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# The influence of oxygen on the induction of radiation damage in DNA in mammalian cells after sensitization by intracellular glutathione depletion

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ABSTRACT

**Treatment of mammalian cells with buthionine sulphoximine (BSO) or diethyl maleate (DEM) results in a decrease in the intracellular GSH (glutathione) and NPSH (non-protein-bound SH) levels. The effect of depletion of GSH and NPSH on radiosensitivity was studied in relation to the concentration of oxygen during irradiation. Single- and double-strand DNA breaks (ssb and dsb) and cell killing were used as criteria for radiation damage. Under aerobic conditions, BSO and DEM treatment gave a small sensitization of 10-20% for the 3 types of radiation damage. Also under severely hypoxic conditions (0.01 μM oxygen in the medium) the sensitizing effect of both compounds on the induction of ssb and dsb and on cell killing was small (0-30%). At somewhat higher concentrations of oxygen (0.5-10 μM) however, the sensitization amounted to about 90% for the induction of ssb and dsb and about 50% for cell killing. These results strengthen the widely accepted idea that intracellular SH-compounds compete with oxygen and other electron-affinic radiosensitizers with respect to reaction with radiation-induced damage, thus preventing the fixation of DNA damages by oxygen. These results imply that the extent to which SH-compounds affect the radiosensitivity of cells <u>in vivo</u> depends strongly on the local concentration of oxygen.**

## SAMENVATTING

Titel: De invloed van zuurstof op de induktie van stralingsschade in DNA in zoogdiercellen na sensibilisering door verlaging van de intracellulaire glutathion concentratie.

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Behandeling van zoogdiercellen met buthionine sulfoximine (BSO) of diethyl maleaat (DEM) heeft een verlaging van de intracellulaire GSH (glutathion) en NPSH- (niet aan eiwit gebonden SH) spiegels tot gevolg. Het effect van de verlaging van de GSH- en NPSH-spiegels op de stralingsgevoeligheid werd bestudeerd als funktie van de zuurstofconcentratie tijdens bestraling. Enkelstreng - en dubbelstreng DNA-breuken (ssb en dsb) en celdood werden als criteria voor stralingsschade gebruikt. Onder aerobe omstandigheden gaf behandeling met BSO en DEM een geringe sensibilisering (10-20%) voor de 3 typen stralingsschade. Ook onder vrijwel anaerobe omstandigheden bij een zuurstofconcentratie in het medium van ca. 0,01 µM zuurstof was het sensibiliserende effect van beide verbindingen op de induktie van ssb en dsb en op het veroorzaken van celdood gering. Bij iets hogere zuurstofconcentraties (0,5-10 µM) echter was de sensibilisering veel sterker (tot ongeveer 90% met betrekking tot de induktie van ssb en dsb en tot ongeveer 50% met betrekking tot celdood). Dit onderstreept de in brede kring geaccepteerde opvatting dat intracellulaire SH-verbindingen concurrenten zijn voor zuurstof en andere electron-positieve stralingssensibilisatoren als het gaat om de reactie met primaire stralingsproducten, waardoor fixatie van DNA schade door zuurstof wordt voorkomen. Dit heeft tot gevolg dat de mate waarin SH-verbindingen de stralingsgevoeligheid van cellen *in vivo* beïnvloeden, sterk afhangt van de lokale zuurstofconcentratie.

Motivering en Toelichting

De dood van een cel t.g.v. blootstelling aan ioniserende straling wordt voornamelijk toegeschreven aan beschadiging van het DNA, het materiaal waarin de erfelijke eigenschappen van de cel in de chromosomen zijn vastgelegd. DNA bestaat uit zeer lange ketenvormige moleculen die zijn opgebouwd uit vele duizenden nucleotiden, de bouwstenen van DNA. Normaliter komt DNA voor in de dubbelstrengige vorm, d.w.z. twee even lange moleculen die om elkaar heen zijn gewonden.

Bij bestraling van een cel ontstaan verschillende soorten beschadigingen in het DNA die men kan indelen in breuken en nucleotideschade. Bij breuken onderscheidt men enkelstreng- en dubbelstrengbreuken, al naar gelang of ter plaatse in één streng slechts een breuk zit, of in beide. Het is niet goed bekend welke beschadigingen het meeste bijdragen tot de dood van de cel en welke beter worden verdragen, bijv. doordat cellulaire herstelsystemen de schade verwijderen. Om hier inzicht in te krijgen moet men de verschillende beschadigingen apart kunnen meten, waarvoor zeer gevoelige methoden nodig zijn, omdat bij biologisch relevante bestralingsdoses het aantal beschadigingen per DNA molecuul zeer gering is in relatie tot de grote lengte daarvan.

Dit rapport beschrijft technieken die de vereiste gevoeligheid bezitten. Maar aangezien de verschillende lesies gewoonlijk in evenredigheid met de stralingsdosis worden geïnduceerd, geven de metingen niet zonder meer uitsluitsel over de relatieve bijdrage van elk type schade tot de dood van de cel. Door echter de bestralingscondities te veranderen kan men de verhouding waarin de diverse lesies worden geïnduceerd, variëren, waarna men de veranderingen in het spectrum van de beschadigingen kan pogen te correleren met verschillen in de letaliteit.

De verhouding tussen aangebrachte enkel- en dubbelstrengbreuken enerzijds en nucleotideschade anderzijds kan worden gewijzigd door de intracellulaire glutathion (GSH) concentratie te veranderen. In het hier beschreven onderzoek met gekweekte cellen werd de GSH-spiegel verlaagd. De mate waarin de verlaging een verandering van het schadespectrum teweegbrengt bleek sterk afhankelijk van de zuurstofconcentratie. In het lichaam kan de zuurstofconcentratie in de weefsels sterk variëren (tot b.v. 1/10 van de concentratie in lucht). Om inzicht te

verkrijgen in de mogelijke effecten van beïnvloeding van de GSH-concentratie *in vivo*, is nagegaan bij welk zuurstofniveau een variatie in de intracellulaire GSH-concentratie maximaal effect heeft op de inductie van stralingsschade in DNA van de levende cel. De resultaten van dit onderzoek en de mogelijke implicaties voor het effect van SH-verbindingen toegepast bij de stralingsbescherming, zijn in dit rapport vastgelegd, in de vorm van het concept voor een publikatie.

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## INTRODUCTION

Much evidence has accumulated on the importance of glutathione (GSH) as an intracellular radioprotector (Révész et al., 1963, Ohara and Terasima, 1969), including reports showing that anoxic bacteria and diploid human cells with low endogenous GSH concentrations are markedly sensitive to low LET radiation, and therefore exhibit a low oxygen enhancement ratio (OER) (Morse and Dahl, 1978; Deschavanne et al., 1981). These results prompted more extensive studies on the effect of a low cellular GSH content, in various cell lines and with different techniques to diminish the intracellular GSH level. Some authors could confirm the effect of GSH depletion on the OER. For example, Bump et al. (1982) found no effect on survival after X-irradiation under aerobic conditions after depletion of intracellular GSH in CHO cells by exposure to diethyl maleate (DEM), but observed a sensitization after irradiation under nitrogen. Clark et al. (1984), also using CHO cells, obtained similar results with buthionine sulphoximine (BSO), an agent that inhibits the enzyme γ-glutamylcysteine synthetase and thus prevents the replenishment of metabolically exhausted intracellular GSH pools. On the other hand, Mitchell et al. (1983) found no appreciable alteration of the OER in V79 Chinese hamster lung cells and A549 human lung carcinoma cells after DEM or BSO treatment. Both agents sensitized under air as well as under hypoxic conditions. Similar results were reported for A549 cells by Biaglow et al. (1983).

The effect of intracellular GSH mostly is explained by its ability to chemically reconstitute the target free radical in critical molecules by hydrogen donation, in competition with the reaction of oxygen with the same free radical (see e.g., Alper and Howard-Flanders, 1956). By formation of peroxy radicals, oxygen would lead to damage fixation, whereas hydrogen donation by a thiol group would result in damage repair. However, the different responses of different cell lines on GSH depletion support the notion that GSH deficiency may also indirectly affect radiosensitivity, for example, when GSH functions as a cofactor in enzyme systems that repair radiation-induced lesions (Malaise, 1983).

Previously we reported about the effects on cellular radiosensitivity of compounds causing GSH deficiency (Vos et al., 1984; 1986). Three cell lines were used (CHO, V79 and Hela), and the effects were studied of both BSO and DEM, which decrease the GSH concentration via different mechanisms. By using diminished cell survival (effect on colony formation), single-strand DNA breaks (ssb) and double-strand DNA breaks (dsb) as criteria for

radiation damage, and by measuring repair of DNA breaks after radiation exposure, we tried to analyze the extent to which primary damage and repair processes are involved in the ultimate survival after GSH removal. In general, GSH depletion resulted in only a small sensitization, if any, when the cells were irradiated under aerobic conditions. Under hypoxic conditions, however, there was a considerable sensitization, in particular with respect to the induction of dsb, albeit with one exception: in HeLa cells DEM did not sensitize with respect to the induction of ssb while the sensitization of dsb induction was only marginal. With regard to cell killing, DEM was somewhat more effective than BSO, in spite of the fact that DEM caused a much smaller reduction of the GSH level. Generally, no effect was found on post-irradiation repair of ssb and dsb.

These experiments had been carried out with cells grown on polystyrene Petri dishes, which are known to contain a large store of dissolved oxygen which diffuses out very slowly (Chapman et al., 1970). This means that liquid in contact with the polystyrene surface, cannot be made completely anoxic within the limited period available for equilibration with nitrogen in a normal experiment with mammalian cells. The question was raised whether variation in the residual oxygen content could lie at the root of ill-understood differences in the results that we obtained in studies on the effects of DEM and BSO on the OER in CHO and V79 cells. Therefore, we decided to study the role of oxygen in irradiations under conditions of GSH depletion. In two cell-lines, CHO and HeLa, the effects of the oxygen concentration on the induction of ssb and dsb and on cell killing were investigated. In order to obtain a really low oxygen content of the medium and to prevent a build-up in oxygen concentration around the cells attached to the plastic, we applied dishes with Mylar bottoms instead of polystyrene, as recommended by Chapman et al. (1970). With cells grown on these dishes we observed that under both aerobic and anoxic conditions there is no or only a marginal radiosensitization due to GSH depletion; at intermediate concentrations of oxygen, however, radiosensitization amounted to much higher values. These results can be described by the simplified model described by Koch et al. (1984) taking into account the competition between endogenous sensitizers and protectors and an exogenous sensitizer, albeit that there is not a strictly first-order dependence of radiosensitization on oxygen concentration.

## MATERIALS AND METHODS

### Cell cultures

HeLa cells and CHO cells were cultured in F10 medium supplemented with 6 % newborn calf serum; 100 IU penicillin and 0.1 mg streptomycin per ml were added. The cell lines were maintained on plastic culture flasks (Costar, Cambridge, Mass.). For survival studies, cells were cloned after irradiation in 6 cm polystyrene Petri dishes (Greiner, Nürtingen, FRG), which were provided with a feeder layer of 150,000 cells of the same cell line 20 h before cloning. Cell suspensions were prepared with a 0.25 % trypsin solution in PBS (8.1 mM $Na_2HPO_4$, 15 mM $KH_2PO_4$, 2.6 mM KCl and 140 mM NaCl). Cells for the feeder layer were irradiated with 40 Gy X-rays. Colonies were counted after 7 days of culturing in a humidified atmosphere of 5 % $CO_2$ in air. Before counting, cultures were stained with methylene blue.

### Preparation of Mylar dishes

In essence, the method described by Barendsen and Beusker (1960) was used, glass cylinders of 3 or 6 cm internal diameter and a height of about 1.2 cm were heated and covered on one flat side with a thin layer of Araldit (Ciba-Geigy, Basel, Switserland). After drying these were placed on Mylar foil (50 μm thick, cleaned with alcohol) and heated at 180 °C for 1 h. Then they were incubated for a few hours in 0.3 M HCl, rinsed with distilled water and sterilized at 170 °C.

### Treatment with BSO or DEM

Details about the mechanisms of depletion of GSH level by BSO or DEM and conditions of treatment have been described previously (Vos et al., 1984; 1986). Briefly, before irradiation log phase cultures on polystyrene or Mylar dishes were exposed to 0.2 mM BSO (for 16 h at 37 °C), or to 1.0 mM DEM (for 1 h at room temperature), both in growth medium. BSO and DEM solutions were washed off immediately after irradiation and subsequently cell suspensions were made for plating or the monolayer cultures were further prepared for studying repair and detection of ssb and dsb. These BSO and DEM treatments had no effect on colony formation of non-irradiated cells and did not induce ssb and dsb.

Determination of intracellular GSH and NPSH

For determination of the intracellular content of non-protein bound thiol (NPSH) and glutathione (GSH), cell suspensions containing about $10^6$ cells were used. Our methods have been described in detail in previous publications (Vos et al., 1984; 1986). Briefly, NPSH was determined with Ellman's reagent and GSH was assessed by high performance liquid chromatography. In table 1 the concentrations of GSH and NPSH in HeLa and CHO cells before and after treatment with BSO or DEM are summarized. These concentrations were derived from the molar amounts per cell, as experimentally determined, and the average cell volume, as estimated under the microscope on trypsinized cultures, of 1.3 pl for CHO cells and 2.9 pl for HeLa cells (1 pl = $10^3$ $\mu m^3$).

Irradiation

Cells in log phase monolayer cultures on Mylar dishes (unless otherwise stated) were irradiated with a Philips 300 kV X-ray machine (dose rate 4 Gy/min) when survival or ssb were studied, and with a $^{60}$Co-γ-source (Gamma cell 200, Atomic Energy of Canada Ltd., Ottawa, Canada) when dsb were investigated (dose rate 50-60 Gy/min). Beam characteristics for the X-rays were 300 kV; 10mA; filtration 1.5 mm Cu (corrected), hvl 2.9 mm Cu, distance to target 24 cm. In case of the X-irradiations, the dishes were placed in a perspex container on a glass-framework, and during γ-irradiation, the dishes were in a brass-container. The containers were constantly on ice except for the short period of irradiation.

Gas-condition

Nitrogen was purified with an oxygen filter (Chromopack, Middelburg, The Netherlands). The oxygen content of the gas leaving the system was measured with a Hersch cell; it was less than 6 ppm. Direct measurements of oxygen in the medium were not possible. A conversion factor was applied, based on the solubility of oxygen at 0 °C (0.002 µM oxygen in the liquid phase per ppm in the gas phase). Higher oxygen contents were obtained by leading nitrogen through (porous) tubes of silicone rubber of varying length through the walls of which oxygen diffused from the outside. The oxygen contents obtained in this way were sufficiently reproducible, as was checked with the Hersch cell, and sufficiently constant to permit a reliable estimation of the oxygen concentration in the solutions in equilibrium with the gas phase.

The humidified gas mixture of $N_2$ and $O_2$ of the desired composition was passed through the container with uncovered dishes with cells for 60 min before and during irradiation, with the container placed on ice, at a flow rate of 1 l/min. The container volume was about 0.4 l. The cells were covered by a fluid layer less than 0.1 mm in thickness to facilitate gas exchange during flushing and irradiation. This means that the time constant (the time required for the oxygen content in the liquid phase to reach 1/e of its final value after a transient change in the gas-phase concentration) is less than 6 sec (Koch, 1984; the flushing for 60 min is necessary mainly to assure complete removal of excess oxygen from the gas phase in the container. At a dose rate of 4 Gy/min the radiochemical oxygen consumption is 1.2 µM/min (Koch, 1984). This corresponds to about 0.1 µM in 6 sec.

Detection of single-strand DNA breaks (ssb) and double-strand DNA breaks (dsb)

For the detection of ssb, alkaline elution was used as described by Van der Schans et al. (1982) and Shiloh et al.(1983). Two days after placing of $3 \times 10^4$ cells on 3 cm diameter Petri dishes, and labelling of the cells with [$^{14}$C]-thymidine (0.025 µCi/ml; 56 mCi/mmol; the Radiochemical Centre, Amersham, UK) a 3-h chase period with fresh medium (F10 + 15 % newborn calf serum) was applied. Cells serving as controls were seeded in 75 $cm^2$ culture flasks and were labelled for 2 days with [$^3$H]-thymidine (0.05 µCi/ml; 18 Ci/mmol, the Radiochemical centre, Amersham, UK). Following irradiation or after the incubation for repair (in F10 + 15 % newborn calf serum, at 37 $^{o}$C), cultures were placed on ice and handled as described by Shiloh et al. (1983). In the experimental set up 32 samples of cells can be analysed simultaneously.

For the detection of dsb, neutral elution was performed according to Bradley and Kohn (1979). The procedure was as described for the alkaline elution by Shiloh et al. (1983), except that the lysis buffer contained 0.05 M glycine, 0.05 M tris-HCl, 2 % sodium dodecylsulphate, 0.025 M EDTA and 0.5 mg/ml of proteinase K (pH 9.6). The same solution without the proteinase K served to elute DNA from the filters. Neutral elution patterns were usually concave, and the actual values of log ($^3$H/$^{14}$C) at fraction 9 were taken as a measure for the number of dsb.

In general, linear dose-effect relations were obtained for the induction by radiation of both ssb and dsb. One Gy of $^{60}$Co-γ-rays or 300 kV X-rays induces $2.5 \times 10^{-10}$ ssb/dalton in DNA in cells irradiated in monolayer in F10 medium under air (Van der Schans et al., 1982), under similar

conditions, 1 Gy of $^{60}$Co-γ-rays is assumed to induce 6 x $10^{-12}$ dsb dalton, on the basis of calculations from the work of Boye (1980) with superinfecting phage λ-DNA molecules in bacteria, irradiated in suspension under comparable conditions.

## RESULTS

### Effect of GSH depletion on the induction and repair of radiation damage in mammalian cells grown on polystyrene Petri dishes and Mylar bottoms

A comparative study was carried out with cells attached to Mylar bottoms and polystyrene Petri dishes, respectively, to examine the importance of oxygen diffusing out of the polystyrene in determining the impact of GSH depletion on radiosensitivity. Figure 1 shows the dose-effect curves for the induction of ssb in CHO cells, measured after irradiation with X-rays under different conditions of oxygenation and GSH depletion. In figure 2 the same is shown for the induction of dsb by γ-rays. The most important difference between Mylar and polystyrene is the fact that under nitrogen (< 6 ppm oxygen in gas outflow) GSH depletion has no effect on the induction of ssb and dsb in cells grown on Mylar, whereas cells attached to polystyrene show strong sensitization for the induction of ssb and dsb. The sensitization observed with cells on polystyrene could be simulated with cells grown on Mylar when these were flushed with a gas mixture containing 100 ppm oxygen instead of < 6 ppm. These results indicate that data obtained with cells attached to polystyrene while irradiated under nitrogen, do not pertain to truly anoxic conditions. Furthermore, they strongly suggest that sensitization by GSH depletion depends on the presence of a certain amount of oxygen.

Additional experiments were performed on the repair of ssb in CHO cells seeded on Mylar-bottomed dishes (data not shown). The results were not different from those obtained earlier with cells grown on polystyrene (Vos et al. 1984; 1986). The time course of rejoining of ssb as well as the fraction non-rejoined after a 30 min repair period (37 °C) were the same, both after irradiation under air and after anoxic exposure. Also with CHO-cells on Mylar, pretreatment with DEM did not affect the rate or extent of ssb-rejoining.

### The induction of ssb as a function of the concentration of oxygen

More detailed studies on the effect of oxygen on sensitization by DEM and BSO were performed with CHO- and HeLa cells grown on Mylar. In figure 3

the induction of ssb in DNA in CHO cells, calculated from dose-effect curves as shown in figure 1, is plotted as a function of the concentration of oxygen in the surrounding medium. The maximum OER which can be derived from these experiments (without pretreatment with BSO or DEM) is about 3.5. Depletion of GSH by pretreatment with DEM or BSO has no or only marginal effect on the induction of ssb both under aerobic and under strongly hypoxic conditions (0.01 μM oxygen initially in medium). At intermediate concentrations of oxygen (0.5-10 μM), however, the sensitization for the induction of ssb is much greater.

In figure 4 the data of identical experiments with HeLa cells are shown. Again, hardly any sensitization is observed under aerobic and strongly hypoxic conditions, whereas a substantial sensitization is seen at intermediate oxygen concentrations, though in a range that lies somewhat higher than with CHO cells. The $K_m$ values (the concentrations for half maximal effect; see Koch et al., 1984) for normal, BSO- and DEM-treated CHO cells are 2.8, 0.75 and 0.45 μM oxygen, respectively, and for normal, BSO- and DEM-treated HeLa cells 7.5, 2.7 and 3.7 μM oxygen.

The induction of dsb as a function of the concentration of oxygen

In figure 5 the induction of dsb in DNA of CHO cells, is shown as a function of the concentration of oxygen in the surrounding medium. The maximum OER (without pretreatment with DEM or BSO) is about 4, which is in agreement with our earlier observations. Again, only at intermediate oxygen concentrations does GSH depletion result in a clearly increased induction of DNA breaks. It should be noted that at 0.1 μM oxygen, there is still no significant sensitization of the induction of dsb, whereas at this oxygen concentration the induction of ssb is markedly enhanced by both DEM and BSO (Figure 3). Probably this has to be ascribed to a lowering of the prevailing concentration by fast oxygen consumption due to the irradiation at high dose rate necessary for the measurements of dsb. The $K_m$ values for normal, BSO- and DEM-treated CHO cells are 2.3, 1.2 and 1.0 μM oxygen, respectively.

The induction of cell killing as a function of the concentration of oxygen

Figure 6 illustrates the killing of CHO cells by X-rays and the effect of GSH-depletion with BSO as a function of the concentration of oxygen in the surrounding medium. The greatest sensitization due to the GSH depletion appears to occur in the range between 1 - 10 μM oxygen, similar to the

effect on ssb induction. The maximum OER (without GSH depletion) in this experiment is about 2.8, which is in reasonable agreement with other values reported in literature (e.g., Shrieve et al., 1985). The $K_m$ values are for normal and BSO-treated CHO cells 7.0 and 3.5 μM oxygen, respectively, which are higher than the corresponding values for induction of ssb in the same cell strain (Figure 3).

## DISCUSSION

The results presented in this paper clearly confirm the importance of GSH in the cell with respect to protection against radiation damage. However, the chemical reconstitution of the target free radical via hydrogen donation by GSH is of importance only when it can compete with the reaction of oxygen with the same free radical in the critical molecules, which is in agreement with the conclusions drawn by other authors from older in vitro studies.

Under severely hypoxic conditions, GSH in the cell seems to be redundant; probably chemical reconstitution occurs without the need of GSH. For the remaining damage, restitution is not possible, neither with nor without GSH. On the other hand, under aerobic conditions oxygen is present in such a large excess, that variation of the GSH concentration does not affect the extent of damage fixation. In terms of the simplified model described by Koch et al. (1984), there is only a small contribution of endogenous sensitizers with respect to the induction of ssb and dsb, since GSH depletion at low levels of exogenous sensitizers (e.g., oxygen) causes no or only marginal enhancement of ssb and dsb induction. According to the same model there is a first-order dependence of radiosensitization on oxygen concentration. Our curves, however, seem to be more in agreement with a higher order dependence, indicating that other factors probably affect the reactions involved.

Our observations of no or only marginal sensitization by GSH depletion under extremely hypoxic conditions (ER = 1.14 for cell killing of CHO, 1.0-1.2 for ssb induction in CHO and HeLa and 1.1-1.3 for dsb induction in CHO) appear to deviate from results reported by Edgren et al. (1985), who found a 1.5 times sensitization of ssb induction after 16 h BSO treatment of $GSH^{+/+}$ human fibroblasts and also a considerable sensitization of ssb induction in V79 cells. On the other hand, Shrieve et al. (1985) and Koch et al. (1984) also using V79 cells, observed only small effects on the radiosensitivity of hypoxic cells (ER = 1,15 - 1,35), well comparable to

our results. In addition, Shrieve et al. (1985) reported that the effect of BSO pretreatment on cell killing of V79 cells was not clearly dependent upon the oxygen concentration at all concentrations tested below 13 µM: significant enhancement (ER = 1.29-1.54). Though our ER's for cell killing of CHO cells are in about the same range (from 1.14 at 0.01 µM oxygen to 1.5 at 3 µM oxygen), the sensitization is substantially smaller at very low than at somewhat higher oxygen concentrations. The fact that the results obtained in the various studies appear to show some discrepancies, might indicate that the concentrations of endogenous sensitizers may vary as a function of cell line or growth conditions.

The sensitizing effect of GSH depletion is more pronounced for the induction of ssb and dsb than for cell killing, particularly in CHO cells where ER's for break induction of up to 1.9 were obtained. Similar observations were made by Koch and Painter (1975) who found an OER of about 4 for the induction of ssb detected by alkaline sucrosegradient sedimentation, which is higher than the OER for cell killing. These observations support the notion that the oxygen-thiol competition model may apply to some types of DNA damage, such as sugar-type radicals, but not to others, e.g., base radicals (O'Neill, 1983). This explains also the earlier data on the protecting effect of cysteamine (Van der Schans et al., 1979): under hypoxic conditions (on polystyrene dishes), cysteamine protects against the induction of ssb in DNA in CHO cells, but not against the induction of base damages that can be detected as γ-endonuclease-susceptible sites. It is also in keeping with the observation that cysteamine protects against cell killing to a lesser extent than against the induction of ssb and dsb (Koch and Painter, 1975; Koch and Howell, 1981; Van der Schans et al., 1982). All these data justify the conclusion that ssb and dsb are not the only causes of cell death. This conclusion undermines the statement of Blöcher and Pohlit (1982) that cell survival can be interpreted on the basis of one unrepaired dsb being a lethal event. Nor is it in agreement with the results of Radford (1985) who found that the level of initially induced dsb correlates with cell killing in mouse L-cells that were exposed to X-rays under different radiation damage modifying circumstances, including the presence of cysteamine under both aerobic and hypoxic conditions.

Beside the effect of GSH, some protection by the other NPSH can not be excluded. This is suggested by the fact that in both cell lines our treatment with BSO decreased the level of GSH by a factor of about 10, whereas the concentration of oxygen required for a given level of radiosensiti-

zation with respect to cell killing and to the induction of ssb and dsb decreased by a factor of 2-4. The latter value is more in agreement with the lowering of the total NPSH level in HeLa cells (with a factor of 1.7 both after treatment with BSO and DEM) and in CHO cells (a factor of 1.7 after DEM treatment and 2.0 after treatment with BSO). The fact that in general the sensitization by DEM is equal to that caused by BSO or even greater, in spite of the smaller effect of DEM on the GSH level, agrees with earlier suggestions (Vos et al., 1986; Biaglow et al., 1983) that 'the mechanisms for DEM-radiosensitization may be independent of GSH and involve reactivity with other cellular NPSH, with protein thiols, as well as chemosensitization'. On the other hand, GSH-depletion by BSO involves the risk of increasing the intracellular cysteine concentration which will counteract the sensitization by low GSH, particulary at very low GSH levels.

The small sensitization by DEM with respect to the induction of ssb and dsb in HeLa cells on polystyrene dishes observed previously (Vos et al., 1984) might be ascribed to the higher original GSH and NPSH levels in HeLa cells as compared to rodent cells. Although the relative decrease of NPSH level in HeLa- and CHO cells was about the same, in HeLa cells the final intracellular concentration is still more than a factor of 2 higher than in CHO; in fact, it is in the same range as in CHO without pretreatment with DEM or BSO (Table 1). This is in keeping with the observation that the control curve for CHO in figure 4 for the induction of ssb as a function of the concentration of oxygen runs between the curves found for HeLa cells after pretreatment with DEM and BSO, respectively. That means that in HeLa cells still enough NPSH should be present to compete with the small amounts of oxygen diffusing from the polystyrene dishes.

Due to the much higher dose rate in case of the γ-irradiations for the dsb measurements, part of the oxygen diffusing out of the polystyrene is removed by the γ-radiation, which explains the earlier observations (Vos et al., 1984; 1986) that, on polystyrene dishes, the OER for dsb induction was higher than that for ssb induction and cell killing. This notion is supported by the results of a comparative study on the induction of dsb in CHO cells on polystyrene dishes by X-rays and γ-rays. With 300 kV X-rays (at a dose rate of 5 Gy/min) an OER of 2 was observed, whereas $^{60}$Co-γ-rays (at a dose rate of 50 Gy/min) gave an OER of 4.0 (unpublished data).

Edgren et al. (1985) and Révész and Edgren (1984) reported a sensitization under anoxic conditions for the induction of ssb in human cells with a depleted GSH level. In addition, they found a delay of repair of ssb, but only after irradiation of GSH depleted cells under aerobic conditions.

These cells were irradiated on glass Petri dishes on which the cells had been seeded only shortly before irradiation, and the doses applied in the repair experiments were rather high. Both facts may have contributed to the rather slow repair of ssb, these authors observed, even in untreated human fibroblasts ($t_{1/2}$ about 13 min, whereas a $t_{1/2}$ of about 2 min is normal; Van der Schans et al., 1982). Evans et al. (1984), who used CHO cells grown in suspension, did not find any change in the rate of repair after pretreatment with DEM.

In general, we conclude that the (limited) information now available suggests that the effects of GSH depletion on the induction of ssb and dsb and on cell killing can be described by a simple competition model involving sensitizers and protectors reacting with target radicals. According to the same model, the extent of the effect of GSH depletion or enrichment depends strongly on the local concentration of oxygen. One has to be aware that in most in vivo situations the local oxygen concentrations are much lower than those usually applied on cells in tissue culture.

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Table 1

The GSH and NPSH concentrations (mM) in HeLa- and CHO cells

| | normal | after BSO | after DEM |
|---|---|---|---|
| HeLa | | | |
| GSH | 7.2 | 0.7 | 2.8 |
| NPSH | 15.1 | 9.0 | 9.0 |
| CHO | | | |
| GSH | 3.1 | 0.3 | 0.7 |
| NPSH | 7.7 | 3.8 | 4.6 |

The numbers are averages of at least 3 independent determinations with an estimated uncertainty of about 15 %. Treatment with BSO: 0.2 mM, 16 h, 37 $^{o}$C; treatment with DEM: 1 mM, 1 h, room temperature.

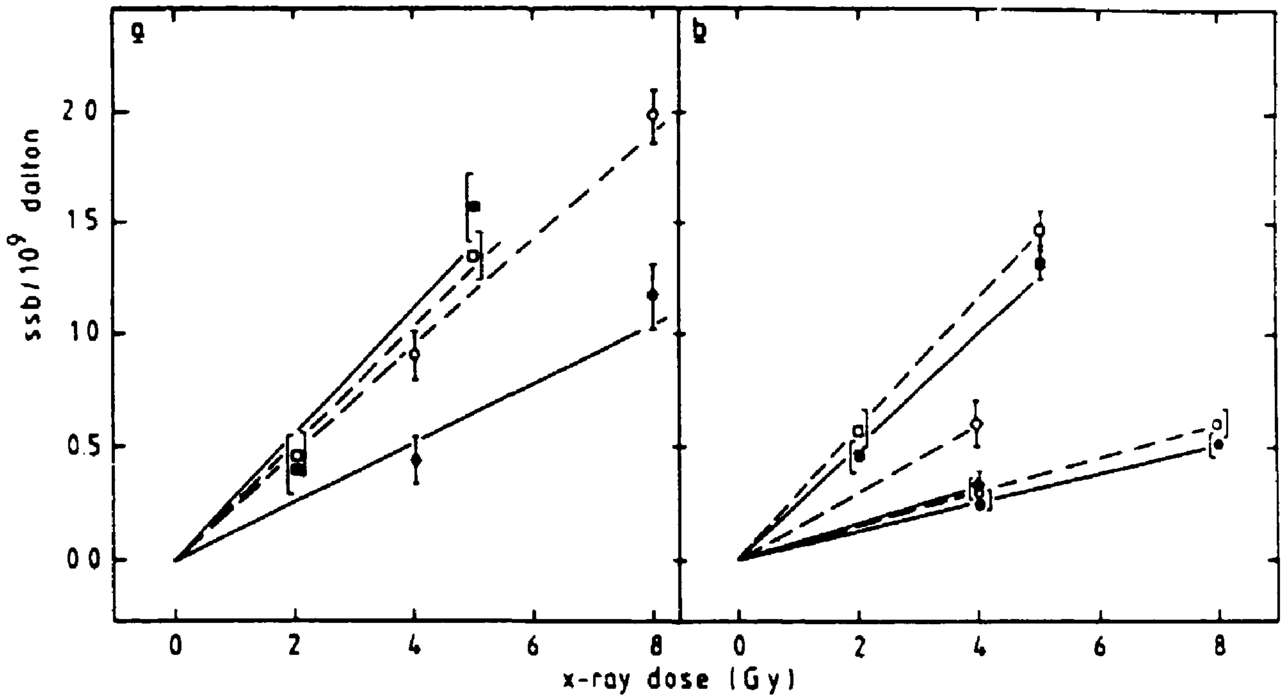


Figure 1

The effect of GSH depletion with DEM on the induction of ssb in DNA of CHO cells by 300 kV X-rays at high and low oxygen concentration. a) Cells seeded on polystyrene Petri dishes; b) cells seeded on Mylar bottoms. Open symbols: cells pretreated with DEM (1 mM, 1h, room temperature); closed symbols: without pretreatment. ■ □: irradiation under air; ● o: irradiation under nitrogen; ◆ ◇: irradiation under nitrogen + 500 ppm oxygen. Ssb were measured with the alkaline elution method. Means + S.E. are shown as calculated from 4 replicate determinations. The straight lines were fitted by eye.

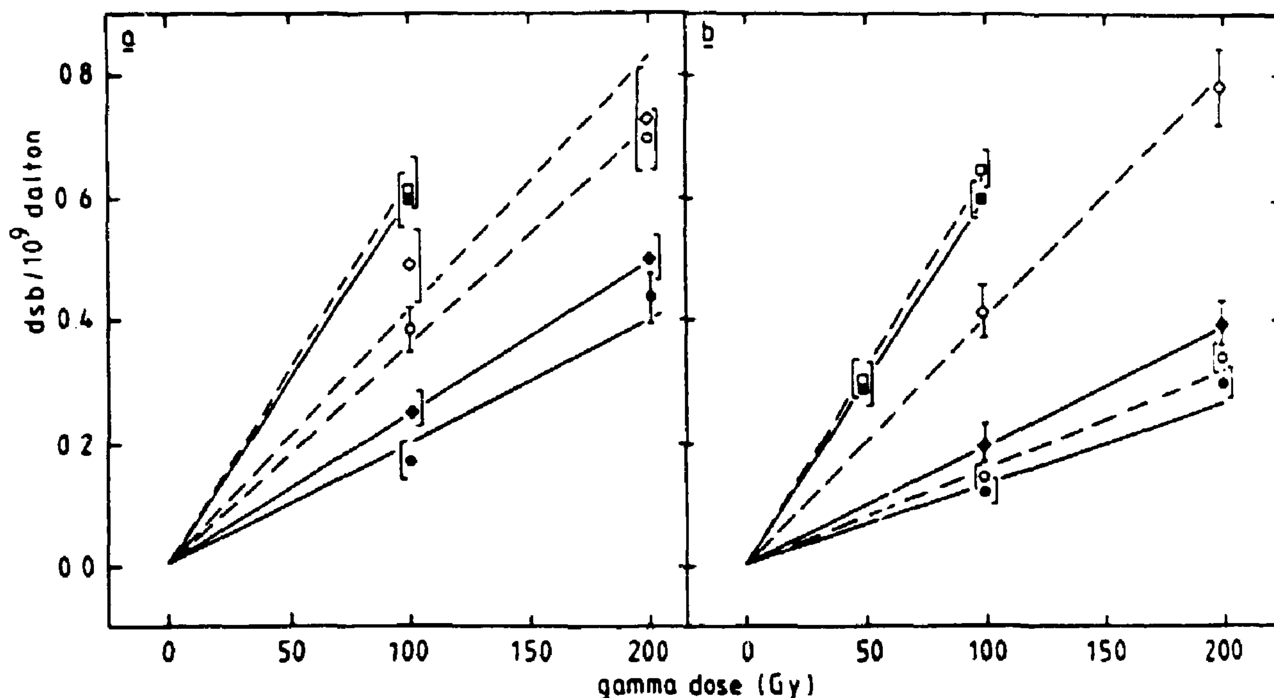


Figure 2

The effect of GSH depletion with DEM on the induction of dsb in DNA of CHO cells by $^{60}$Co-γ-rays at high and low oxygen concentration. a) Cells seeded on polystyrene Petri dishes; b) cells seeded on Mylar bottoms. Symbols as in figure 1. Dsb were measured with the neutral elution method. Means + S.E. are shown as calculated from 4 replicate determinations. The straight lines were fitted by eye.

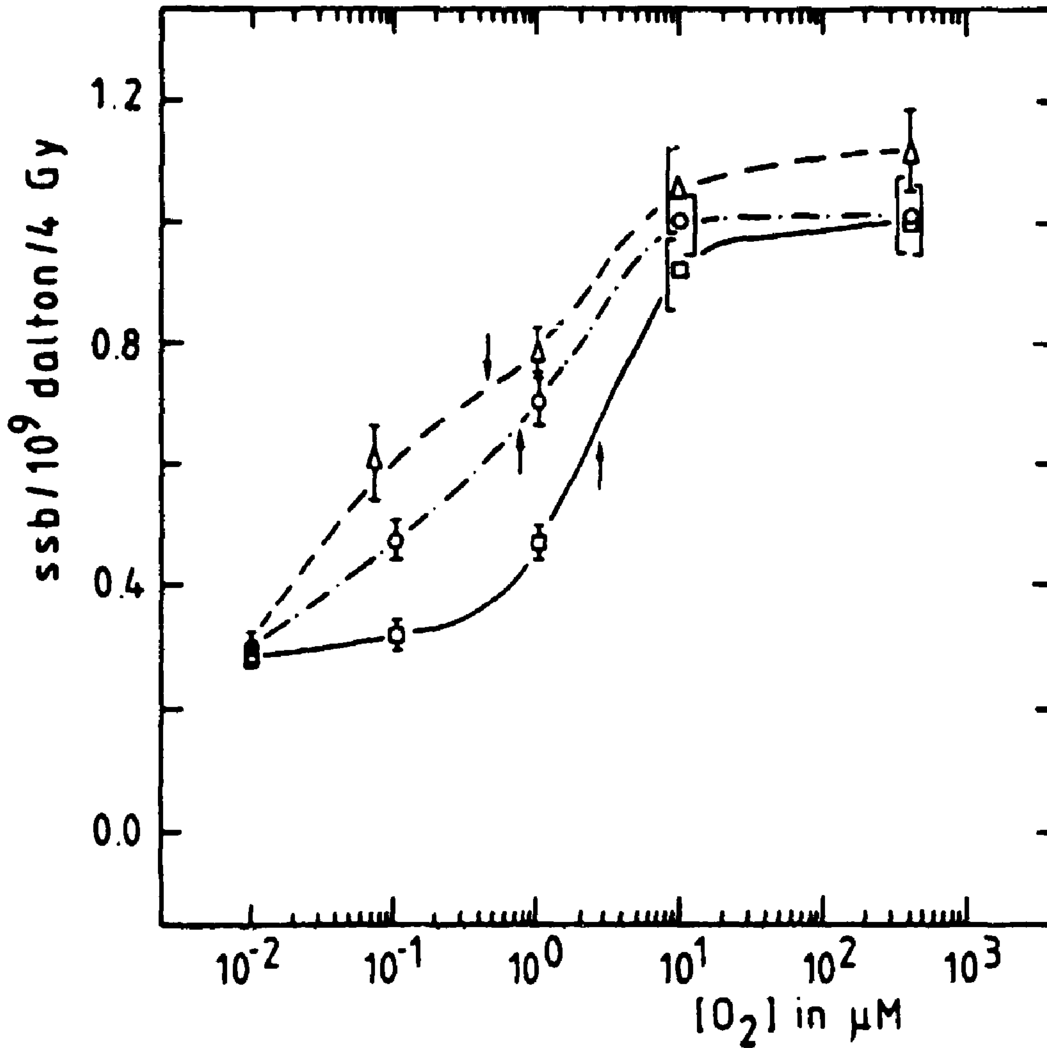


Figure 3

The sensitization of CHO cells by GSH depletion for the induction of ssb in DNA by X-rays in dependence on the oxygen concentration. (△) GSH depletion with DEM (1mM, 1 h, room temperature; (○) GSH depletion with BSO (0.2 mM, 16 h, 37 °C); (□) control. The $K_m$ for each curve is indicated by the arrows. Each point represents the averaged slope (+ S.E.) of the dose-effect curves obtained with all determinations of two independent experiments, similar to those presented in figure 1. Curves were fitted by eye.

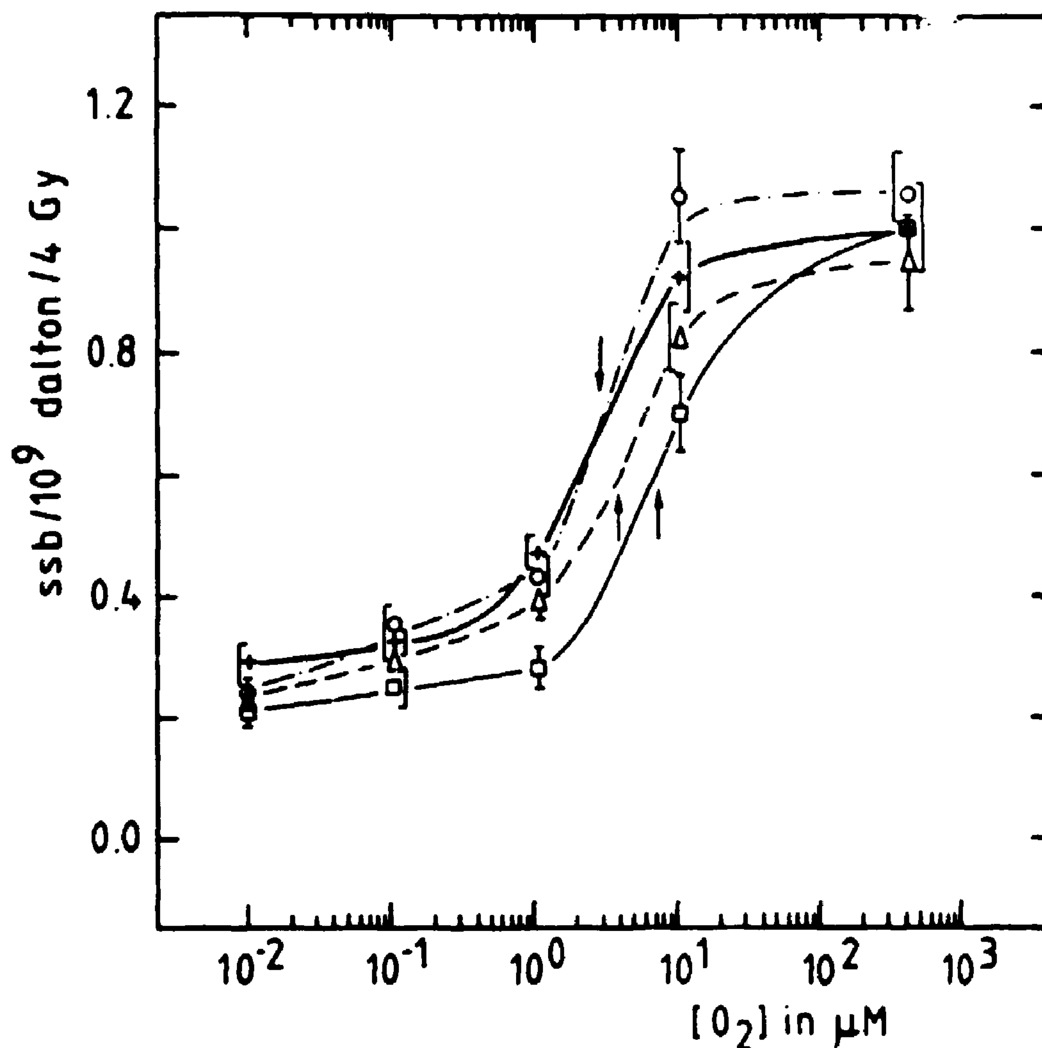


**Figure 4**

**The sensitization of HeLa cells by GSH depletion for the induction of ssb in DNA by X-rays in dependence on the oxygen concentration. (△) GSH depletion with DEM (1mM, 1 h, room temperature; (○) GSH depletion with BSO (0.2 mM, 16 h, 37 °C); (□) control. For comparison the control curve for CHO cells of figure 3 is included (+). The Km for each curve is indicated by the arrows. The points were derived as in figure 3.**

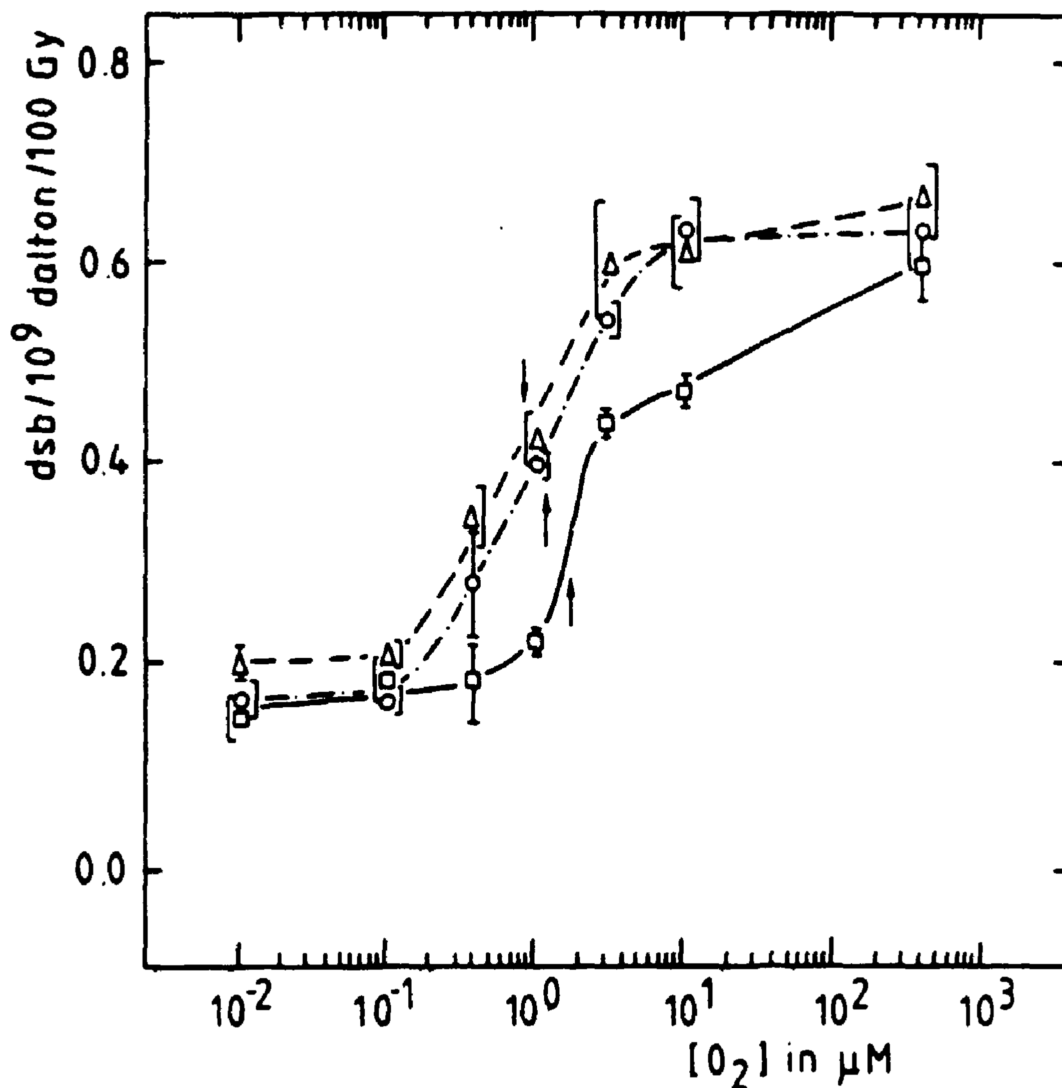


Figure 5

The sensitization of CHO cells by GSH depletion for the induction of dsb in DNA by γ-rays in dependence on the oxygen concentration. (△) GSH depletion with DEM (1mM, 1 h, room temperature; (○) GSH depletion with BSO (0.2 mM, 16 h, 37 °C); (□) control. The Km for each curve is indicated by the arrows. The points were derived from the averaged slopes of dose effect curves as presented in figure 2. The vertical bars represent standard errors of the mean of 4 independent experiments.

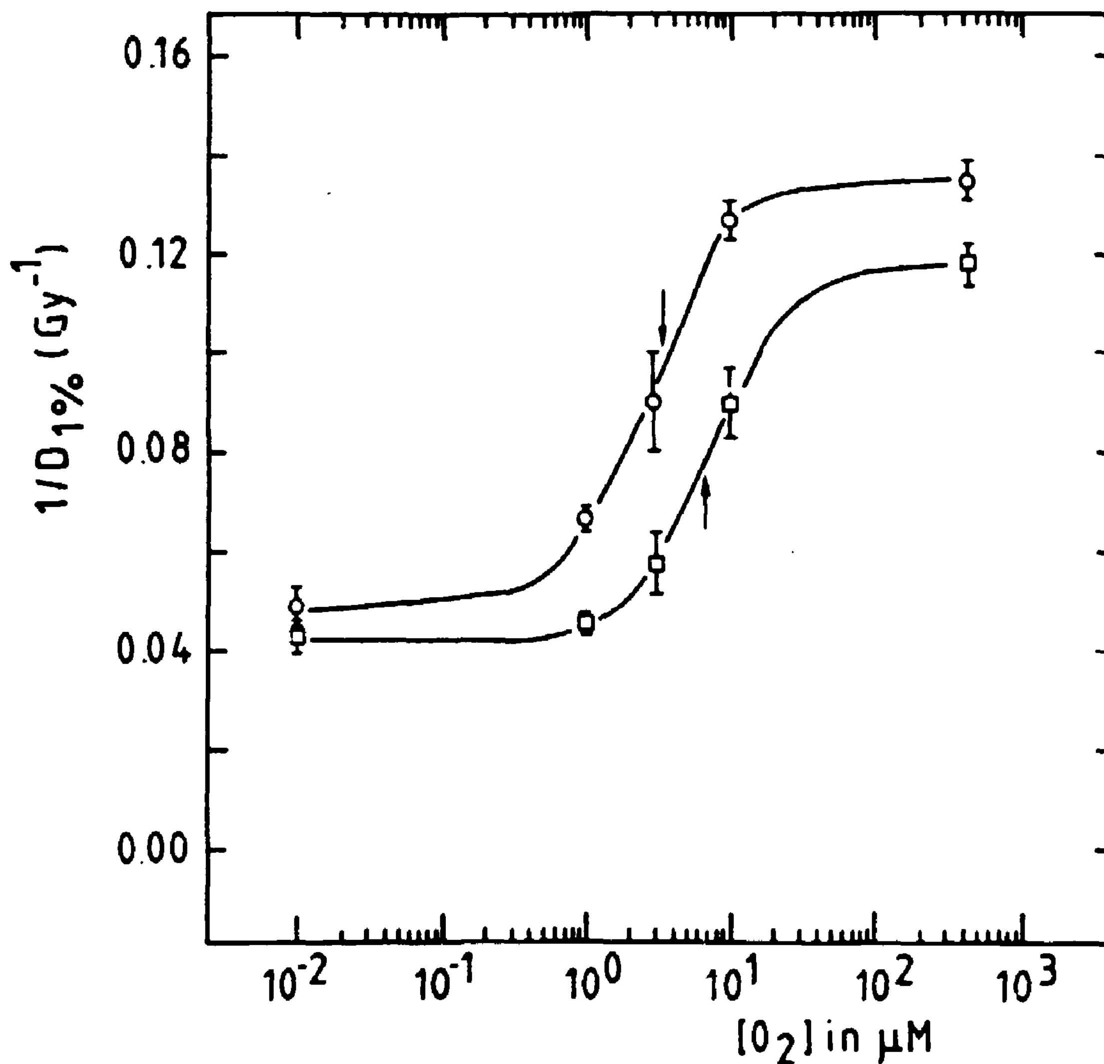


Figure 6

The effect of GSH depletion on the radiosensitivity of CHO cells to X-rays in dependence on the oxygen concentration. The radiosensitivity is expressed as the reciprocal of the one percent survival dose ($D_{1\%}$). (O) GSH depletion by BSO (0.2 mM, 16 h, 37 °C); (□) control. The vertical bars represent errors estimated from the range of variation of the corresponding survival curves.

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