_2} both decrease, Q_L^{air} , $Q_L^{N_2}$, RQ and WQ increase. These facts show that the treatment with 0.01% OX inhibits the respiration of Ehrlich ascites tumor cells but enhances glycolysis.

Fig. 4 Effect of OX on the succinic dehydrogenase activity of HeLa cell (24hr. treatment of OX)

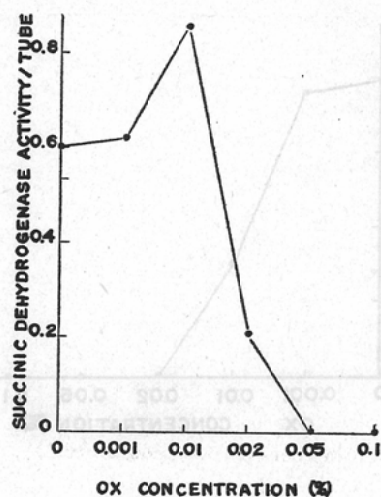


Fig. 5 Effect of OX on the pH of culture medium of HeLa cell

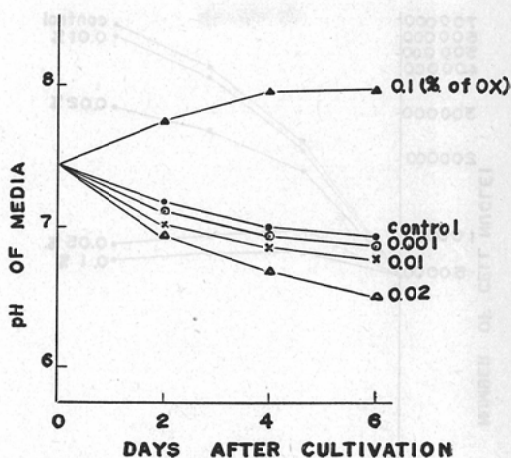


Table 2. Effect of OX on the respiration and anaerobic glycolysis of liver slices (mouse)

System	QO ₂	QCO ₂	Q _L ^{N₂}	RQ
Control	2.75	1.98	0.66	0.72
+glucose	3.40	2.56	0.50	0.76
+glucose + OX original (0.01%)	2.49	1.76	0.92	0.71

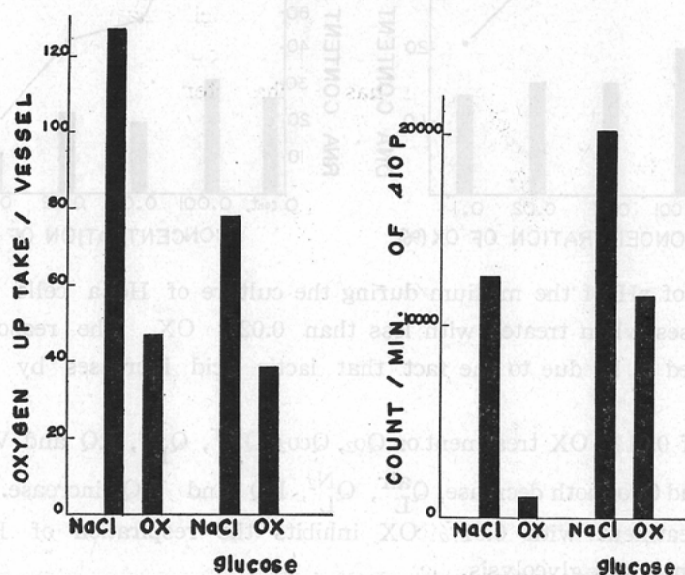
Fig. 6 Effect of OX on the oxygen up take and incorporation of ³²P into Δ10 P fraction of Ehrlich ascites tumor cells

Fig. 7 (a). A chromatograph of acid soluble fraction of ascites tumor cells (wet. 1.95g). control.

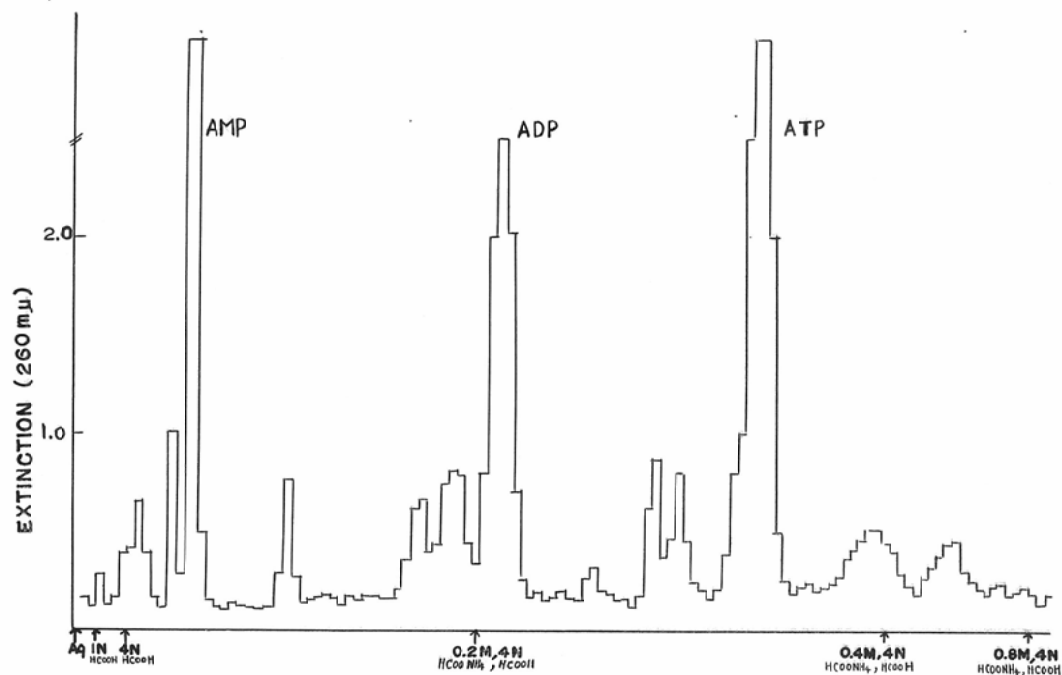
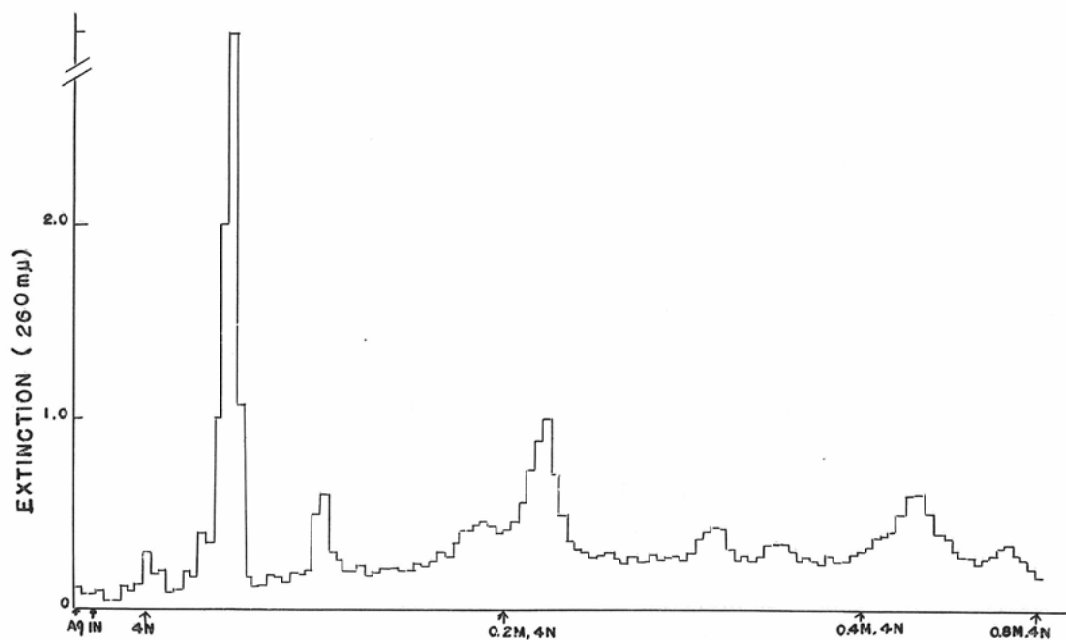


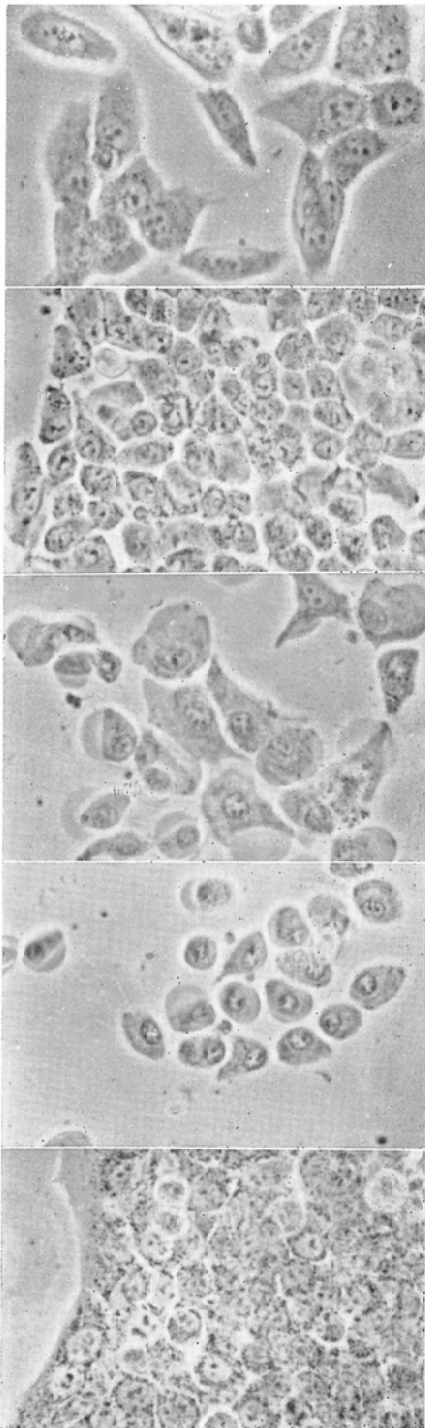
Fig. 7 (b) A chromatograph of acid soluble fraction of ascites tumor cells (wet. 2.4g). 3hr. after administration of OX (0.2 cc 2%) into peritoneal cavity of tumor bearing mouse.



Morphological change of HeLa cell attacked by various concentration of OX

3 hr.

9 hr.



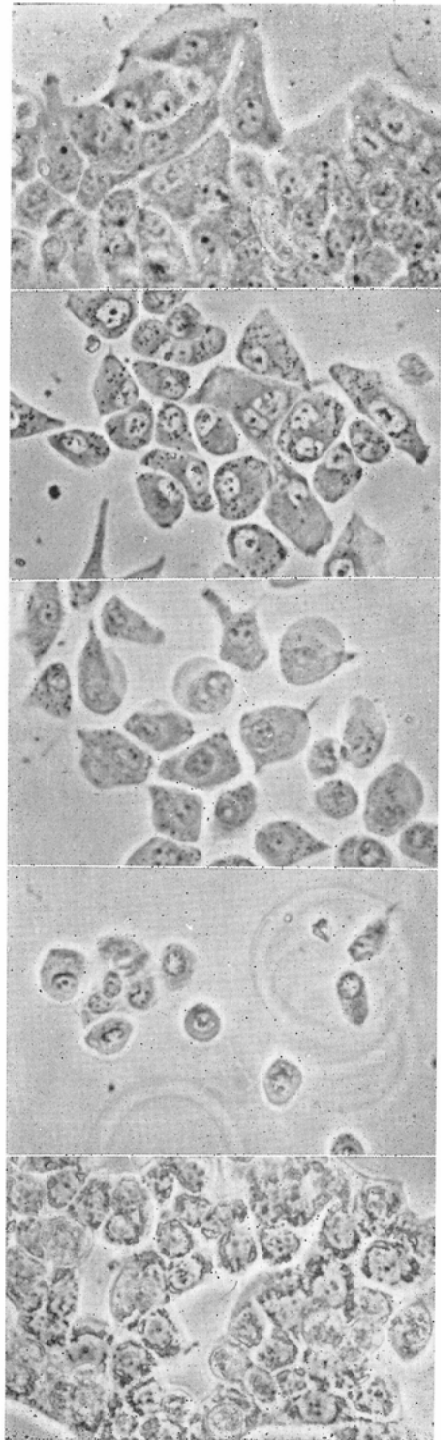
Control

0.1% OX

0.05% OX

0.02% OX

0.01% OX



The effect of OX on the respiration and anaerobic glycolysis of mouse liver slices are as shown in Table 2. Q_{O_2} and Q_{CO_2} decrease a little. RQ decreases contrary to the case of tumor cells and $Q_L^{N_2}$ increases like the case of tumor cells.

The effect of 0.01% on the oxygen consumption and the incorporation of P^{32} into $\Delta 10$ P fraction of Ehrlich ascites tumor cells is as shown in Fig. 6. They are absolutely inhibited with OX and in such cases the inhibition of oxygen consumption by OX is weaker compared to that of P^{32} incorporation into $\Delta 10$ P. Moreover oxygen consumption decreases when glucose is added as substrate but the inhibition of the incorporation of P^{32} into $\Delta 10$ P by the addition of OX is ameliorated considerably. This shows that OX has a character as an uncoupling agent of oxidative phosphorylation.

On the basis of these observation, actual contents of nucleotide phosphate compounds in the tumor cells in tumor bearing mice were determined by column chromatography. 0.3 ml of 2% OX emulsion was injected into the peritoneal cavity of tumor bearing mouse. The tumor cells collected three hours after injection and used for the chemical analysis. The result are shown in Fig. 7 (a) and (b). It becomes clear that by the treatment with OX, the ATP fraction of tumor cells decreases remarkably.

IV. Discussion

0.02% OX restrains 50% of the growth of HeLa cells 6 days after treatment and decreases the amounts of DNA and RNA in HeLa cells per culture tube 2 days after treatment to 75% and 66%, respectively.

0.01% OX has little to inhibit the proliferation of HeLa cells but it acts remarkably on the energy metabolism of Ehrlich ascites tumor cells.

OX inhibits the formation of ATP by uncoupling oxidative phosphorylation but on the contrary stimulates the formation of lactic acid. Such characters of OX resemble to that of fatty acid which has been reported by Scholfield¹¹⁾ and Utsumi et al¹²⁾. namely, fatty acid including more than C_{10} chain has a character as an uncoupling agent to oxidative phosphorylation such as DNP. Also OX has a similar character to endogenous uncoupling factors from rat liver cytoplasm because according to Lehninger¹³⁾ and Pressman and Lardy¹⁴⁾ they cause the swelling of mitochondria, uncouple oxidative phosphorylation and stimulate the activity of the latent mitochondrial ATPase.

V. Summary

1. OX substance, unsaturated fatty acid fraction from X-irradiated rabbit, restrains the proliferation of HeLa cells and decreases the amounts of DNA and RNA of the cells per culture tube.

2. OX inhibits the respiration and enhances the glycolysis of Ehrlich ascites tumor cells. To liver slices of mice, OX also inhibit its respiration and enhances glycolysis but RQ decreases contrary to the case of cancer cells.

3. OX uncouples the oxidative phosphorylation of cancer cells.

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