Jpn. J. Cancer Res. (Gann), 79, 523–528; April, 1988

Oxic and Hypoxic Cells in a Murine Squamous Cell Carcinoma

Yoshihiro TAKAI,[*1, *3] Kiyohiko SAKAMOTO[*2] and Shigefumi OKADA[*1, *4]
[*1]*Department of Radiation Biophysics, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 and* [*2]*Department of Radiation Research, Faculty of Medicine, Tohoku University, 2-1 Seiryocho, Sendai 980*

[^{125}I]Iododeoxyuridine labeling of a squamous cell carcinoma and follow-up of ^{125}I activity at the tumor *in situ* revealed that the ^{125}I activity remained at a constant level from the 24th to the 100th hour post-labeling and then decreased with a half time of about 200 hr. Autoradiographic studies with [^{3}H]thymidine showed that the tumor cells were labeled around capillaries, spread through the corded structure (the cord) and finally reached the necrotic regions. One could speculate that the constant ^{125}I period represents the transit time of the labeled cells through the cord and that the decline occurs mostly in the necrotic regions. X-Irradiation shortened the constant period of ^{125}I activity by about 24 hr and accelerated the declining rate in a dose-dependent manner. When tumors were made hypoxic by clamping the legs, the declining rate decreased significantly. When misonidazol (a hypoxic radiosensitizer) was administered before X-rays, the declining rate increased to a level higher than that of the oxic tumors. From the time course studies, it was suggested that the tumor cells immediately after ^{125}I-labeling were oxic, that they became gradually hypoxic during their transit through the cord and that they became anoxic when they reached the necrotic regions.

Key words: Oxygen effect — X-irradiation — Cell loss — ^{125}I-labeling of tumor — Misonidazol

Thomlinson and Gray[1] in 1955 proposed a corded structure of solid tumors. The corded structure was made up of a capillary in its center, surrounded by tumor cells of about 60–140 μm thickness (tumor cord)[1,2] and then zones of necrotic cells. Here, oxygen would be supplied from capillaries, then would diffuse through the tumor cells, be metabolized by the cells, and be exhausted at the edge of the cord to form necrotic regions. It was thought that the tumor cells near capillaries would be oxic, while the cells near necrotic regions would be hypoxic.[1,2] Since hypoxic cells were known to be radioresistant, a strategy for radiotherapy has been to search for means of efficient killing of radioresistant hypoxic cells in a tumor mass.[2,3] The oxygen tank, hypoxic radiosensitizers, high LET radiations and hyperthermia have been some of the approaches used.[2,3]

In the present murine squamous cell carcinoma, our early TD50 (50% tumor dose) assay showed that the clonogenic cells in the tumor were made up of 80% oxic and 20% hypoxic cells.[4,5] The subsequent study compared the radiosensitivity of tumor DNA in air-breathing and nitrogen-asphyxiated animals, and showed its hypoxic fraction to be about 80%.[6] If the tumor DNA includes DNA of both clonogenic and non-clonogenic cells, and if all the oxic cells were clonogenic, one may say that the tumor would be made up of 20% of oxic clonogenic cells and 80% of hypoxic cells, of which only 5% would be clonogenic and the remaining 75% would be non-clonogenic.

In 1970s, Begg,[7] Dethlefsen[8,9] and Porschen *et al.*[10–12] developed the *in situ* ^{125}I-IUdR[*5] labeling method of tumors in animals to study the declining pattern (cell loss pattern) of ^{125}I activity at the tumor site with or without X-irradiation. In the present study, combinations of ^{125}I-IUdR labeling, X-irradiation, clamping or unclamping of tumor-bearing legs, and administration of misonidazol (a hypoxic radiosensitizer) were used to study quantitatively the kinetics of oxy-

[*3] Present address: Department of Radiology, Faculty of Medicine, Tohoku University, 2-1 Seiryocho, Sendai 980.

[*4] Present address: Radiation Biology Center, Kyoto University, Yoshida-konoecho, Sakyo-ku, Kyoto 606.

[*5] Abbreviation: ^{125}I-IUdR, [^{125}I]iododeoxyuridine.

genated (oxic) cells in a corded structure of a solid tumor, including a distribution of oxic and hypoxic cells in the cord and the transition of oxic to hypoxic cells in relation to their movement through the cord.

Materials and Methods

Animals and Tumors Male or female mice of WHT/Ht strain, 12 to 15 weeks old, were obtained from an inbred colony in this laboratory. Tumors were a transplantable keratinizing squamous cell carcinoma, originally obtained from Dr. H. B. Hewitt (Gray Laboratory, Mount Vernon, UK) and maintained in the laboratory. Tumors were transplanted on right legs of animals. Methods of transplantation were described previously.[4, 5]

Administration of ^{125}I-IUdR and *in situ* Measurements Tumor-bearing mice were kept on solid mouse food and 0.1% NaI solution for three or four days before the ^{125}I-IUdR administration and thereafter. Each mouse was given 20 μCi of ^{125}I-IUdR (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham) intraperitoneally. Each mouse was kept in a separate plastic cage, replaced every day with a new one. When counting ^{125}I activity, each animal was placed in a brass tube with two holes. Two legs were pulled through the holes and tied on a plastic cross bar. A tumor-bearing right leg or a non-tumor-bearing left leg was placed at a fixed distance from the NaI crystal of a scintillation counter (Model TDC 50, Aloka Co. Ltd., Tokyo). X-Rays from ^{125}I were counted by the counter in the energy region of 18 to 34 keV. The brass tube (1.5 mm thickness) was sufficient to block X-rays from other portions of the animal body. The radioactivity of a tumor-bearing leg and that of a non-tumor-bearing leg were counted. The difference, after calibration for tumor size, was taken as the radioactivity in the tumor. The volume calibration curve was previously obtained by placing thin glass balls of various sizes, containing known ^{125}I activity, at the fixed position where the legs were placed in relation to the scintillation counter.

X-Irradiation Four mice in brass tubes were placed in the 10×10 cm^2 irradiation area under the X-ray machine, such that only the tumor-bearing legs were in the area and the rest of the bodies were covered by a lead plate. X-Irradiation was carried out with a Shimadzu X-ray therapy machine (Model SHT 250 M-3, Shimadzu Manufacturing Co. Ltd., Kyoto) at 250 kVp, 20 mA, 0.5 mm Cu and 1.0 mm Al filter, and TSD of 40 cm at a dose rate of 1.55 Gy/min. Radiation doses received at the four tumors varied by less than 2% and the doses received by the bodies in the brass tubes were about 7% of the doses on the legs. Mice were irradiated without anesthesia at 1, 24, 48 and 72 hr after injection of ^{125}I-IUdR. In some experiments, the right thigh with a tumor was clamped by tying a rubber band at the hip joint to make the tumor hypoxic.

Autoradiography Tumor-bearing mice were injected intraperitoneally with 50 μCi of [^{3}H]-thymidine (specific activity of 5 Ci/mmol, New England Nuclear Co., Boston) and killed by cervical dislocation at 1, 24, 48 and 72 hr after injection. Each tumor was excised and fixed in Bouin's solution. A fixed tumor was embedded in a paraffin block and sectioned at 4 μm thickness. Slides carrying deparaffinized sections were dipped into nuclear emulsion (Sakura NR-M2 emulsion, Konishiroku Photo Co. Ltd., Tokyo), dried in room air, exposed for 4 weeks, developed and stained with hematoxylin-eosin.

Results

^{125}I-IUdR Activity in Non-irradiated Tumors A whole-body scintigram of a mouse killed at the 73rd hour after ^{125}I-IUdR injection is shown in Fig. 1. ^{125}I activities were concentrated in the abdomen and at the tumor site on the right leg, but not in the thyroid. Examina-

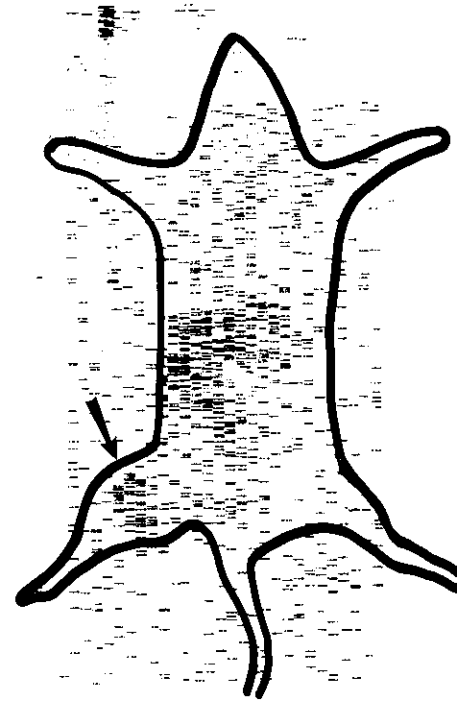

Fig. 1. A whole-body scintigram of a tumor-bearing mouse killed after intraperitoneal injection of 100 μCi of ^{125}I-IUdR. The arrow indicates the tumor site on its right leg.

tion of ^{125}I activities in various tissues after dissection of the animal confirmed the high ^{125}I activities in the intestine and tumor.

^{125}I-IUdR Retention Pattern (Cell Loss Pattern) of Non-irradiated Tumors The full line with open circles in Fig. 2B shows the ^{125}I activity at the tumor site in relation to the time after ^{125}I-IUdR injection. Immediately after the injection, the very high initial ^{125}I activity decreased rapidly, then leveled off at the 24th hour, and thereafter remained constant up to the 100th hour. The activity subsequently declined slowly at a rate of 0.26% per hour (a half time of abut 196 hr). In order to follow the movement of DNA-synthesizing 'labeled' cells in the tumor, [^{3}H]thymidine was injected into animals instead of ^{125}I-IUdR and autoradiographs of tumors were examined at various times after injection. Figure 3 shows that heavily labeled cells appeared around the capillaries 1 hr after injection (Fig. 3A) and that most of the labeled cells were near or in necrotic regions at 72 hr post-injection (Fig. 3D). The radius of the

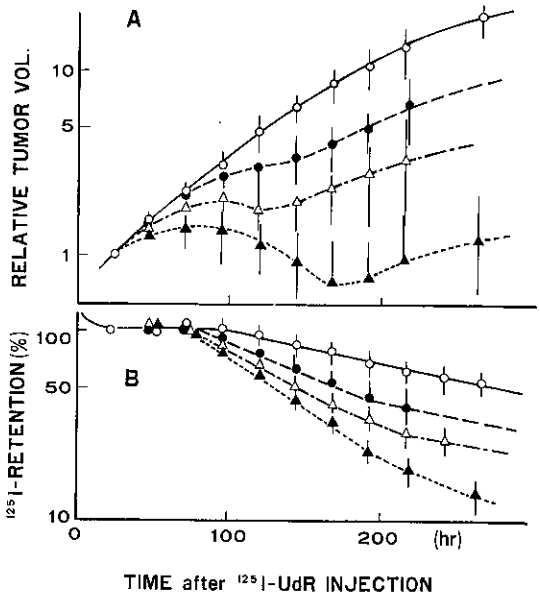

Fig. 2. Volume change (A) and ^{125}I retention curve (cell loss pattern) (B) of squamous cell carcinoma of X-irradiated animals. ○, 0 Gy; ●, 5 Gy; △, 10 Gy; ▲, 15 Gy.

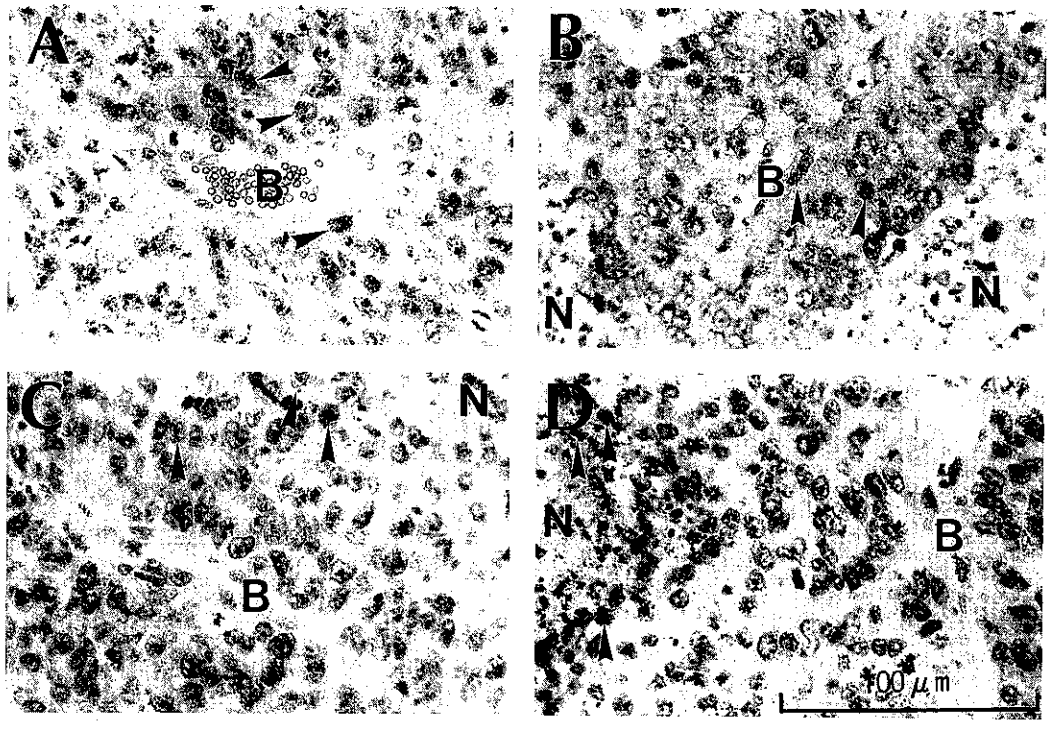

Fig. 3. Autoradiographs of tumors of non-irradiated animals in relation to the time post-injection of [^{3}H]thymidine. Arrows indicate heavily labeled cells, B indicates a blood vessel and N a necrotic region. A, the 1st hour; B, the 24th hour; C, the 48th hour; D, the 72nd hour.

Table I. Distribution of Labeled Cells (%) in Tumor Corded Structure in Relation to the Time Post-injection of [^{3}H]Thymidine

Post-injection time (hr)	Percent of labeled cells[a]		
	Zones[b] from blood vessel to necrotic region		
	I	II	III
1st	49±3	26±2	0
24th	35±4	29±10	15±3
48th	28±8	22±7	22±2
72nd	19±6	16±3	32±8

a) The cells with more than 5 grains on their nuclei were counted as labeled cells. In each of three zones (see footnote *b*)), the number of counted labeled cells was divided by the total number of counted cells to give percent of labeled cells.

b) Tumor cord structure from blood vessels to necrotic regions was divided into three parts. I. The first zone was from blood vessel to 40 μm toward the necrotic zone. II. The second zone was the area between 40 μm and 80 μm. III. The third zone was 80 μm away and over, including the necrotic region.

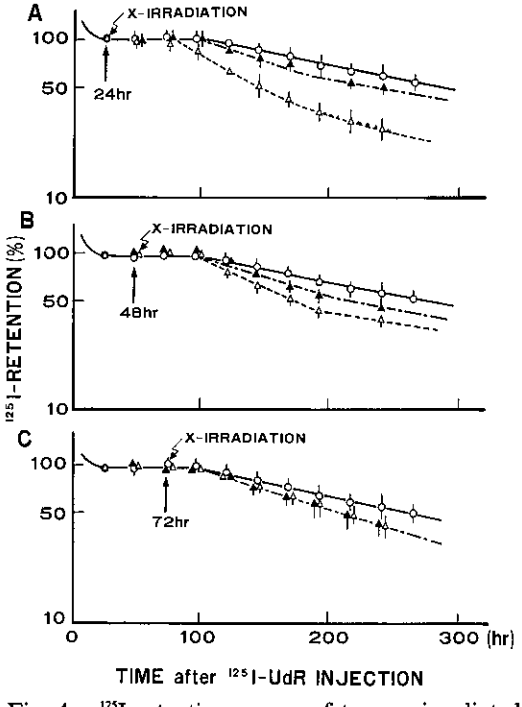

Fig. 4. ^{125}I retention curves of tumors irradiated with 10 Gy of X-rays under unclamped or clamped conditions. ○, Non-irradiated tumors in unclamped legs (control); △, irradiated tumors under unclamped condition; ▲, irradiated tumors under clamped (hypoxic) condition. The arrows indicate the time of X-irradiation.

tumor cord from blood vessels to the necrotic region was divided into three parts, inner, middle and outer zones. The table shows the transition of the heavily labeled cells from the inner zone to the outer zone with time. About 30% of the labeled cells migrated into the outer zone in 72 hr at a migration rate of about 100 μm/72 hr or 1.4 μm/hr. It is interesting that the decline of ^{125}I activity (Fig. 2B) seemed to occur when most of the labeled cells moved into the necrotic region (Fig. 3D).

Effects of X-Rays on Volume Change and ^{125}I Retention of Tumors Figure 2A shows that tumors kept increasing their volume for a while even after X-irradiation, followed by a temporary decrease and then a rise again. Figure 2B shows that, after X-irradiation, the ^{125}I activity of the tumors remained constant and then started to decrease. The constant periods of X-irradiated tumors were about 24 hr shorter than that of non-irradiated tumors, but were the same for all three doses. The declining rates, however, were dose-dependent. It is interesting that the ^{125}I activity of irradiated tumors decreased much earlier than

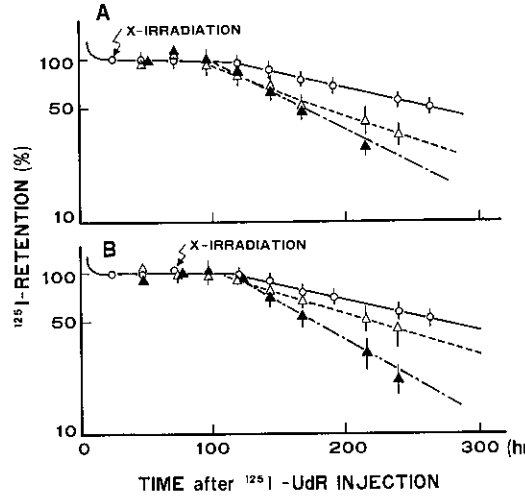

Fig. 5. ^{125}I-IUdR retention curves of tumors irradiated with or without misonidazol. A. Tumors were irradiated at the 24th hour post-injection of ^{125}I. B. Tumors were irradiated at the 72nd hour. ○, Non-irradiated control without misonidazol; △, tumors irradiated with 10 Gy of X-rays; ▲, tumors irradiated with 10 Gy of X-rays 30 min after injection of misonidazol (0.5 mg/g body wt.).

the decrease of tumor volume and that the ^{125}I activity continued to decrease even when the tumor volume started to increase (Fig. 2).

Effects of X-Rays on ^{125}I Retention of Unclamped and Clamped Tumors Tumor-bearing animals with or without clamping were X-irradiated at the 24th, 48th or 72nd hour after ^{125}I-IUdR injection. Figure 4 shows that the cell loss rate (the declining rate) of the unclamped legs was clearly faster than that of the clamped legs at the 24th hour. The rate of unclamped legs became closer to that of clamped ones at the 48th hour and similar to that of the clamped ones at the 72nd hour. This means that the tumor cells became gradually hypoxic with time.

Effects of Misonidazol on ^{125}I-Retention in X-Irradiated Tumors Misonidazol had little effect on the ^{125}I-retention curves of non-irradiated tumors. In the case of X-irradiated tumors, the declining rate in the presence of misonidazol was slightly higher than that without misonidazol when X-irradiation was given at the 24th hour (Fig. 5A). The rate was significantly higher when X-irradiation was given at the 72nd hour than that without misonidazol (Fig. 5B). The misonidazol enhancement ratios were 1.26 at the 24th hour and 1.70 at the 72nd hour. It should be noted that the increased declining rate in the presence of misonidazol at the 24th hour was the same as that at the 72nd hour (Fig. 5).

Discussion

Transition of the Cells in the Corded Structure of Non-irradiated Tumors Autoradiographic studies (Fig. 3) and the ^{125}I retention curve of non-irradiated tumors (Fig. 2B) suggested that the cells were radioactively labeled around capillaries, spread through the corded structure without loss of ^{125}I activity and finally reached the necrotic regions. The constant period of ^{125}I activity may represent the transition time of labeled cells through the tumor cord and the decline may occur at around the time when the labeled cells reach the necrotic regions. Similar findings were reported by Bosiljanoff *et al.*[12] in sarcoma 180.

It is interesting that the ^{125}I retention curves of duodenum, a normal tissue,[9] showed a constant period of ^{125}I activity for 48 hr, followed by a decline. Here, the constant level could represent the transit time for the epithelial cells to move from crypts to villi and the declining part might reflect the exfoliation process of the labeled cells from the tips of villi to the lumen.

Transition of Oxic Cells to Hypoxic Cells in Tumor Cord The cell loss rates (declining rates) with or without clamping (Fig. 4) and those with or without misonidazol treatment (Fig. 5) were plotted against the time interval between ^{125}I-IUdR administration and X-irradiation (Fig. 6). The results in Fig. 6 suggest the following. (i) Tumor cells were mostly oxic at the 0th and 24th hours, became partially hypoxic at the 48th hour and anoxic at the 72nd hour (open circles in Fig. 6). (ii) The cell loss rates of tumor cells (open triangles in Fig. 6) showed that misonidazol radiosensitized the cells at the 72nd hour as much as at the 24th hour. This provided further evidence that the 72nd hour cells were more hypoxic than the 24th hour cells. Radiosensitization by misonidazol was previously found in sarcoma 180[10] and adenocarcinoma.[11] (iii) When tumor cells were made acutely hypoxic by clamping legs, the cell loss rates at the 24th and 48th hours slowed down to the same level as those at the 72nd hour

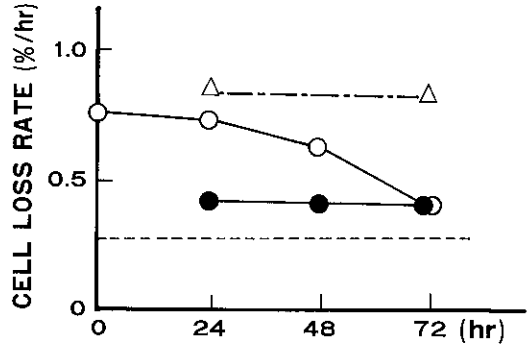

Fig. 6. Relationship between cell loss rate and time after ^{125}I-IUdR injection. Cell loss rates are given by the slopes of the declining parts of the retention curves (Figs. 4 and 5). Time scale on the abscissa is the interval between ^{125}I-injection and X-irradiation. ○, Irradiated tumors in unclamped legs; ●, irradiated tumors in clamped (hypoxic) legs; △, irradiated tumors in unclamped legs with misonidazol administration; ----, non-irradiated tumors in unclamped legs (control).

(closed circles in Fig. 6). Here, the 72nd hour cells might be called 'chronically hypoxic' as they were hypoxic without clamping.

This is perhaps the first experimental confirmation of the cord model of Thomlinson and Gray[1] *in vivo* with a time scale, demonstrating how oxic cells become hypoxic as they transit through the tumor cord toward necrotic regions. It should be noted that the distribution pattern in the present squamous cell carcinoma might not represent those of all solid tumors, as a different pattern was obtained in another tumor, a fibrosarcoma (data not shown).

Acknowledgments

The authors acknowledge support of this work by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture. They thank Mrs. Masako Miyata for taking care of the animals.

(Received Oct. 15, 1987/Accepted Feb. 4, 1988)

References

1) Thomlinson, R. H. and Gray, L. H. The histological structure for some human lung cancers and the possible implication for radiotherapy. *Br. J. Cancer,* **9,** 539–549 (1955).
2) Hall, E. J. Oxygen effect. *In* "Radiobiology for the Radiologist," 2nd Ed., pp. 81–92 (1978). Harper & Row, Publishers, Hagerstown, Maryland.
3) Fowler, J. F. New approaches for the improvement of radiotherapy. *In* "Proceedings of 7th International Congress of Radiation Research," ed. J. J. Broerse, G. W. Barendsen, H. B. Kal and A. J. van del Kogel, pp. 13–22 (1980). Martinus Nijhoff Publishers, Amsterdam.
4) Hewitt, H. B., Chan, P. S. and Blake, E. R. Survival curves for clonogenic cells of murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int. J. Radiat. Biol.,* **12,** 535–549 (1967).
5) Hewitt, H. B. and Sakamoto, K. The comparative survival of clonogenic cells of a murine epithelioma after irradiation in mice breathing air, oxygen and carbon dioxide or hyperbaric oxygen. *Br. J. Radiol.,* **44,** 457–463 (1971).
6) Ono, T., Sakamoto, K. and Okada, S. Radiation sensitivity of DNA molecules *in situ* in normal and neoplastic tissues of mice. *Acta Radiol. Oncol.,* **19,** 285–291 (1980).
7) Begg, A. C. Cell loss from several types of solid tumour: comparison of ^{125}I-iododeoxyuridine and tritiated thymidine methods. *Cell Tissue Kinet.,* **10,** 409–427 (1977).
8) Dethlefsen, L. A. An evaluation of radioiodine-labeled 5-iodo-2′-deoxyuridine as a tracer for measuring cell loss from solid tumors. *Cell Tissue Kinet.,* **4,** 123–138 (1971).
9) Dethlefsen, L. A. Reutilization of ^{131}I-5-iodo-2-deoxyuridine as compared to ^{3}H-thymidine in mouse duodenum and mammary tumor. *J. Natl. Cancer Inst.,* **44,** 827–840 (1970).
10) Porschen, W., Gartzen, J., Gewehr, K., Muhlensiepen, H., Webeer, H. J. and Feinendegen, L. E. *In-vivo* assay of the radiation sensitivity of hypoxic tumour cells; influence of γ-rays, cyclotron neutrons, misonidazol, hyperthermia and mixed modalities. *Br. J. Cancer,* **37** (Suppl.), 194–197 (1978).
11) Porschen, R., Porschen, W., Muhlensiepen, H. and Feinedegen, L. E. Cell loss from viable and necrotic regions measured by ^{125}I-UdR. *Cell Tissue Kinet.,* **16,** 549–556 (1983).
12) Bosiljanoff, P., Porschen, J. F. and Piepenbring, W. *In-vivo* Untersuchung uber die relative Strahlenempfindlichkeit hypoxischer tumorzellen. *Strahlentherapie,* **153,** 178–189 (1977).