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# Reoxygenation of Tumours in "Sandwich" Chambers

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**Abstract**—*The process of reoxygenation of tumour tissue was studied in the rhabdomyosarcoma BA1112 following single doses of* 10 *and* 20 Gy *of* X-rays. *The analytical system consisted of the evaluation of the fluorescence response of the hypoxia-induced conversion of NAD to NADH. The tumours were grown for this purpose in thin, transparent "sandwich" chambers. The results indicate that, only after a dose of 20* Gy *reoxygenation of the tumour tissue takes place. There was an indication of an increase during the first day postirradiation which was followed by a dip at* 2 days *and a 2nd peak at* 3.5 days *after a dose of* 20 Gy.

## INTRODUCTION

A LARGE amount of evidence indicates that the oxygenation of tumours is improved after irradiation. This effect is called reoxygenation, and has been shown to occur in experimental systems based on tumour cell viability such as tumour cell survival as the endpoint [1], tumour cure [2, 3] and tumour volume regrowth time [4–6]. At least one tumour, however, does not show any appreciable reoxygenation after irradiation [7]. Improvement in oxygenation by radiation treatment has also been inferred from results obtained with other methods such as the determination of the tissue oxygen tension [8], tumour blood flow [9, 10] and morphological analysis of the blood vessel pattern, including angiography [11–14]. Despite the many investigations in this field, no clear picture has yet emerged concerning the mechanisms involved in the process of reoxygenation. Possible factors that have been suggested are a radiation-induced decrease in oxygen consumption, increased blood circulation, tumour shrinkage and migration of tumour cells [15]. The present experiments were conducted with a system in which the degree of oxygenation of the tumour tissue is derived from the *in situ* fluorescence of NAD(H). For this purpose, a "sandwich" observation chamber was developed. It appears that radiation-induced modifications of the oxygenation status of the tumour tissue is dose-dependent and their intensity changes with time.

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## MATERIALS AND METHODS

The tumour used in these investigations is a rather poorly differentiated sarcoma originating from the Rhabdomyosarcoma BA 1112 [16]. This tumour is isogeneic with the WAG/Rij strain of rats and no antigenic or immunological differences between tumour and host has ever been demonstrated. The animals used for these experiments are 12-week-old female WAG/Rij rats, with a body weight of about 140 g. The "sandwich" system is shown in Fig. 1 and its preparation is as follows. As the first step, the subcutis of a rat is inflated with about 30 ml of air. This causes a gentle separation of the skin, including a part of the subcutis, from the underlying tissues of the back of the animal. A 1-day interval before the next step allows the restoration of the circulation in the elevated skin. Then the surface of the skin of the left lateral side is opened and an "Algire" type of observation window [17] is embedded between the skin and a sheet of subcutis that was bluntly separated from the skin. The observation window is moulded from "Delrin" (Du Pont Chemicals) and resembles a ship's port hole. The wound is closed with a clip. The sheet of subcutis has now become situated between the glass coverslip of the observation window on one side and the air in the air pouch on the other. The circulation is allowed to restore for 7 days. After this period, a midline incision is made in the caudal surface of the air pouch causing the air pouch to deflate. A piece of optical grade mica measuring 25 × 50 mm with rounded corners is

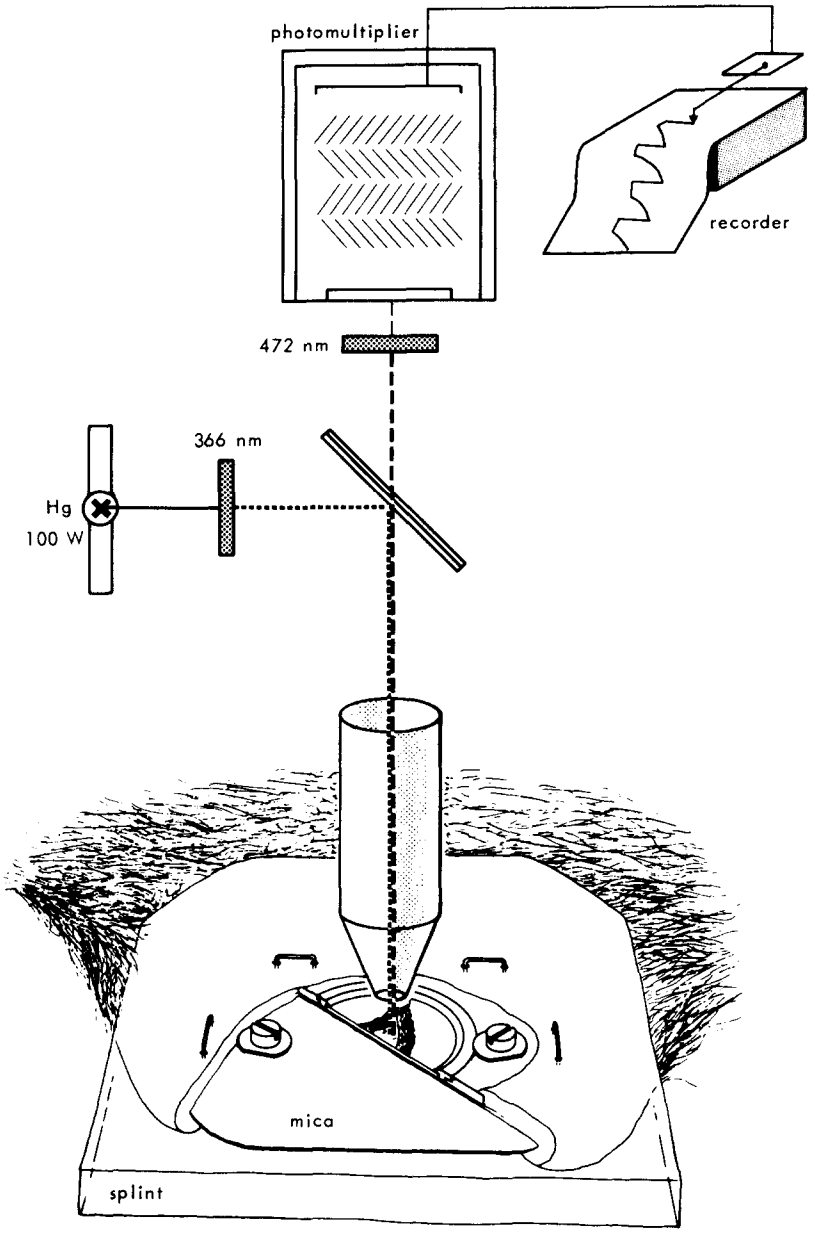


*Fig. 1. Diagram of the "sandwich" tumour system and the fluorescence measuring system.*

inserted into the air pouch. The observation window assembly (which is still embedded below the skin) with its inner lining of the sheet of subcutaneous tissue is gently positioned in the middle of the sheet of mica. Then a plastic splint which is preformed from a 0.25 mm thick sheet of Hostaphan (Kalle A. G., West-Germany) is placed over the emptied pouch. While the splint, the window and the mica are being kept in place in such a way that the centers of these three components roughly correspond, the entire assembly is stapled together by means of four stainless steel staples. An industrial type of pneumatic stapler is required for this and the straight staples are driven through the assembly while the latter is being supported by a piece of cork. The staples (which were driven into the cork) are freed and their ends are bent by means of pliers. The splint is symmetrical and the assembly therefore is such that a fold of skin, including the sheet of subcutis, remains stretched in the midline of the back of the animal.

After another 3–4 days for recovery of the circulation, the skin covering the observation window is removed. On the next day, the removable inner ring to which the coverslip is

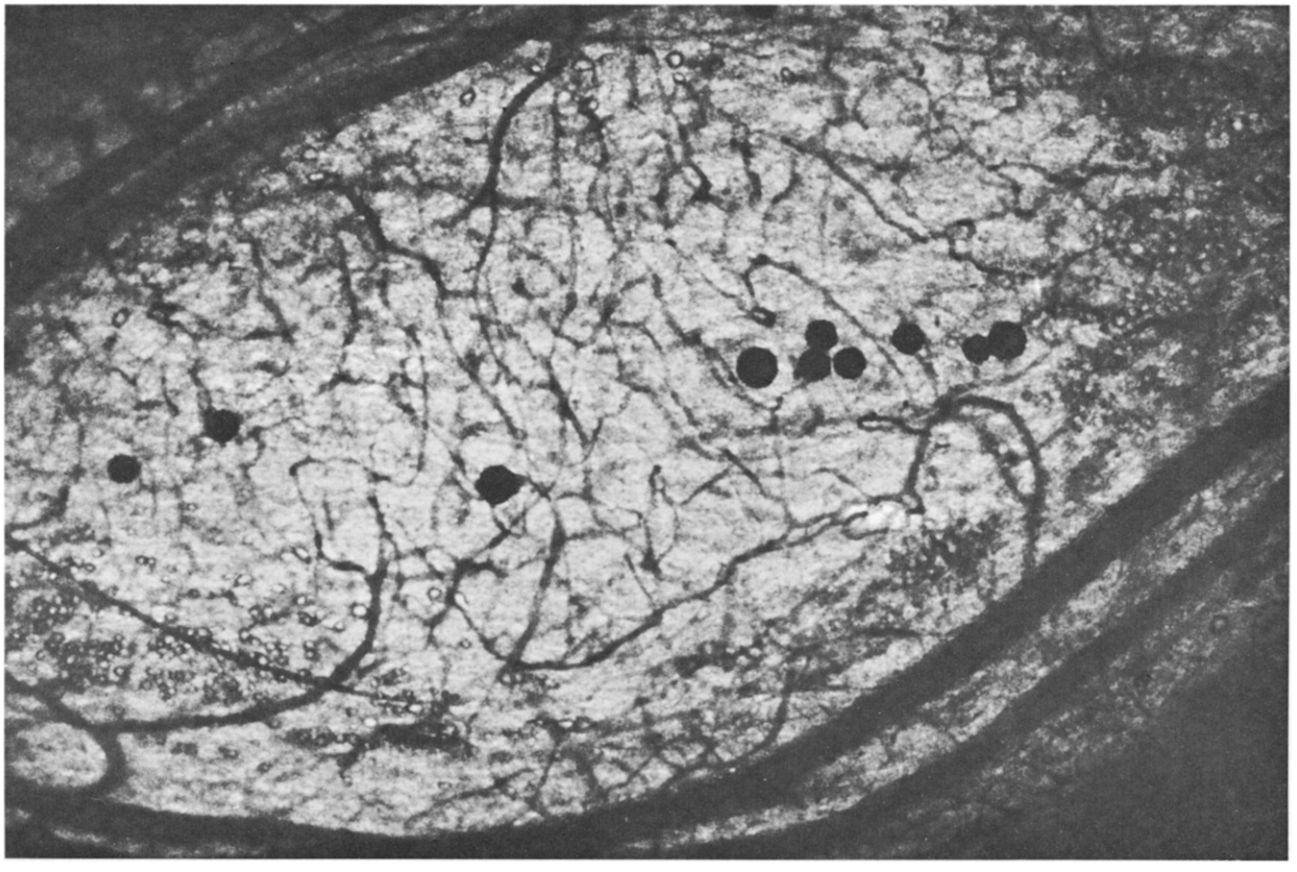

*Fig.* 2. *Microphotograph of a "sandwich" tumour of about* 3 mm *diameter. The tumour tissue is transparent but the blood vessels are visible. The average diameter of the "markers" (carbon microspheres) is* 80 μm.

attached is opened and a tiny piece of tumour tissue is transplanted in the afore-mentioned subcutaneous sheet. About 10 carbon microspheres of approximately 80 $\mu$m diameter (3M, St. Paul, Minnesota, USA) are pushed into the tumour inoculum. these serve as "landmarks" in the growing tumour. The inner ring is closed and is temporarily prevented from falling out by means of an extra bit of plastic sheet (not shown in Fig. 1) which is placed between the splint and the inner ring. From this moment, the animals are kept at an environmental temperature of between 35° and 36°C. This is necessary because the temperature of the tumour will obviously be mainly determined by the environmental temperature. Once the tumour is growing well, an area of skin corresponding with the observation area is removed from the contralateral skin fold. This allows the tumour, which is now enclosed only between the coverslip and the mica to be transilluminated in a direct way. In practice, the animals are placed on a simple holder which can be placed directly on the stage of a stereomicroscope or a Leitz "Orthoplan" compound microscope. The latter is used for fluorescence microscopy.

Fluorescence measurements were performed with the Leitz "ploemopak" dichroic system and an 11 × objective diameter. Measuring area 100 $\mu$m diameter. The excitation wave length was 366 nm and the emission wave length, 472 nm. The S-20 photomultiplier was operated at 0.9 kV and required cooling to −28°C in order to reduce its dark current to negligible values (0.1 nA). For the determination of the NAD(H) response to hypoxia, the animal was intubated and force-ventilated with a "Starling" type respiration pump. The respiration volume for a frequency of one stroke per minute was adjusted according to the data given by Guyton [18]. The tumour temperature was kept at 36°–37°C by means of an electronic temperature controlling air blower.

A series of standardized "hypoxic cycles" [19] was induced via a system containing a series of gas valves that opened and closed automatically under the control of a timing clock. The desired concentration of oxygen was adjusted with calibrated flow meters. Ten-minute cycles in which the animals were alternatively ventilated with a 3% $O_2$, 97% $N_2$ mixture for 3 min and 100% $O_2$ for 7 min were used. The 3% $O_2$, 97% $N_2$ mixture gives such an intense drop in tumour perfusion that its effect equals that of complete anoxia. Changes in the fluorescence were recorded on a digital meter and on a chart recorder. Three sites per tumour were subsequently analysed for the hypoxic response of the NAD(H) fluorescence. The results presented in this paper are based on 396 "control" determinations and a total of 218 determinations at various times after irradiation.

## RESULTS

The record of a typical determination is demonstrated in Fig. 3. Soon after hypoxic ventilation, the intensity of the fluorescence increases due to conversion of NAD into NAD(H). This is in accord with earlier findings in normal tissues and tumours [19]. At present, it is not possible to translate the increase in fluorescence for any given tumour site into the actual oxygen concentration in that area. This is due to possible differences in composition, and refractory index, of the tumour tissue, variations in focusing, tumour thickness, reflections of the cover slip, etc. One can assume that, if a given site was already hypoxic under aerobic respiratory conditions for the animal, then no increase in fluorescence would be expected from hypoxic ventilation. The increase in NAD(H) fluorescence would therefore be scored as "zero". With this hypothesis in mind, an index which is based on the relative increase in the fluorescence per site was derived. The values derived from recordings as shown in Fig. 3 are corrected for instrumental bias, i.e., dark current of the photomultiplier and autofluorescence of the optics. A wide range of values for the "relative increase" was found; these varied from below 0.1 to about 0.6. In the "sandwich" tumour system, any given site can be accurately localized by the combined use of a grid in the eyepiece and the microspheres in the tumour tissue. This makes it possible to follow the NAD(H) response of any given site in the tumour tissue over the follow-up period of about one week. This is illustrated in Fig. 4. It follows that, in unirradiated tumours, a continuous slight increase in the NAD(H) response occurs over the one week period of observation. After a dose of 20 Gy, an increase in response can be noted on the first day after irradiation; the response apparently drops below the normal range during the second day after irradiation. Then a temporarily increased response seems to occur after three days and this is followed by a gradual decrease. After a dose of 10 Gy, no obvious increase in response can be observed, but there is a general decrease over the follow-up period of about one week.

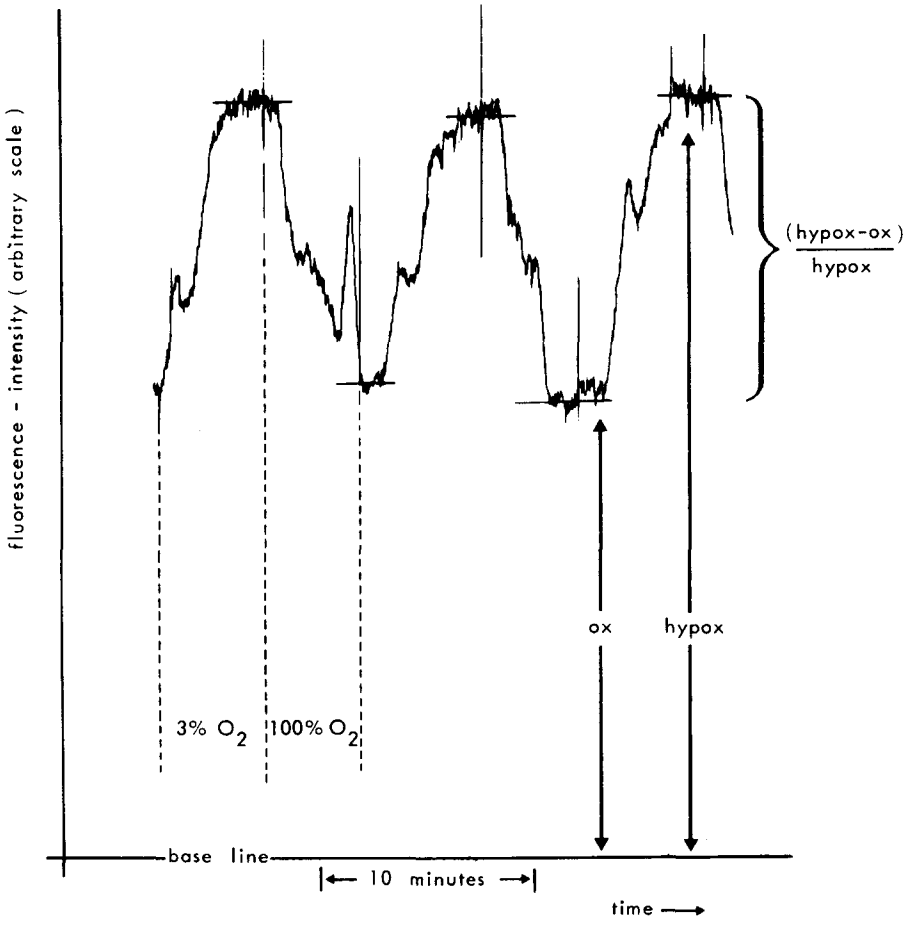


*Fig. 3. Example of a series of 3 hypoxic cycles. The response of the fluorescence to hypoxia is expressed as a ratio. The "baseline" represents the fluorescence value determined if no "sandwich" tumour was present in Fig. 1.*

## DISCUSSION

The experiments described in this paper were performed to gain some insight into the process of reoxygenation. The term reoxygenation as used by Van Putten and Kallman in 1968 [1] refers to a return of the proportion of hypoxic cells to a pre-irradiation level. Changes in the proportion of hypoxic cells in those experiments were determined by assays based on the surviving fraction of cells after irradiation of the tumours *in vivo* in air or nitrogen. These cell survival assays are very relevant with regard to the question of how many viable hypoxic cells are present in the tumours. However, tumour cell hypoxia is a condition which depends upon the physiological relationship between the tumour cells and the microcirculation and it must be possible to investigate tumour cell hypoxia by physiological means. The frequently used polarographic electrodes are inevitably too thick (minimum thickness, 200 $\mu$m) and cause distortions in the microcirculatory pattern. For this reason, the present method in which an impression of the local tissue oxygenation can be derived by optical means only was developed. Although NAD(H) itself is not an absolute indicator for the tissue oxygen tension, its place in the respiratory chain, together with its response to hypoxia, makes it a useful indicator for the oxygenation status of the tumour tissue [19].

The results shown in Fig. 4 confirm that a temporary improvement in the oxygen status of the tumour tissue can be obtained by a dose of 20 Gy. In effect, the time-sequence of the undulating pattern of Fig. 4a is very similar to the one found by Thomlinson for the rat fibrosarcoma $RIB_5$ after 15 Gy [5]. It shows that the oxygenation state of the tissue can change quite rapidly after a sufficiently high dose of irradiation. This is in agreement with findings of Kallman [20], Howes [2], Thomlinson [5] and Durand and Sutherland [21]. It should be noted, however, that the single dose of X-rays required for this

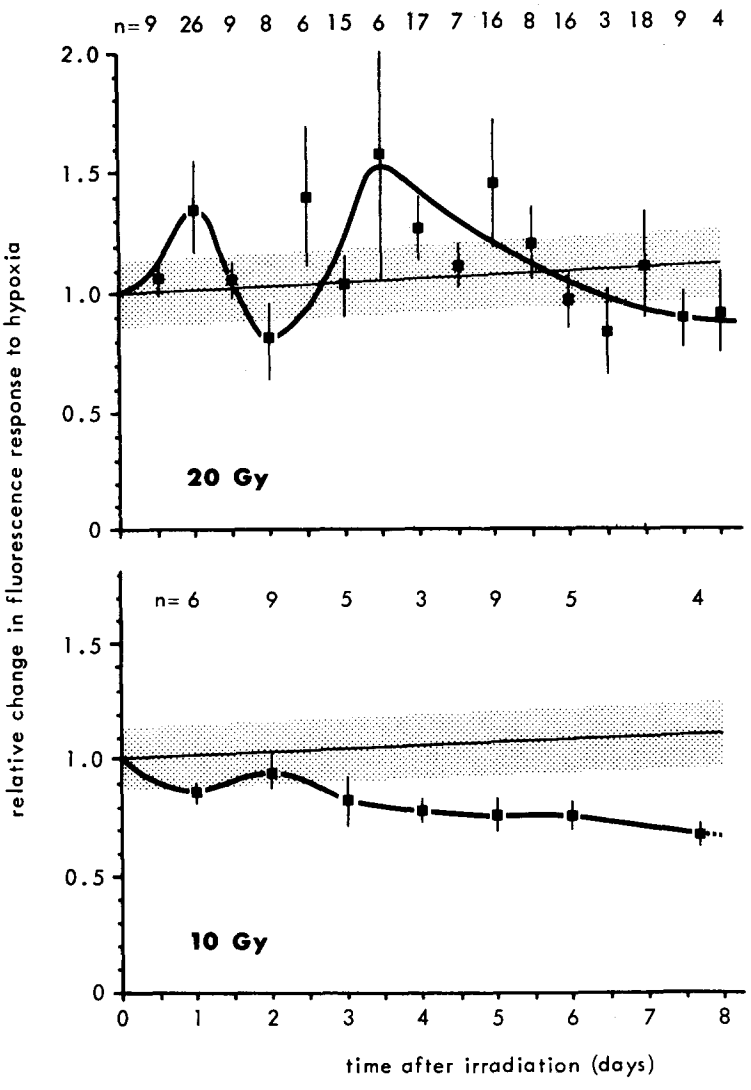


*Fig. 4. The effect of irradiation on the hypoxic NADH response, expressed as change per site. The numbers indicate the total of sites investigated: the bars show standard errors of the mean* (S.E.M.). *The shaded areas represent the mean* ± S.E.M. *of the pooled control values.*

rapid reoxygenation is very much higher than the usual daily fractional dose of about 2–3 Gy as used in clinical radiotherapy. No data are yet available on the rate of reoxygenation in our "sandwich" system during a course of fractionated radiotherapy. The results of cure-rate experiments with fractionated radiotherapy of this tumour (BA1112) indicated that reoxygenation may very well occur in the time between fractions, although the fractional dose must be rather high [3].

The availability of oxygen to tumour cells depends on a combination of factors, including the oxygen concentration in the blood vessels, the oxygen diffusion rate and the respiration rate of the tumour cells [22, 23]. The possible mechanisms involved in reoxygenation have been discussed by Kallman [15] and by van Putten [24]. They include: (a) a decreased tumour cell respiration rate; (b) changes in the circulation; (c) shrinkage, causing a smaller tumour cell volume to be nourished by the vascular system; and (d) migration of tumour cells [15]. The process of reoxygenation is most likely the result of a combination of the factors mentioned. This implies that one cannot expect all tumours to behave in the same manner. For a better understanding, it may be advisable to divide the process of reoxygenation into a few well-

defined phases, for instance, early phase (1st day), intermediate phase (2nd and 3rd days) and late phase (>3 days). In the present investigations, the increase in the oxygenation in the first phase might be attributed to decreased respiratory activity [25, 26] or increased circulation. We have made notes on the appearance of all sites investigated and no consistent change in the circulation parameters was observed during the early phase. This means that the most likely explanation for the early reoxygenation should be sought in the context of a temporarily decreased cell respiration. This possibility was suggested by Thomlinson [27] and has recently been confirmed by Durand and Sutherland [21] and by Clement *et al.* [26]. The present results where a dose of 20 Gy facilitates the hypoxic conversion of NAD into NAD(H) seem to indicate that the biochemistry of the respiratory chain is largely unaffected. The possibility that a decrease in cell respiration bears a relationship with the radiation-induced mitotic block might be considered. This remains as speculation, however, because no data are known to us concerning changes in the respiration rate of tumour cells during a radiation-induced mitotic delay.

The transient slight decrease in fluorescence response during the second phase (about 2–3 days postirradiation) may be correlated with a rearrangement of the hypoxic and well-oxygenated compartments as a result of radiation induced cell death with cell lysis in this phase.

At about 3.5 days postirradiation, the data of Fig. 4 suggest a nonsignificant temporary increase in the fluorescence response. This may be due to an improvement in the microcirculation of the tumour tissue. On visual observation, the impression was indeed gained that some areas showed a slightly increased vascular density, while the circulation appeared to have a somewhat higher flow rate, in wider vessels, in some areas. This transient improvement in the oxygenation around 3.5 days after 20 Gy (Fig. 4), which can be seen as a 3rd phase, is followed by a general deterioration of the fragile "sandwich" chambers. This made it necessary to limit the observation period to about one week. It should be noted that some of the factors mentioned here with regard to the microcirculation in these small tumours might have been studied by means of fluorescence angiography [28]. This technique, however (which always leaves traces of fluorescent dyes in the tissue), inevitably interferes with NAD(H) determinations. Further developments will therefore have to concentrate upon the measurements of factors such as the velocity of the erythrocytes in this tumour [29]. In addition, methods have very recently become available to determine the actual oxygen concentration in this type of preparation via the oxygen-induced quenching of the fluorescence of pyrene butyric acid [30]. By combining these methods, it may be possible to more accurately determine the sequence and the location of hypoxic cells in tumours after irradiation in the future. The present experiments should therefore be seen as the first step in the study of the physiology of reoxygenation in the "sandwich" tumours.

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