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RADIATION SENSITIVITY OF DNA MOLECULES IN SITU IN NORMAL AND NEOPLASTIC TISSUES OF MICE

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Sensitivity to radiation in various tissues in man and in animals is known to vary a great deal, depending upon the type of tissues (CLEMENDSON & NELSON 1960). In a previous report on air-breathing live mice, one sensitive tissue, the thymus, and one resistant tissue, the liver, were found to show similar in situ sensitivities in their DNA molecules (ONO & OKADA 1974). This raised the question whether all DNA molecules in all tissues of mice may have the same sensitivity regardless of the tissue sensitivity. The purpose of the present investigation was to extend the previous work into two other normal tissues (spleen, cerebellum) and one malignant tissue (a squamous cell carcinoma), and to assess the role of oxygen by comparing the DNA sensitivities in various tissues from air-breathing live mice and nitrogen-asphyxiated dead mice. The nitrogen asphyxiated death was used to create a hypoxic state in tissues of animals, as previously demonstrated by CATER (CATER & PHILLIPS 1954, CATER 1960) and used in animal experiments by DESCHNER & GRAY (1959), and HEWITT & WILSON (1959, 1961).

Materials and Methods

WHT/Ht mice aged 9 to 14 weeks were used throughout the experiments. Each mouse was confined in a plastic box (4.0 cm×4.0 cm×4.5 cm) and irradiated at 33 Gy/min (measured by Fricke's ferrous sulfate dosimeter) with gamma rays from a 148 TBq of ^{137}Cs source (the Atomic Energy Research Center, University of Tokyo). For hypoxic irradiation, mice were killed by nitrogen asphyxiation, kept at room temperature (5-20 °C, varied with the season) from 30 to 80 min (CATER & PHILLIPS, CATER) to allow tissues to become hypoxic, and irradiated at room temperature. Under these experimental conditions, the sedimentation profiles of DNA were the same, independent of the time between killing and irradiation and of room temperature.

Within 3 min after whole body irradiation, tissues were removed and soaked in ice-cold Fischer's medium with 10 per cent horse serum. Tissues (spleen, thymus and cerebellum) were cut into pieces with a pair of scissors and sieved through stainless mesh of pore size of 125×125 microns. A cerebellar cell suspension was centrifuged once or twice at 50 G for 1 min to remove large capillary debris.

A squamous cell carcinoma transplanted subcutaneously in armpits of mice (HEWITT et coll. 1967, HEWITT & SAKAMOTO 1971) was used. Most tumours were from 0.1 to 0.3 g, although occasionally, tumours as large as 1.2 g were used and necrotic parts were removed with a pair of forceps as much as possible. The remainder of the tumour was then dispersed to prepare a cell suspension as described. Finally, tumour cells were spun down at 400 G for 2 min and resuspended. Liver cell suspensions were prepared by the method of BRANSTER & MORTON (1957).

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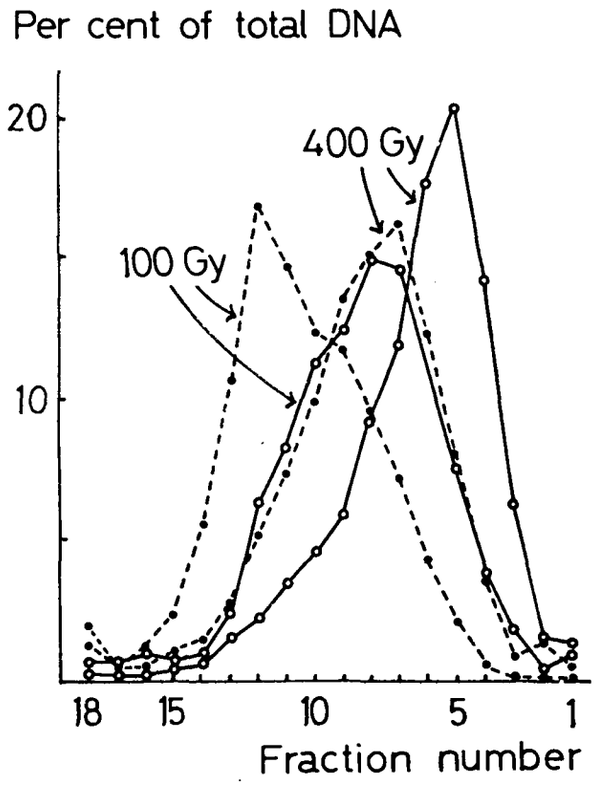

Fig. 1. Alkaline sucrose gradient sedimentation profiles of spleen DNA after 100 Gy and 400 Gy of whole body irradiation in situ under oxygenated (living) or hypoxic (dead) conditions. Sedimentation direction is from right to left. Solid lines with open circles indicate DNA irradiated under oxygenated conditions, and the dotted lines with closed circles under hypoxic conditions. Each profile represents one spleen from one animal. The profiles, here, are four typical samples. The profile (not shown) of splenic DNA from a non-irradiated animal overlapped with that of the profile with 100 Gy under hypoxic conditions shown in this figure.

A cell suspension was adjusted to a cell concentration of 4 to 8×10^6 cells/ml. A half ml of the suspension was gently layered over a sucrose gradient solution in a nitrocellulose centrifuge tube. The sucrose gradient was made by placing 1.5 ml of a 25 per cent sucrose solution on the botton of a nitrocellulose centrifuge tube as a cushion, followed by 34 ml of a 20 to 5 per cent sucrose gradient (0.9 M NaCl, 0.1 N NaOH and 0.01 M Na_2EDTA) and finally 0.5 ml of a lysing solution (0.5 N NaOH and 0.1 M Na_2EDTA) as a top layer (ONO & OKADA 1974). For liver cells, 0.3 ml of a cell suspension was applied over 0.7 ml of the lysing solution on the sucrose gradient.

After layering a cell suspension on the sucrose gradient, the gradient was kept at 29 °C for 3 hours to complete lysis. Then, they were centrifuged at 25000 rpm for 165 min in a SW 27 rotor in a Beckman L3-50 Ultracentrifuge. For liver cells, the centrifugation time was 270 min. The DNA sedimentation profile was analyzed by a fluorometric method (ONO & OKADA 1973), and the average molecular weight of DNA was calculated as described previously (ONO & OKADA 1974).

Results and Discussion

The alkaline sucrose gradient centrifugation patterns of splenic DNA after 100 Gy and 400 Gy of irradiation under oxic or hypoxic conditions appear in Fig. 1. The number average molecular weights of the data in Fig. 1 were 6.3×10^7 daltons under oxic and 11.1×10^7 daltons under hypoxic conditions after 100 Gy, and 3.1×10^7 and 6.3×10^7 daltons under oxic and hypoxic conditions after 400 Gy of gamma irradiation. The sedimentation profile of the non-irradiated control (not shown in Fig. 1) was very similar to that of 100 Gy under hypoxic conditions in Fig. 1 and its average molecular weight was 1.3×10^8 daltons. The reciprocals of the number average molecular weights, which were proportional to the number of single-strand scissions, were plotted against the radiation dose (Fig. 2a). The regression analysis of normal and hypoxic experiments reveals that the efficiency of DNA breakage is 60 single-strand breaks/10^{12} daltons DNA/Gy (or 172 eV/s.s.b.) under normal conditions and 19 single-strand breaks/10^{12} daltons DNA/Gy (or 545 eV/s.s.b.) under hypoxia. Fig. 2b to d shows the results with thymus, cerebellum and liver. The dotted lines in Fig. 2b and 2d under oxic conditions are based on the previously reported data (ONO & OKADA 1974) with some additional points. All other experiments are new data where each point represents one tissue from one mouse.

In the liver (ONO & OKADA 1974), no data at doses over 400 Gy under normal conditions were available because most mice exposed to the higher doses died during the irradiation. The breakage efficiency of hepatic DNA under hypoxic conditions (41 s.s.b./10^{12} daltons/Gy or 252 eV/s.s.b.) showed a slight reduction from those (62 s.s.b./10^{12} daltons/Gy or 167 eV/s.s.b.) under normal conditions, in contrast to the results of spleen, thymus and cerebellum. It is stated that the time required to rejoin 50 per cent of single-strand breaks range from 40 min to 2 hours in all the normal tissues analyzed, thymus and liver (ONO & OKADA 1974), and cerbellum and spleen (ONO & OKADA 1978). Thus, the extent of DNA

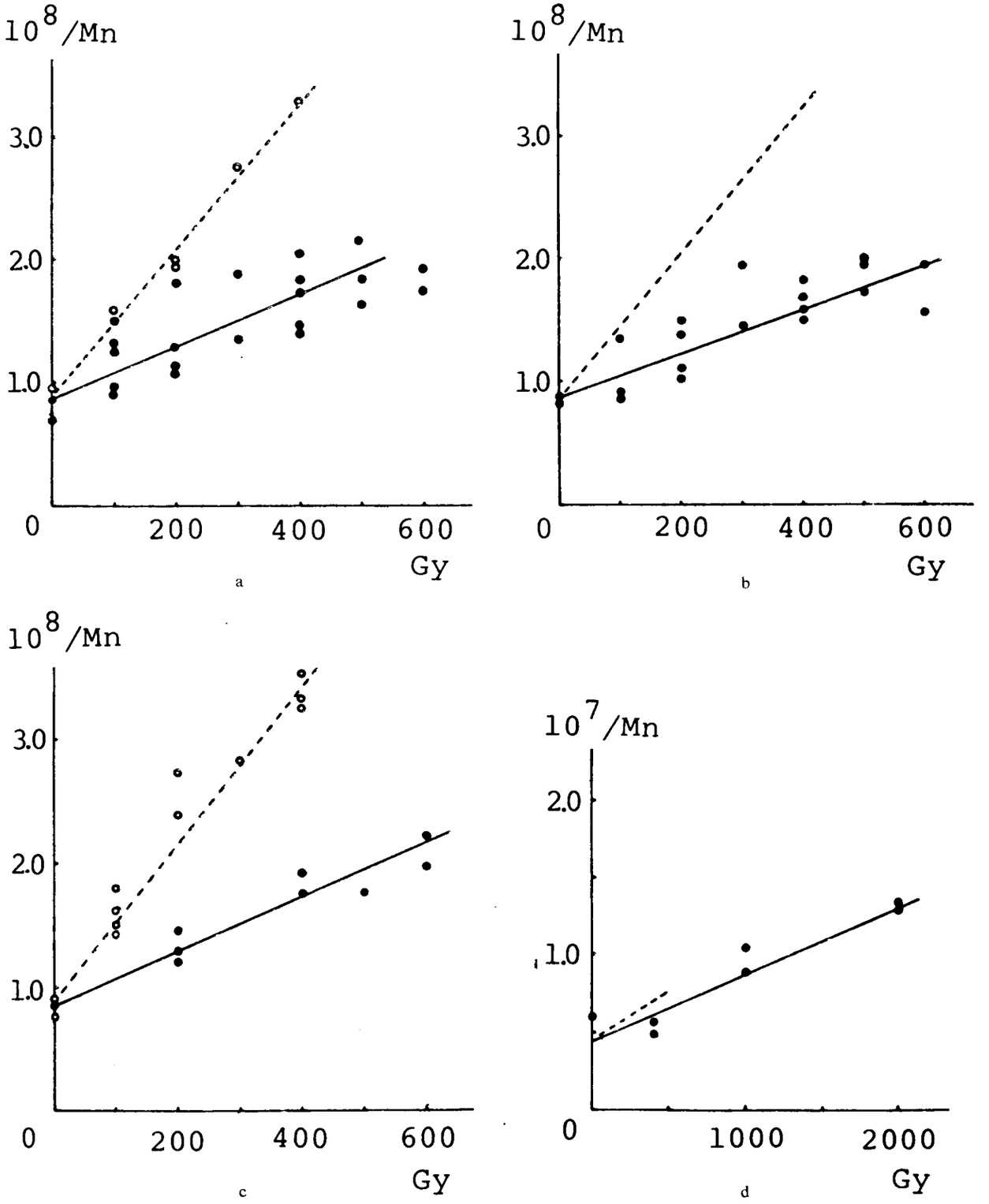

Fig. 2. Dose response curve for single-strand breaks in a) spleen, b) thymus, c) cerebellum and d) liver DNA in situ. The reciprocal of the number average molecular weight, which is proportional to the number of single-strand breaks, is plotted against dose. Each point represents one animal. The lines represent the regression curve. The dotted line with open circles is that of the oxic conditions and closed circles that of the hypoxic conditions. Dotted lines in (b) and (d) are the results of regression analysis with some additional data to the previously published curves (ONO & OKADA 1974).

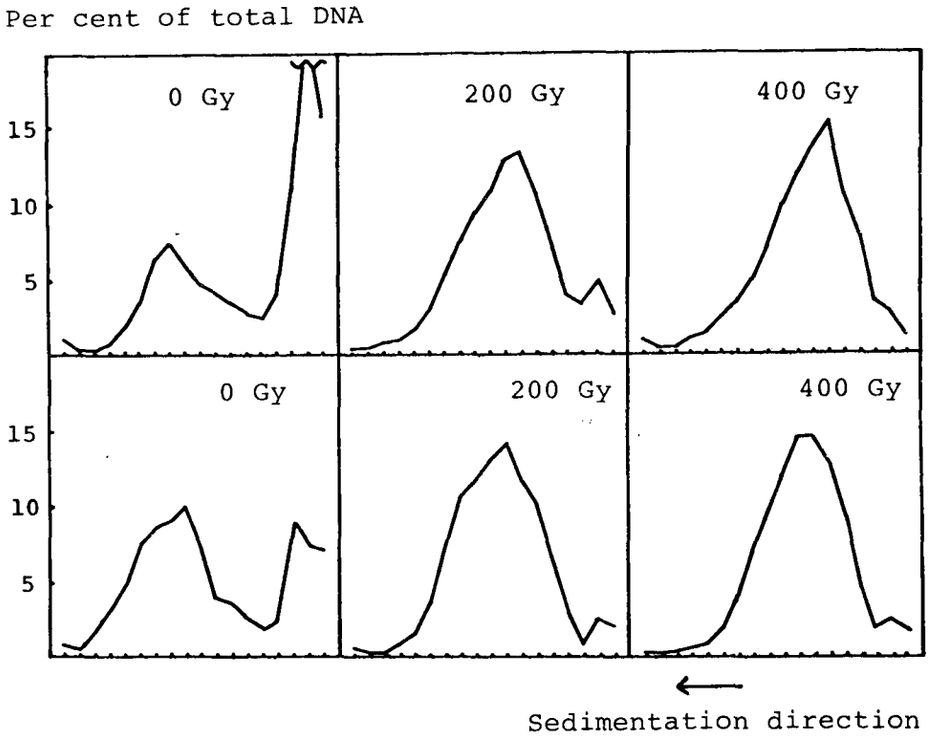

Fig. 3. Sedimentation profiles of irradiated or non-irradiated tumour DNA under oxic (upper curves) or hypoxic (lower curves) conditions. Tumour volume was 0.1 to 0.3 g. Left top panel is the sedimentation pattern of tumour DNA without a centrifugation procedure for purification of the cell population before lysis of cells. Top three fractions were neglected for the calculation of average molecular weight.

scissions rejoined during and after irradiation till death would amount at most to 8 per cent. In other words, the observed break efficiencies would be more than 92 per cent of the initial breaks induced by the irradiation.

It is added that the DNA size in liver of non-irradiated mice was found to be significantly lower than those observed in other tissues with exception of spermatozoa (ONO & OKADA 1977). Previously (ONO & OKADA 1974, 1978, ONO et coll. 1977), several attempts have been made to find whether autolysis of DNA during cell preparation and other steps would cause a reduction of DNA size. For example, a DNAase inhibitor was added during the cell preparation (ONO & OKADA 1978). Or the time interval between cell preparation and cell lysis was altered (ONO & OKADA 1974, ONO et coll.). None of these affected the DNA size. Moreover, the DNA size of old mice was found to be smaller than that of young adult mice (ONO et coll., ONO & OKADA 1978). Thus, the small DNA size might be real. It is also pointed out that the estimation of DNA breakage efficiency under oxic conditions is similar to those of other tissues, and that the estimation of the breakage efficiency obtained from the slope of the dose response curve would not be affected by the small DNA size of non-irradiated liver.

In a squamous cell carcinoma, the sucrose gradient pattern of DNA was made up of one major peak in the center of the gradient and another peak near the top of the gradient. The top peak represents DNA of molecular weight of less than 10^6 daltons, found in the top three fractions of the gradients. Its DNA content varied greatly from one sample to another. A typical example is shown in the top panel of the left column of Fig. 3 (oxic, 0 Gy). Since this DNA may have come from autolysis of cells in a necrotic region of tumours (ONO & OKADA 1974, WILLIAMS & LITTLE 1975), a cell suspension was centrifuged at 400 G for 2 min several times before applying it to a sucrose gradient. The procedure removed most of the top peaks of low molecular weight DNA (cf. the rest of panels in Fig. 3). When calculating the average molecular weight, the top three fractions were excluded. Fig. 4 shows that the dose response curve of tumour DNA from live mice was slightly higher than that of nitrogen asphyxiated mice. It is added that the size of tumours (0.1 g to 1.2

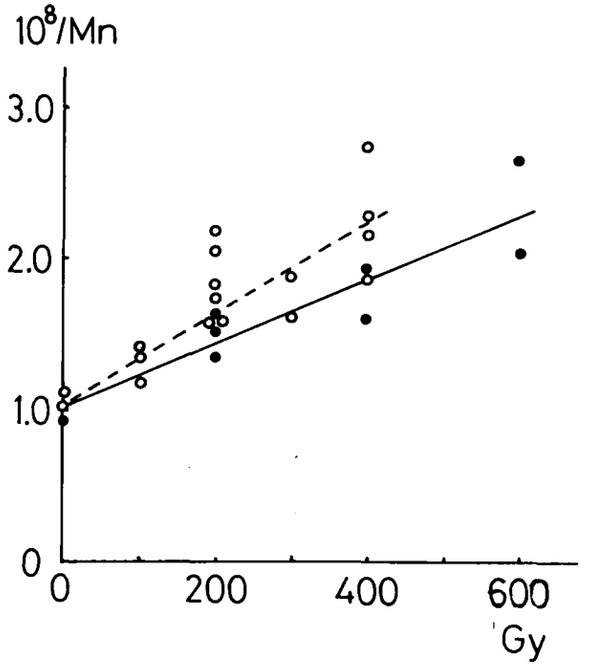

Fig. 4. Dose response curve of tumour DNA in situ under oxygenated (○) and hypoxic (●) conditions. Each point represents one animal. The lines are the results of regression analysis. The standard deviations for oxic and hypoxic conditions appear in the Table.

g) seemed not to influence the break efficiency of tumours.

Since the rejoining time of the single-strand breaks of the tumour is unavailable, the extent to which the break efficiencies estimated for tumours would be affected by a possible rejoining during and after irradiation is unknown. However, it may be speculated that the observed break efficiencies of tumours was 70 per cent or more of the initial breaks induced by irradiation, from the following considerations: (1) If the rejoining rate of the tumour is as slow as those of 4 normal tissues (ONO & OKADA 1974, 1976, 1978) the observed efficiencies would represent more than 92 per cent of the initial breaks. (2) If the rejoining is as fast as that (the half time of 10 min at 100 Gy) of testis (the fastest among 5 tissues in this strain of mice; ONO & OKADA 1976), the observed breaks would be 72 per cent of the initial breaks. (3) The carcinoma is known to contain an hypoxic component to a significant extent (HEWITT et coll., HEWITT & SAKAMOTO). Such a hypoxicity might slow the rejoining rate further (MODIG et coll. 1974). If so, more than 72 per cent of the initial breaks might be accounted for as the observed breaks.

One of the target molecules responsible for the reproductive death of mammalian cells is probably DNA (OKADA 1971a). Several experiments have been reported in which a correlation between the DNA break efficiencies and cellular radiation sensitivity is demonstrated. Some of them are oxygen effect (MODIG et coll., LENNARTZ et coll. 1973, CHAPMAN et coll. 1974), bromodeoxyuridine sensitization of radiation (SAWADA & OKADA 1971), and protection by cysteamine and other compounds (SAWADA & OKADA 1970, ROOTS & OKADA 1972, ROOTS & SMITH 1974). Since at least two modes of cell death, reproductive and interphase death (OKADA 1971b), are known to occur in somatic cells of various tissues, this brings about the question how the sensitivity of DNA molecules in various tissues would relate to the modes of cell death. The present results are summarized in the Table. They show that DNA sensitivity in normal tissues of live mice are all in the same order of magnitude and that there is no relationship of DNA sensitivity to cellular proliferative capacity, or to tissue sensitivity or the modes of cell death.

The DNA sensitivity of tumours in mice were found to be significantly lower than those of 4 normal tissues even after a maximum correction of a possible fast rejoining.

In nitrogen asphyxiated mice, the radiation sensitivity of normal tissues with the exception of liver and of the carcinoma was found to have decreased to the same level of 17 to 22. Since nitrogen asphyxiation makes tissues hypoxic (DESCHNER & GRAY, CATER & PHILLIPS, CATER) and since the squamous cell carcinoma in situ is found to contain a significant portion of hypoxic cells (THOMLINSON & GRAY 1955, HEWITT et coll., HEWITT & SAKAMOTO), the relatively low DNA sensitivity of the squamous cell carcinoma in live mice could be attributed to its low hypoxicity. In other words, hypoxicity of the tumour seemed to be demonstrable at molecular level.

In the squamous cell carcinoma, the oxygenated portion was estimated by the TD50 method to be around 82 per cent and the hypoxic portion about 18 per cent (HEWITT et coll., HEWITT & SAKAMOTO). If it is assumed that the break efficiency of DNA in oxygenated tumour cells is 60 and 22 in hypoxic cells (Table), the observed break efficiency of 29 in carcinomas of air-breathing mice would give an oxygenated component of 18 per cent and a hypoxic component of 82 per cent. The ratio of the oxygenated versus hypoxic components by the

Table

Comparison of DNA radiation sensitivity in various tissues of the mouse under oxic and hypoxic conditions. DNA radiation sensitivity in eV/s.s.b. units can be obtained by dividing 10360 with the number of s.s.b./10^{12} daltons of DNA/Gy

Organ	Number of single-strand breaks/10^{12} daltons of DNA/Gy (±SD)		OER (±SD)
	Oxic	Hypoxic	
Spleen	60± 7	19±1	3.2±0.4
Thymus	59± 5	17±3	3.6±0.7
Cerebellum	58± 8	21±3	2.8±0.5
Liver	62±16	41±4	1.5±0.4
Tumour	29± 4	22±4	1.3±0.3

TD50 method seems to be quite different from the values estimated by the present DNA break efficiency assay. One possible explanation for the difference is that the TD50 method is based upon the clonogenicity of tumour cells, while the DNA sedimentation method would include clonogenic and non-clonogenic cells for their measurement. The difference in the estimation of oxygenated and hypoxic components by two methods may be evaluated by assuming a high cloning efficiency of the oxygenated component versus a low cloning efficiency of the hypoxic component. For example, if a relative cloning efficiency of 1 is assigned to the oxygenated component, the relative cloning efficiency of the hypoxic component would be 0.048 (0.18×0.18/0.82×0.82) or 4.8 per cent of the oxic component.

The oxygen enhancement ratios of normal tissues were estimated from comparison of DNA sensitivity of air-breathing mice and nitrogen asphyxiated dead mice. The oxygen enhancement ratios of spleen, thymus and cerebellum were found to be about three (Table). They were comparable to the ratios estimated from DNA single-strand scissions in cultured mammalian cells (CHAPMAN et coll., MODIG et coll.), rat thymocytes in vitro (LENNARTZ et coll.) and mouse testicular cells in vitro (ONO & OKADA 1976). The oxygen enhancement ratios of tissues in situ have been previously estimated only in a limited number of tissues in which either clonogenicity or cell loss could be measured. For example, intestinal crypt cells gave a ratio of 2.6 (HORNSEY 1970), the spleen 2.1 (HORNSEY 1967), the testis 1.8 to 2.5 (HORNSEY et coll. 1971), etc. The present method utilizing alkaline sucrose gradient centrifugation offers another means to estimate the oxygen enhancement ratios of various tissues in situ. The method can be applied to any tissue in which the present sucrose gradient method is applicable.

A low oxygen enhancement ratio of liver is somewhat different from those of tumour and testis (ONO & OKADA 1976, 1977). The low ratio in liver is attributed to a high DNA scission efficiency of the hypoxic condition, while those of tumour and testis are attributed to low efficiencies under the oxic conditions. It is possible that the liver, being full of red blood cells, would be one organ difficult to make hypoxic.

SUMMARY

The sensitivity to radiation of DNA molecules in situ in various tissues was estimated by alkaline sucrose gradient centrifugation. The sensitivity of DNA in liver, thymus, spleen and cerebellum was found to be of the same order of magnitude. The oxygen enhancement ratios of DNA in spleen, thymus and cerebellum were all approximately three. Sensitivity of DNA molecules in the tumour was about half of that in normal tissues, probably because of its hypoxicity.

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