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# Breakage of Chromosomal DNA with Reductones in Foodstuffs

## 1. The Action of L-Adrenaline

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One of aromatic reductones, adrenaline (25 $\mu$M) causes double strand breakages in chromosomal DNA of rat fetal lung cell strain cultured in Eagle MEM medium after the 5 hour-contact.

Prior to double strand breakages of chromosomal DNA, single strand ones are observed by sucrose density gradient ultracentrifugation. These chromosomal alterations are not recovered during 30 hours after the removal of the reductone.

## INTRODUCTION

Reductones, produced in process of food-manufacturings, have DNA- and RNA-depolymerizing potencies (Yamafuji et al., 1970, 1971).

Aromatic reductones are the first ones that are discovered their strand-breaking abilities of DNA and RNA ***in vitro*** (Murakami and Yamafuji, 1970). L-Adrenaline, one of sympathomimetric catecholamines, also belongs to aromatic reductone and it is enzymatically synthesized from tyrosine in foodstuffs by mammals.

These aromatic reductones depolymerize nucleic acids, and these potencies are remarkably enhanced by the co-existence with $Cu^{2+}$ (Yamafuji and Murakami, 1968). The mechanism of phosphodiester breakage in nucleic acids with the reductone has been circumstantially researched (Murakami and Yamafuji, 1970; Iiyama ***et al.,*** 1973) and effects of produced strand-scission is discussed with regard to the initiation of DNA-dependent RNA polymerase reaction (Yamafuji *et al.*, 1972).

The present paper describes the aromatic reductone also causes single and double strands breaks of chromosomal DNA in mammalian cells.

## MATERIALS AND METHODS

Cells: Rat fetal lung (RFL) cells were grown in Eagle MEM medium, supplemented with 0.0292 % of glutamine and 10 % of calf serum. Sterilization of medium was performed by the use of membrane filter (pore size, 0.45 $\mu$). Seeded cells ($10^5$) amounted to confluency to make full sheet at 37°C during 4 days in TD 15 flask.

***Trypsinization of cell sheet :*** To transplant the cells, cell sheet was treated with

sterilized trypsin solution (200 U/ml) dissolved in sterilized phosphate buffered saline (PBS) (Dulbecco et al, 1954) for 5 to 10 minutes at room temperature. As the excess trypsinization was injurious to cells, the treatment was performed under the observation with inverted microscope to obtain the most suitable phase in which intercellular bondages took apart. Trypsinized cells were collected in a small glass tube and centrifuged at 2,000 rpm for 5 minutes. Precipitated cells were suspended in the medium as to give $10^5$ cells per ml. Collected cells were never washed, as remained trypsin was adsorbed and inactivated with calf serum in newly added medium.

*Labelling of cellular DNA with $^3$H-thymidine :* To vigorously proliferating cells of the 3rd day, 2 $\mu$Ci of $^3$H-thymidine was added, and incubation of the mixture was proceeded for 4 hours at 37°C. Then, the medium containing $^3$H-thymidine was discarded to stop the incorporation of radioactivity. Prolongation of isotope-labelling gave the same results to the sedimentation analyses. Incorporation of $^3$H-thymidine into cellular DNA in slightly alkaline medium (pH 8.0) was remarkably hindered as compared with in neutral one (pH 7.2)

*L-Adrenaline treatment:* Labelled cells ($10^5$/ml) were incubated with sterilized 25 $\mu$M or 2.5 $\mu$M of adrenaline (final concentration) for various durations at 37°C. Treatment of cells with 100 $\mu$M of the drug for 6 hours functioned cytocidally. The cell sheet was then trypsinized and floating cells were cellected by the low speed centrifugation, and washed with cold PBS. Cells ($10^5$/0.2 ml) suspended in cold PBS were immediately analyzed by the use of sucrose gradient centrifugation.

Sucrose gradient ultracentrifugation was performed in two ways : (1) With neutral sucrose gradient centrifugation in a Spinco SW-39 swinging bucket rotor ; 10 to 30 % sucrose gradient, buffered with 0.01 M Tris-HCl-1 M NaCl at pH 7.2 (4.4 ml) was prepared in a nitrocellulose tube: at the bottom of the tube, 0.2 ml of 80 % sucrose was layered as cushion to protect over-sedimentation of isotope-labelled material; to isolate near-intact DNA from mammalian cells, we used a method in which 2 % sodium dodecyl sulfate (SDS) solution at pH 7.2 was employed for lysing the cells ; at the top of 10 to 30 % sucrose gradient, 0.2 ml of SDS solution was added, followed by the introduction of $10^5$ cells suspended in 0.2 ml of PBS solution. After standing for 20 minutes at 37°C, the gradient was centrifuged in the swinging bucket rotor SW-39 at 30,000 rpm for 3 hours. (2) With alkaline sucrose gradient centrifugation in the same rotor; on the top of a 4.4 ml of alkaline sucrose gradient (10 to 30 % sucrose in 0.3 N NaOH, 0.01 M EDTA and 0.5 M NaCl) accompanying 80 % sucrose cushion, 0.2 ml of 0.5 N NaOH containing 0.1 M EDTA was layered; cells ($10^5$) in 0.2 ml of 0.8 % NaCl-0.038 % KCl-0.01 % $Na_2HPO_4$ (Sambrook *et al.*, 1968) were carefully layered into the alkaline layer on the top of the gradient. The gradient was stored at 4°C for 16 hours to complete the liberation of DNA. Storing temperature must be carefully maintained. Then the mixture was centrifuged in a Spinco SW-39 rotor at 30,000 rpm for 1.5 hours at 10°C.

*Fractionation of sucrose gradient and radioactivity measurement :* The high viscosity of the DNA tends to create considerable turbulence in the tube collection. To avoid this difficulty, fractions were collected from the top by pumping saturated sucrose solution in which 0.1 % of sodium fluorecein was added to clarify the

bottom fraction. The tube content flowed through polyethylene tube and was guided into fractionation tubes, thus content was fractioned to 20.

To each fraction tube, 0.2 ml of 0.25 % bovine albumin was added to stabilize labelled acid-precipitable material. One ml of 5 % trichloroacetic acid (TCA) solution was pipetted into the mixture under cold condition, then kept at 0°C for 20 minutes. Acid-precipitable material was collected by low speed centrifugation. Precipitate was washed twice with 2 ml of cold 5 % TCA. Washed precipitate was dissolved in 0.4 ml of 2 N ammonia water. The method for liquid scintillation counting of radioactivity in the acid-precipitable material was described in a previous report (Murakami *et al.*, 1971).

RFL cellular DNA derived by these methods sedimented normally as a single distinct peak in sucrose gradients. The sedimentation constants were roughly estimated to be 300S for neutral and 400S for alkaline sucrose solution.

The determination of sedimentation behavior of mammalian DNA by this method permitted a sensitvie detection of strand breaks.

*Relative growth determination of the cells :* To determine the growth rate of cells, ordinary, counting of trypsinized cell numbers on hematometer was repeated as time passed. This method is complicated and still further exposed to the chance of bacterial infections. Moreover, it is already known that trypsin treatment affects on physiological state of cells.

We devised more simple, physiologically inert and statistically manageable method, compared to the former. Under the bottom of TD-15 flask were marked 5 circles which had 1 mm-inner diameter. An inverted microscope we used had such a wide field as 1.2 mm of diameter that the circle was successfully coincided with microscopic field. Cell numbers in 5 circles were counted at suitable intervals. From these data, standard error on counting cell numbers was calculated.

*Chemicals:* L-Adrenaline was purchased from Merck, West Germany. $^{3}$H-Thymidine (5.0 curies/m mol) was obtained from Radiochemical Center, Amersham. Eagle MEM powder was a product of Nissui Seiyaku Co., Tokyo. Glutamine was received from Wako Pure Chemcial Co., Trypsin was a product of Mochida Seiyaku Co.

## RESULTS AND DISCUSSION

The sedimentation pattern of double stranded DNA derived from RFL cells which treated with 2.5 $\mu$M or 25 $\mu$M of L-adrenaline at 37°C for 6 hours was obtained after centrifugation of neutral sucrose gradient at 30,000 rpm for 3 hours. As shown in Fig. 1, the pattern of untreated celluler DNA exhibited a sharp main peak at the 9th fraction from the bottom of centrifuge tube.

By the incubation with 25 $\mu$M of adrenaline, the main peak of radioactivity shifted to the 12th fraction, indicating double strand scission of chromosomal DNA. Radioactivity of DNA derived from the treated cells was also condensed in a single peak. It is suggested that the molecular weight of double strand-broken DNA is comparatively mono-dispersal. Adrenaline of 2.5 $\mu$M never brought about the transition of sedimentation peak under neutral condition.

The double strand breaks of cellular DNA was never observed in case of 4 hour-treatment with 25 $\mu$M of adrenaline. Centrifugal pattern of 5 hour-treat-

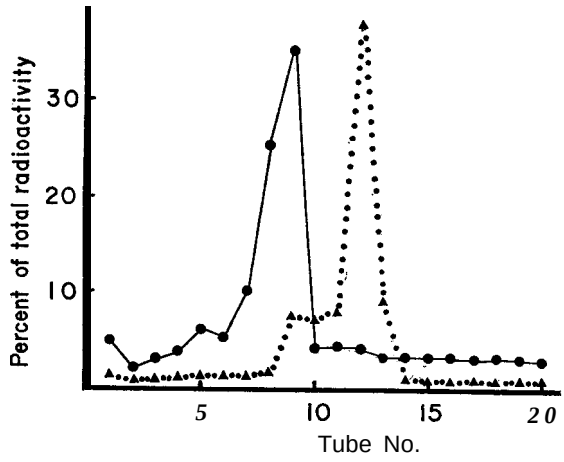



Fig. **1.** Sedimentation pattern of double strands of DNA derived from REL cells treated with 25 $\mu$M of L-adrenaline. Fractions collected were numbered from bottom to top of the centrifuge tube. ●—● untreated control ; ▲...▲ treated with adrenaline for 6 hr.

ment showed the occurrence of double strand breakages, giving the same profile as in Fig. 1.

Prior to double strand breakages, single strand breaks were found by 1 hour-treatment with adrenaline. Fig. 2 illustrates the alkaline sucrose gradient pattern of DNA derived from treated cells. One-hour treatment gave new two peaks which resulted in single strand breaks.

By the treatment for 3 hours, single strand breaks were accelerated and

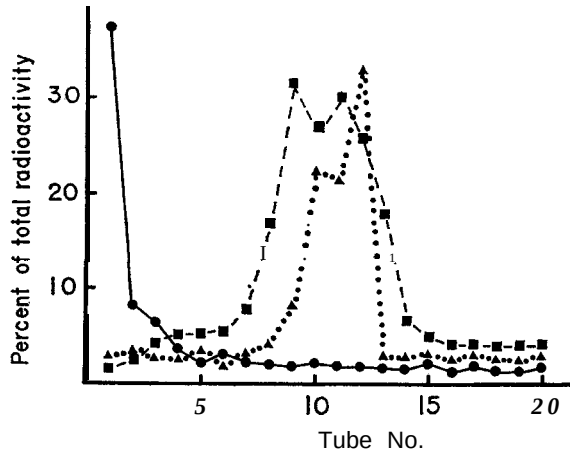



Fig. 2. Sedimentation pattern of single strands of DNA derived from RFL cells treated with 25 $\mu$M of L-adrenaline. Fractions collected were numbered from bottom to top of the gradient. ●—● untreated control ; ■---■ treated with 25 $\mu$M of adrenaline for 1 hr ; ▲...▲ for 3 hrs.

main radioactive peak of resulted fragmentation situated to the 12th fraction, indicating that produced fragments were further nicked.

These findings clarify that single strand scissions take place prior to double strand breaks. Such strand breaks should give remarkable alteration to the intracellular organization of DNA in treated cells. In spite of many single and double strands breaks, treated cells could proliferate, as shown in Fig. 3.

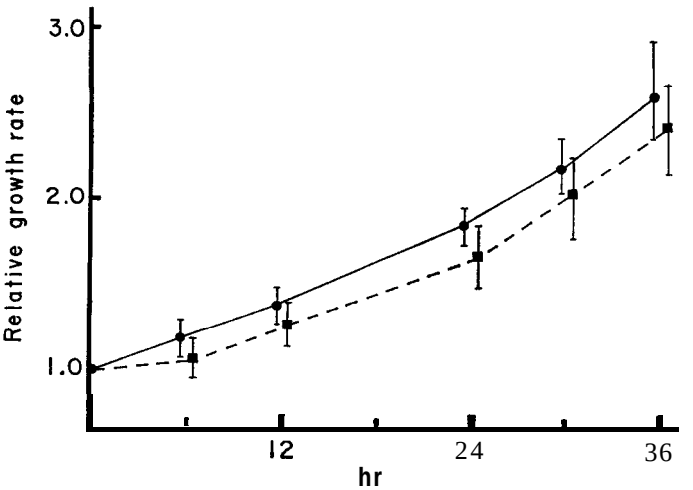


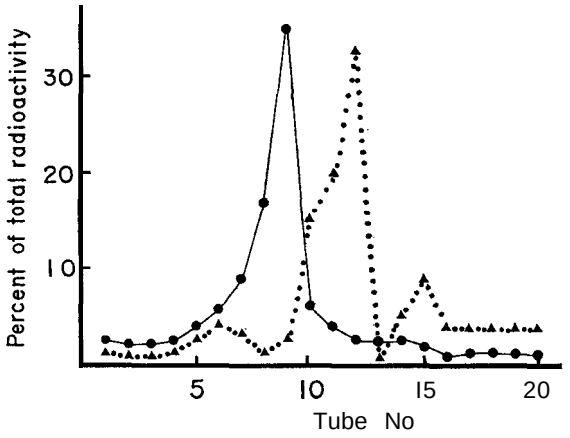

Fig. 3. Relative growth rate of L-adrenaline-treated RFL cells. ●—● untreated control : ■---■ treated with 25 $\mu$M of adrenaline for 6 hr. From time 0 to 6, cells were dipped in Eagle MEM medium containing adrenaline. then the medium was exchanged for Eagle MEM without adrenaline. The medium of untreated control was also exchanged for new medium at the same time.

The relative growth rate of treated cells during 36 hours after the 6 hour-treatment with adrenaline was nearly equal to that of control. Between time 0 to 6 the cells were dipped in 25 $\mu$M of adrenaline, and showed lower growth rate than that of control, thus indicating cytostatic action of this drug. Despite of the action, no decrease of cell numbers was found by the treatment with adrenaline. It is suggested that this drug has no cytocidal potency to the cells under this condition.

By the withdrawal of adrenaline from medium, cells grew so vigorously as to be compared with normal cells. These results indicate that chromosomal DNA of RFL cells is nicked to give smaller sedimentation coefficient.

Strand scission of mammalian DNA by nitroquinoline-N-oxide (Andoh and Ide, 1972), X-ray (Terasima and Tuboi, 1969) or bleomycin (Terasima et al., 1970) had been found. These injuries were rapidly repaired within a few minutes or hours. The scission of RFL cellular DNA by adrenaline could not be repaired even after 36 hours. This evidence is obviously shown in Fig. 4.

The cell cycle of REL cells is estimated to be about 26 hours. Fig. 3 also shows that during 36 hour-cultivation, nearly all of the treated cells undergo mitosis. Then the shifted single peak of adrenaline-treated cellular DNA was consisted




Pig. *4.* Conservation of doule strand breaks in chromosomal DNA of RFL cells, ● -●; untreated control A . . . A; treated with 25 $\mu$M of adrenaline for 6 hrs., then cultured in Eagle MEM medium without adrenaline for 36 hrs.

of DNA molecules of newly proliferated daughter cells. This indicated that REL cell DNA which was disintegrated by adrenaline could replicate and newly proliferated daughter cells maintained those disintegration of DNA. These nickings in chromosomal DNA sholud cause recombinations reciprocally or non-reciprocally. The chromosomal alteration of RFL cells treated with adrenaline should reflect to the development of genetic information. So some enzyme activities or susceptibility to certain viruses of RFL cells should change by the treatment with adrenaline.

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