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Glutathione Depletion by DL-Buthionine-*SR*-sulfoximine (BSO) Potentiates X-Ray-Induced Chromosome Lesions after Liquid Holding Recovery

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The impact of intracellular glutathione depletion on chromosome damage induced by X irradiation under aerobic conditions was investigated in two different cell lines, Ehrlich ascites tumor cells (EATC) and Chinese hamster ovary cells (CHO-K1). Thiol-depleted cell cultures in plateau phase were obtained by prolonged incubation in growth medium containing DL-buthionine-*SR*-sulfoximine (BSO), a specific inhibitor of γ-glutamyl-cysteine synthetase. Cells were then assayed using the procedures of G. L. Ellmann (*Arch. Biochem. Biophys.* **82,** 70–77 (1959)), F. Tietze (*Anal. Biochem.* **27,** 502–522 (1969)), and J. Sedlack and R. H. Lindsay (*Anal. Biochem.* **25,** 192–205 (1968)) for non-protein bound SH (NPSH), glutathione (GSH), and total SH (TSH). In both cell lines GSH was reduced to less than 10% of controls at higher BSO concentrations around 1 m*M*, whereas TSH and NPSH were affected to only 40–60%. In EATC pretreated with up to 1 m*M* BSO for 72 h, increased levels of spontaneously occurring micronuclei were found. At BSO concentrations above 200 μ*M*, both cell lines showed a potentiation of chromosome lesions scored as micronuclei and induced under aerobic X irradiation when liquid holding recovery in the original nutrient-depleted medium was performed; the extent of chromosome damage eventually reached that which could be obtained by application of β-arabinofuranosyladenine (β-araA), known to inhibit DNA repair processes by blocking DNA polymerases. It is therefore suggested that GSH depletion causes impairment of repair of lesions leading to chromosome deletions and subsequently to micronuclei. In contrast to CHO cell cultures, EATC showed a reversion of the potentiation effect as indicated by a decrease in the micronucleus content during prolonged incubation in the presence of BSO in the millimolar range. This effect could not be correlated to the remaining GSH content of less than 10% but may be due to some accumulation of unknown NPSH components. Since addition of L-cysteine to EATC cultures pretreated with BSO decreased the micronucleus content, cysteine/cystine or a related thiol within the NPSH fraction may be involved in the reestablishment of repair. Thus at least in one cell line, a rather complex response to BSO administration indicated that not only GSH but also other thiols may determine the level of chromosome damage after liquid holding recovery.

INTRODUCTION

DL-Buthionine-*SR*-sulfoximine (BSO) is one compound in a series of analogs of methionine sulfoximine, the most effective inhibitor of γ-glutamyl-cysteine synthetase. This enzyme plays an important role in the proposed γ-glutamyl cycle synthesizing glutathione from amino acids (*1, 2*). BSO is generally less toxic to cultured cells and to animals than other thiol-depleting drugs because of its high specificity (*3, 4*). BSO

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application results in glutathione depletion within the cells and thus leads to a state similar to that which occurs in oxoprolinuria, a rare genetic disease characterized by a low GSH content (*5*). Since lack of GSH or thiols in general sensitizes to hypoxic irradiation (*6*) and thus may help to overcome the difficulties in the radiation treatment of hypoxic tumor parts, radiotherapists and other scientists have developed a great interest in the effects of BSO administration.

Some results concerning the effects of BSO on the *aerobic* radiation response have questioned the initial enthusiasm for this drug in the improvement of radiation therapy (*7, 8*). Our own results on the ability of Ehrlich ascites tumor cells (EATC) to repair potentially lethal damage to colony formation when exposed to BSO have indicated that the change in the aerobic radiation response (loss of the shoulder in the survival curve) might be related to a change in the repair capacity.[1]

The role of GSH or other thiols in various repair processes is as yet unclear. It has been reported that GSH-deficient cells obtained from oxoprolinuria patients show an impairment in the repair of DNA single-strand breaks (*9*). GSH depletion by diethylmaleate (DEM), but not by BSO treatment, caused some deficiency in both single and double-strand break repair after hypoxic irradiation (*10*). After uv treatment a reduced capacity for repair of potentially lethal damage in GSH-deficient fibroblasts was found (*11*), and recovery of cells treated with chemicals was impaired when BSO was present before and during repair (*12*). However, in CHO cells treated with DEM and depleted in GSH to less than 10%, no effect on DNA strand-break repair could be detected (*13*).

In the present investigation, the main emphasis was on the BSO-induced effects after liquid holding recovery. Among the many end points of radiation action which can be studied *in vitro,* damage to chromosomes was thought to allow some initial insights into the impact of GSH depletion on repair, since liquid holding alters the extent of chromosome aberrations due to events which presumably involve repair at the DNA and chromosome level (*14, 15*). However, to separate liquid holding effects from initial radiation effects, the results presented must be based on the response without any repair contribution. Unless all molecular processes which contribute to liquid holding recovery are known, a "reference" value may be obtained only when certain drugs are used, like β-arabinofuranosyladenine (β-araA), which interferes with repair events involving DNA polymerase action, thus potentiating the extent of damage induced by X irradiation. The use of the term "potentiation" instead of "repair inhibition" throughout this work should therefore avoid a biased interpretation of results, leaving the subject of GSH impact on repair to more detailed studies at the macromolecular level.

MATERIALS AND METHODS

Cells and Culture Techniques

A suspension line (*16*) of Ehrlich ascites tumor cells with a similar radiosensitivity *in vitro* and *in vivo* was maintained by daily passage in A2 medium with 20% horse serum. To obtain partially synchronized

[1] H. Schorn and U. Bertsche, Radiosensitization of EAT Cells by Glutathione Depletion with BSO. Abstract of presentation at 18th Annual Meeting, European Society of Radiation Biology, Zürich, 1984.

cell cultures, EATC were seeded in petri dishes at an initial concentration of 1×10^5 cells/ml and incubated at 37°C and 6% CO_2 for 72 h. A plateau-phase culture of about 1.8×10^6 cells/ml was obtained. This population contained approximately 60–65% G1 cells, 25–30% S cells, and 10–15% G2/M cells, as measured by DNA flow cytometry.

Chinese hamster ovary cells (CHO-K1; Flow Lab) were grown in 25-cm^2 flasks in Eagle's minimal essential medium plus 15% fetal calf serum. Cells were incubated at about $1.3 \times 10^4/cm^2$ for 80 h, after which controls reached $1.4 \times 10^5/cm^2$. Unlike EATC, these controls showed a rather high content of G2/M cells in flow cytometric measurements: approximately 35% were G1, 23% S, 42% G2/M, and about 2% mitotic cells. A further increase in incubation time of 15 h reduced the G2 content to about 30% but made the culture more sensitive to prolonged BSO administration. The time 80 h was therefore chosen, although plateau-phase conditions with partial synchronization in G1 could not be obtained as in EATC. Within that period, CHO cultures doubled on the average 3.5 times and EATC cultures four times.

BSO (obtained from Prof. Dr. O. Vos, TNO Rijswijk) was dissolved in distilled water (stock solution, 20 m*M*). The dissolved drug was assayed by chromatography using a Silica gel system and the ninhydrin reaction for chemical integrity. For both EATC and CHO, BSO was added to the nutrient medium at the beginning of incubation when the cell cultures were in exponential growth. In the standard protocol, BSO was present during the whole growth period, during X irradiation, and during the subsequent liquid holding recovery period; i.e., X irradiation and liquid holding were performed in the same medium in which the cells had grown to plateau phase. Since this medium was depleted of nutrients and likewise of thiols (C medium), the same state of GSH depletion was present during the liquid holding recovery period and during X irradiation. To ensure that BSO by itself did not change the radiation response, controls were performed where BSO was added immediately before irradiation and removed immediately afterward by washing the cells twice in balanced salt solution. This control was compared with respect to X irradiation and liquid holding recovery to a control with no BSO treatment.

To compare the response of GSH-depleted cultures with a reference characterized by potentiation of damage in some samples treated with BSO during growth to plateau phase and during X irradiation, β-araA was added immediately after irradiation to the C medium at a final concentration of 100 μ*M*. This drug is known to interfere with DNA α- and β-polymerases (*17*) and to inhibit the repair of potentially lethal damage as measured by macrocolony formation (*18*). Reference values have been measured only where BSO *and* β-araA were present in the C medium during liquid holding recovery. BSO was not removed from these samples, because a change to a BSO-free liquid holding medium could have induced the buildup of GSH and other thiols within the cells which could interfere with the action of β-araA. BSO by itself is not thought to act synergistically with β-araA in the radiation response, because earlier experiments have shown that the potentiation of damage by β-araA in EATC was the same whether BSO was present or not.

After allowing for liquid holding recovery, cell cultures were washed twice in BSS and incubated in fresh nutrient medium to initiate thiol synthesis and subsequent transition into the cell cycle. It was possible that some BSO was still present in the cells, at least in those samples where it had been applied at rather high concentrations (1 m*M* and more). Growth experiments have shown, however, that the presence of small amounts of BSO does not interfere substantially with cell division and multiplication of cell cultures.[1] The test system applied here requires only that the majority of treated cells pass through mitosis to express chromosome damage in interphase I (postirradiation interphase). This is guaranteed in any case and for all cells which did not experience strong membrane damage or interphase death. Microscopic observation of cells treated over prolonged periods with BSO but not with X irradiation revealed no extensive morphological damage to cell nuclei once the drug had been removed and the culture had been allowed to multiply in fresh nutrient medium. The percentage of dead cells in EATC after 72 h exposure to BSO at various concentrations did not exceed 6% in any case, as judged by morphological appearance and by the trypan blue exclusion test. By this and other evidence not reported here in detail it could be excluded that prolonged exposure to BSO even at concentrations in the millimolar range exerted a toxic influence on most of the cells in the culture. This statement is generally also true for CHO-K1 cells in culture, although CHO cells are much more sensitive to prolonged exposures to BSO. A shorter exposure time might have rendered these cells less sensitive; however, such a modification would have interfered with the aim that the average doubling number of the culture in the presence of BSO should be similar in both cell lines.

Thiol Assay Procedures

After growth of cell cultures to plateau phase under the influence of BSO, samples were suspended in nutrient medium, centrifuged, added to 0.5 ml phosphate buffer (pH 6.8) containing 1 m*M* EGTA, and centrifuged at 2500*g* for 10 min at 4°C.

Cell pellets were washed in phosphate buffer, and 1 ml of 5% (v/v) sulfosalicylic acid was added while vortexing. Solutions were frozen and thawed three times under a stream of nitrogen and the protein precipitate was separated by centrifugation. Supernates were neutralized and the glutathione content was determined using procedures described by Ellmann (*19*) and Tietze (*20*) with slight modifications. The total and protein-bound sulfhydryl (TSH and PSH) contents were measured by the method of Sedlak and Lindsay (*21*). The difference TSH minus PSH gave somewhat higher but more reproducible non-protein bound sulfhydryl values than the estimation of NPSH according to Ellmann (*19*), especially in the low thiol range. Values were referenced to total protein mass, as determined by the method of Lowry *et al.* (*22*). If reference was made to the cell number measured by a Coulter counter (where lysed cells are not included in the count), values were about 5–10% higher, depending on the concentration of BSO previously administered.

Flow Cytometry

Cell cultures were suspended in nutrient medium and centrifuged. The cell pellet was dissolved in salt solution containing RNAse (10 mg/liter), Na-citrate (1000 mg/liter), NaCl (584 mg/liter), Nonidet-P40 (0.3 ml/liter), and ethidium bromide (10 mg/liter) and left for at least 1 h at room temperature to achieve optimal staining (*23*). DNA histograms were obtained from these samples with a flow system (30 L, Ortho Instruments) using an argon laser at 488-nm excitation wavelength. The histograms were evaluated assuming Gaussian peak distribution under the G1 and G2 + M peaks, and the low DNA mass background was measured at channel numbers reaching from the low channel threshold to some arbitrary channel number below the G1 peak where noise levels were reached.

Irradiation Procedures

X irradiation was always performed with aerobic cell cultures. For EATC, cell suspensions were transferred from 10-ml petri dishes to 3-ml dishes after careful pipetting and mixing. The original plateau-phase cell density was maintained, and a sufficient supply of oxygen was guaranteed. For CHO-K1 monolayers, cells were treated at least 2 h before irradiation with dispase (Boehringer, Mannheim) and replated at the original plateau-phase cell density in small petri dishes which fit the X-ray equipment. The use of dispase instead of trypsin guaranteed a mild treatment of cells during detaching and no interference with subsequent radiation response. Other samples of CHO were plated at very low concentrations (about 1000 cells per ml) onto cleaned and sterilized glass cover slips which had been placed in petri dishes which fit the X-ray equipment. They were irradiated no sooner than 2 h after plating, left in the same medium during the liquid holding time period, and then treated according to the procedures of the microcolony assay system (Bertsche, in preparation).

Irradiation was performed with an industrial X-ray tube at 150 kV tube voltage, filtered with 0.8 mm aluminum, at a dose rate of approximately 5 Gy/min at room temperature. The dosimetry was checked by ionization chambers and calibrated using a modified $FeSO_4$ dosimeter system (*24*).

Micronucleus Test and Microcolony Assay

The micronucleus test as described by Heddle (*25*) and Schmid (*26*) scores chromosome or chromatid damage resulting in acentric fragments which are excluded from the main nucleus and appear as small nuclei in the cytoplasm. To allow expression of micronuclei in treated cultures, cells must have passed through a post-treatment mitosis and must appear morphologically intact in interphase I. Normally, cell multiplication was monitored by Coulter counting and cell fixation was performed when multiplication indicated that cells were in interphase I or II. Some cells, either severely damaged or unable to divide, did not pass mitosis even after prolonged incubation. By various methods reported elsewhere it was estimated that this fraction did not exceed 30% of the total population (EATC).

Micronuclei in all size classes up to one-third of the diameter of the main nucleus (to exclude multinucleated events) and at any position within the cell were scored, providing that they could be classified by specific DNA staining (Hoechst 33258 or DAPI, Sigma Chemical, München) as separate entities.

In the microcolony assay system (Bertsche, in preparation) cells attached to glass cover slips of 3 cm ϕ were fixed in Carnoy's fixative (ethanol/acetic acid, 3/1) after washing in BSS, stained with Hoechst 33258 or DAPI (40 μg/ml), passed several times through Xylol, and placed in a drop of Entellan (Merck) on a glass slide. After overnight drying, samples were scored in a fluorescence microscope at 365-nm excitation wavelength and long pass emission at 395 nm.

Whereas in the micronucleus test only micronuclei could be scored, in the microcolony assay system chromatin bridges between daughters in a microcolony may also be counted. The number of micronuclei or chromatin bridges found was related to the total number of scored cells, excluding those which apparently were damaged or lysed. Thus the mean number of micronuclei/bridges was evaluated. In some cases, the distribution of micronuclei (number of cells with one, two, or three micronuclei) was also determined.

RESULTS

Growth in the Presence of BSO

The effect of prolonged BSO treatment on the final cell number of CHO-K1 and EATC is shown in Fig. 1. Final cell number after 80 h (CHO-K1, upper diagram) or 72 h growth (EATC, lower diagram) was evaluated by Coulter counting. In a semi-logarithmic plot versus BSO concentrations ranging from 0 to 2 mM the measured

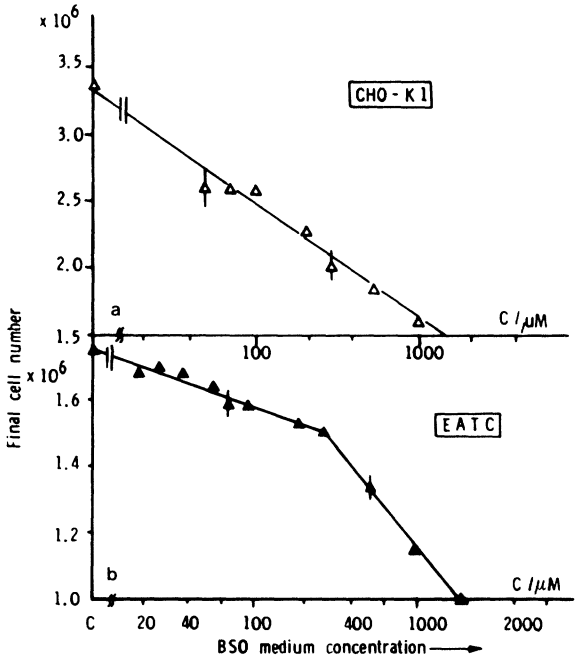

FIG. 1. Final cell number of CHO-K1 (a) and EATC cultures (b) after 80 or 72 h growth, respectively, in the presence of various BSO concentrations. (a) The total number of cells per 25-cm^2 flask, and (b) the cell concentration per ml medium in 10-ml petri dishes are given. Error bars are always below ±5% of indicated values.

points are either on a single line, as for CHO, or on a curve which may be approximated by two lines with different slopes, as for EATC. From these data it can be seen that in CHO cells the influence of BSO is similar in impact to the second component in EATC observed at higher concentrations. Therefore, at lower BSO concentrations, EATC proved to be less sensitive than CHO-K1.

DNA Histograms and Cell Cycle Distribution

DNA histograms measured by flow cytometry after prolonged BSO treatment are shown in Fig. 2. For each cell line, all histograms were measured in a single run. The example presented in Fig. 2 shows the general trends seen in EATC as well as in CHO-K1: (i) an increase in the low DNA mass background at low channel numbers, and (ii) minor alterations in the G1, S, and G2 + M compartments. In Fig. 3, the relative EATC cell content in G1, S, or G2 + M phase is plotted against applied BSO concentrations. In CHO-K1 (data not shown) the fraction of G1 cells decreased from 35% to about 30% at 500 μM BSO with a concomitant increase of cells in S phase, whereas the fraction of G2 + M cells remained constant at 44% throughout the range of BSO concentrations. Thus the influence of BSO on cell cycle distribution during prolonged exposure was negligible in the low concentration range ($C < 100$ μM BSO) and significant only at higher concentrations above 200 μM. These higher concentrations did affect cellular growth by reducing the mean number of doublings by one; i.e., cells were not able to pass through either the third or fourth mitosis after the initial setup.

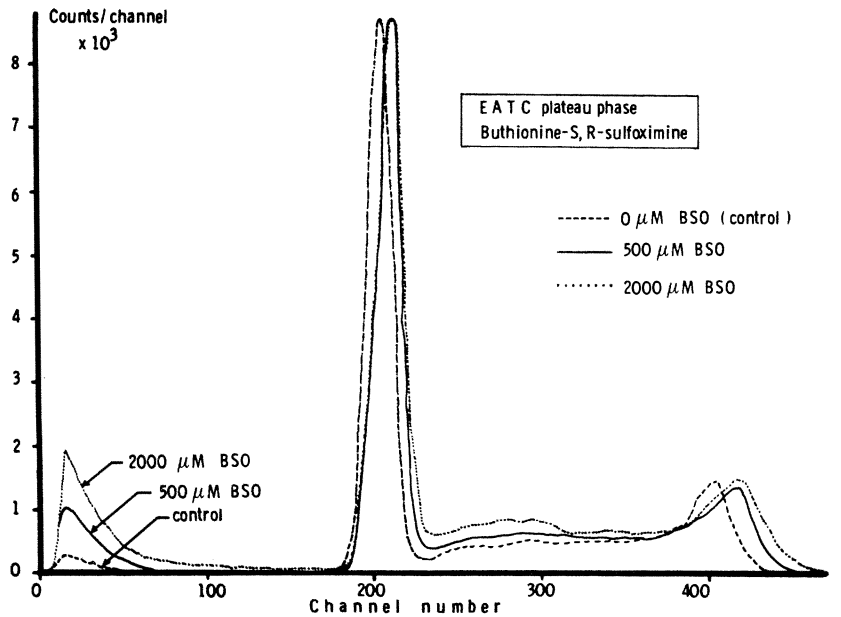

FIG. 2. DNA histogram for plateau-phase EATC treated with either 0, 500, or 2000 μM BSO for 72 h during growth to plateau phase. The low DNA mass peak is centered around a channel number corresponding to about 5% of DNA mass of the G1 peak (linear amplification).

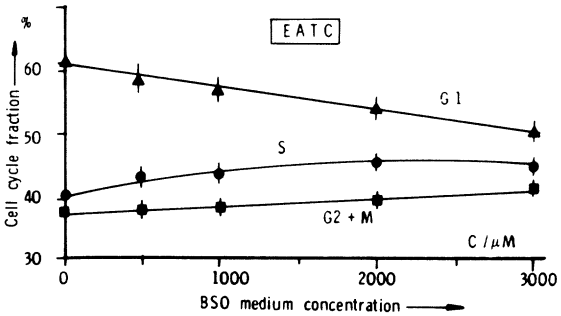

FIG. 3. Distribution of EATC in the cell cycle after growth to plateau phase in the presence of various BSO concentrations.

This growth inhibition does not necessarily indicate that BSO is toxic, since low GSH levels may prevent further passage through the cell cycle.

Low DNA Mass Background and Spontaneous Micronucleus Induction

In the DNA histograms a variability in the region of low DNA mass was found for cultures treated with BSO. The counts in this region increased in general with increasing BSO concentration. Such an effect has been observed before in cell cultures which completed cell division after treatment with ionizing radiation and has been attributed to the occurrence of micronuclei with a mean DNA mass of around 3% of the main G1 cell nuclei (*27*). To test whether BSO application may induce chromosome breakage and micronuclei formation without irradiation, cells were scored for the presence of micronuclei after prolonged exposure to BSO (Fig. 4). As expected from flow cytometric data, EATC revealed a significant increase in the mean number of micronuclei per cell from 0.01 ± 0.003 to 0.04 ± 0.01. In contrast, CHO-K1 showed a constant content of micronuclei up to 500 μM BSO, although DNA histograms from CHO cultures were similar to those from EATC in the region of low DNA mass. Thus the increase in low DNA mass in CHO cultures must be attributed to other events such as nuclear

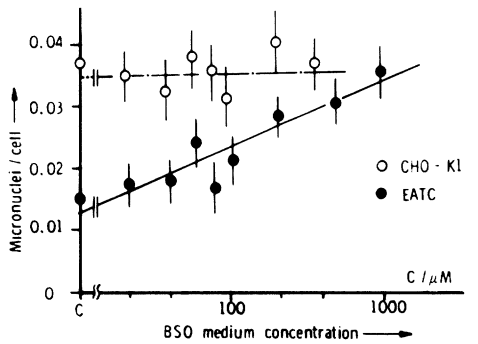

FIG. 4. The mean number of micronuclei/cell scored in unirradiated EATC or CHO-K1 cells after BSO pretreatment for 72 or 80 h, respectively. Error bars correspond to 95% confidence limits. In the case of EATC (black dots) a linear regression line has been drawn through the points (log–linear plot).

pycnosis or lysis which may release small DNA pieces. Microscopic observation revealed that such events (which are ignored in the micronucleus test) were elevated in number in CHO cells with increasing BSO, whereas they remained very low in EATC cells throughout the whole range of applied drug concentrations.

Thiol Levels after BSO Administration

From previous detailed measurements using different mammalian cell lines (*28*), it is known that BSO enters the cell interior rapidly and reacts with its target enzyme in a short time compared with the cell cycling time. GSH depletion must therefore depend on both turnover and distribution into daughter cells in an exponentially growing culture. Since the enzyme binding constant of BSO may be less than 100 μM (*3*), simple distribution should deplete GSH to less than 10% of controls within three cell doublings. In Fig. 5, where the relative levels of TSH, NPSH, and GSH are plotted against applied BSO concentration, a fall of GSH to less than 5% in CHO and to less than 10% in EATC can be seen. Repeated measurements of GSH in BSO-treated cells

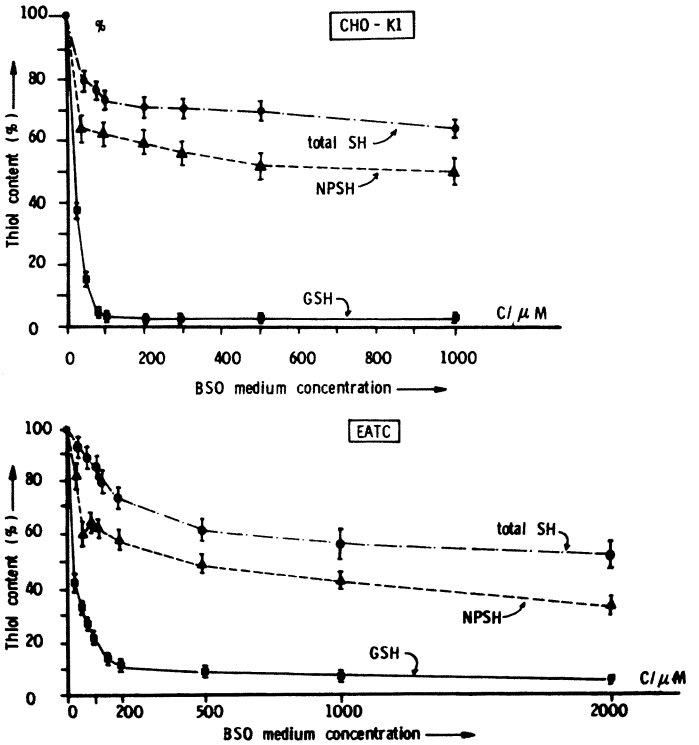

FIG. 5. Thiol content (total SH, NPSH = non-protein bound SH, and GSH = glutathione) of BSO-treated and control plateau-phase cultures analyzed according to modified procedures of Ellmann (*19*), Tietze (*20*), and Sedlak and Lindsay (*21*). Upper diagram: CHO-K1 cells; lower diagram: EATC. Error bars correspond to experimental errors for up to three different determinations per point.

also showed that the remaining GSH in EATC is stable within about 30% of the final value. The terminal depletion of GSH in CHO-K1 is somewhat lower than in EATC but also significantly above zero. Thus even high concentrations of the drug did not fully deplete the internal GSH, in contrast to other cell lines in which GSH declined to 1% or less (*28, 29*). In our hands, an incomplete thiol reduction is not due to our measurements, since in HeLa cells the same method resulted in terminal GSH values of around 1% of the untreated controls (*30*).

The fraction of GSH within the non-protein bound SH was determined in exponentially growing and plateau-phase EATC as well as in cells treated with BSO at 500 μM (Table I). The data show that NPSH declines by a factor of 3.2 during growth from the exponential to confluent state in the absence of BSO, and that GSH makes up more than 80% of the NPSH. The decline in NPSH is partly due to the different amount of G1 and S-phase cells (*31*), but more so to a shortage of thiol supply in the plateau phase, where thiols are used up at a faster rate than could be compensated by synthesis. All values shown in Fig. 5 are related to control cultures in plateau phase and not to controls in the initial setup, i.e., at the beginning of exponential growth. Comparison to the higher content of GSH at the onset of growth would have yielded the values for the terminal level of GSH of less than 5% for EATC and less than 2% for CHO.

Since GSH represents more than 80% of the non-protein bound SH in controls at plateau phase of growth, the NPSH content should show a similar decline with increasing BSO concentrations as the GSH content. As seen in Fig. 5, this is not the case. The maximum depletion of NPSH was approximately 40% for EATC. A detailed examination of the data presented in Fig. 5 indicates that during GSH depletion by

TABLE I

Changes in Non-protein Sulfhydryl (NPSH) and Glutathione (GSH) Content in Ehrlich Ascites Tumor Cells in Plateau Phase of Growth after Various Pretreatments

Treatment	*NPSH (μM of 10^8 cells)*	*GSH (μM of 10^8 cells)*	*GSH as percentage of NPSH*
Exponential growth, no BSO application	1.17 ± 0.02	1.09 ± 0.03	93 ± 3
Plateau phase of growth, no BSO application	0.37 ± 0.03	0.32 ± 0.03	86 ± 10
Plateau phase of growth, 500 μM BSO for 72 h	0.26 ± 0.04	0.023 ± 0.005	8.8 ± 2.5
Plateau phase of growth, 2 mM L-cysteine for 4 h, no BSO application	0.85 ± 0.04	Not measured	
Plateau phase of growth, 2 mM L-cysteine for 4 h, 500 μM BSO for 72 h	0.49 ± 0.03	Not measured	

Note. Values refer to cell number and not to protein content. 10^7 to 5×10^7 cells were taken for each measured sample.

BSO another component within the NPSH fraction may have been built up: with 60 μM BSO NPSH declined by 50% (C_{50} = 60 ± 10 μM). If this decline had continued up to 120 μM BSO, in parallel with the fall of GSH, an NPSH content around 25% of controls would have been expected, instead of the 60% observed. From considerations of the γ-glutamyl cycle, one of the NPSH components in question may be cysteine/cystine, since cysteine derived from peptidase action on cysteinyl-glycine is processed by γ-glutamyl-cysteine synthetase to γ-glutamyl-cysteine (*1*). A blockage of the synthetase by BSO would result in the accumulation of cysteine within the cell which may be oxidized to cystine. Evidence for cysteine/cystine accumulation during the course of GSH depletion by BSO has been reported elsewhere (*32*). On this basis, it was decided to investigate a possible effect of cysteine addition to cultures treated with BSO (see below). No attempt has been made in the present work to measure cysteine/cystine or other NPSH components directly since this would have required other biochemical techniques.

Micronucleus Induction in X-Irradiated Cells Pretreated with BSO

In EATC or CHO cells which were depleted of GSH by prolonged BSO treatment, X irradiation induced a pattern of micronuclei formation which was dependent on the BSO concentration. Figure 6 shows a typical result: the mean number of micronuclei/cell detected after 2 Gy and a liquid holding period of 4 h is plotted as a function of the BSO concentration present during growth to plateau phase. Under the influence of β-araA, which potentiates chromosome damage and micronuclei formation, a high and rather constant number of micronuclei/cell was found. This curve may also serve

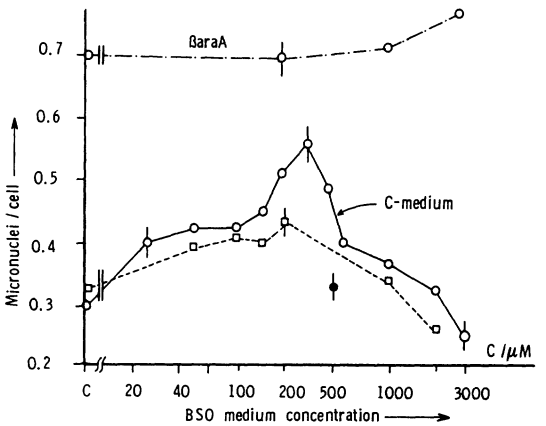

FIG. 6. The micronucleus content (mean number of micronuclei/cell) in EATC treated with various concentrations of BSO, then X-irradiated (2 Gy) and left in liquid holding medium for 4 h before transition through the cell cycle was initiated. The liquid holding medium was either conditioned medium (C medium, containing BSO), C medium plus β-araA (β-araA), or balanced salt solution (BSS). In BSS no BSO was present. Representative error bars indicate 95% confidence region. A control value (black point at C = 500 μM BSO) was included to show the effect of BSO when added immediately before irradiation and washed out immediately after irradiation, in contrast to the other values obtained after prolonged pretreatment with BSO.

as an "aerobic control," indicating that aerobic irradiation, irrespective of BSO treatment, induced a constant response (the significant increase at $C = 3000\ \mu M$ may be due to some action of β-araA on S cells, since cells in the S compartment accumulate during prolonged BSO exposure; see Fig. 3). Another control shown in Fig. 6 is the value measured in the absence of BSO in which the number of micronuclei/cell (0.31 ± 0.02) is more than twofold lower than with β-araA. Since in EATC a mean of 0.5 micronucleus per cell coincides with one lethal hit in terms of macrocolony formation,[2] a reduction in the mean value by a factor of 2 means that about twice as many cells survive under optimal liquid holding conditions when compared to β-araA. However, when the cell culture was pretreated with BSO in a concentration range around 400 μM, the C medium curve showed that instead of 0.31 ± 0.02 micronucleus/cell, a number (0.56 ± 0.035) of events were induced which indicated that the overall chromosome damage was increased by at least 80%. If, however, the liquid holding medium was BSS without BSO, the response at 400 μM was much less significant. This could have been due to a buildup of GSH during liquid holding, since a change to a BSO-free medium could have diminished the intracellular concentration of BSO in such a way that *de novo* synthesis of GSH from existing precursors was possible.

Another effect which becomes evident from Fig. 6 is the decline in micronuclei at BSO concentrations larger than about 500 μM. This decline is significant and reproducible, and is reflected also in an increase in survival of the same EATC line (Schorn and Bertsche, in preparation). At 2000 to 3000 μM, the decline has led to a significant lowering of the micronucleus content below that of untreated controls in BSS and C medium. No such decline is observed in the β-araA control, indicating that β-araA plus BSO pretreatment did not act synergistically to alter the maximum potentiation which can be obtained by β-araA. We conclude therefore that the decline at higher BSO concentrations is neither an artifact of BSO itself nor an artifact due to a shift in the sensitivity of a subpopulation in the culture, but that intrinsic changes in liquid holding recovery occurred which were mediated by such amounts of BSO.

If the response around 400 μM BSO is a potentiation of X-ray damage by impairment of the repair capacity, it should be reflected in the distribution of micronuclei in the scored cells. It has been demonstrated previously that repair of lesions leading to micronuclei is indicated by a shift of the micronucleus distribution to lower mean values and impairment of repair to higher mean values (*33*). In Fig. 7, the fraction of cells with one, two, or three micronuclei is plotted against the concentration of BSO administered prior to X irradiation (C medium as liquid holding medium). Only a moderate increase from 19 to 26 ± 2% at 50 μM BSO was observed in the fraction of cells with one micronucleus, followed by a return to control values at higher concentrations. This effect probably includes the increase in micronucleus induction seen in unirradiated controls (Fig. 4). Another response around 350 ± 100 μM is observed in the fraction of cells with two or more micronuclei; the effect is most pronounced in the fraction with three micronuclei. Thus, although the absorbed X-ray dose was the same in each sample, an accumulation of cells with several events occurred depending

[2] M. Krämer, *Theoretische und experimentelle Untersuchungen zur Erzeugung, Reparatur und Fixierung von Strahlenschaden in lebenden Zellen.* Diploma-Thesis University Frankfurt/M, 1985.

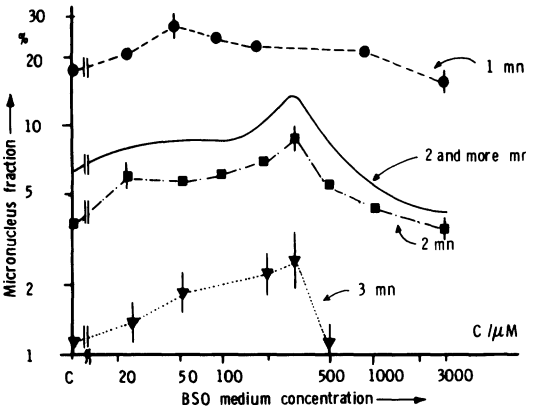

FIG. 7. The distribution of micronuclei in EATC samples, scored by H33258 staining and fluorescence microscope. The fraction of cells with either one, two, or three micronuclei is given as a function of BSO concentration in prolonged pretreatment. The full line shows the sum of fractions containing two or more micronuclei. Representative error bars indicate experimental errors determined from repeated measurements.

on the BSO pretreatment. If this pretreatment had led to an impairment of the repair capacity, it would have modified the final radiation response so that some cells showed more accumulation of damage than found in the control. The other possibility, that such an accumulation is merely the consequence of a limited toxic response at higher BSO concentrations, may be excluded since control values were reached again at very high amounts of BSO. It also seems unlikely that the initial radiation response is significantly altered, since such alterations would appear predominantly in the fraction of cells with one micronucleus.

In CHO-K1 cells (Fig. 8), similar experiments showed a steady increase in micronuclei with BSO concentration but showed no evidence for a reversion at higher amounts of BSO. Similar to EATC results, potentiation was strongest in the fraction of cells with two or more micronuclei (Fig. 8b). Damage reached the level exerted by β-araA exposure when higher amounts of BSO were administered. Interestingly, in both EATC and CHO, the potentiation of damage by β-araA was 2.4-fold over that of the control without BSO, indicating that the amount of chromosome damage which can be either expressed or repaired under these circumstances is similar in both cell lines.

In CHO cultures pretreated with BSO the induction of chromatin bridge formation was also assayed. Since this assay required the plating of a low number of cells (see Material and Methods) and development into microcolonies, the results were not directly comparable to the high cell density experiment. However, the data indicate at least quantitatively a similar response of bridge formation to BSO pretreatment, i.e., a potentiation at higher drug concentrations (data not shown).

If the mean numbers of micronuclei/cell at each BSO concentration are referred to the relative GSH content, CHO cells showed a potentiation effect only below 10% of GSH, whereas in EATC at 10% GSH the response was at the maximum (data not shown).

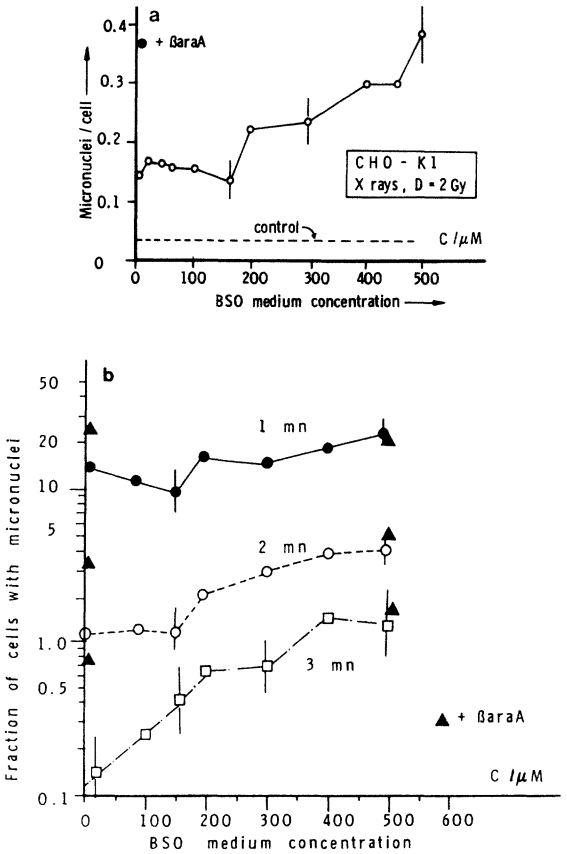

FIG. 8. Micronucleus induction in CHO-K1 cell cultures pretreated with various concentrations of BSO for 80 h, X-irradiated with 2-Gy, and left for 4 h in conditioned medium (C medium, containing BSO) or C medium plus 100 μM β-araA. (a) The mean number of micronuclei/cell. (b) The micronucleus distribution. Indicated are the fractions bearing either one, two, or three micronuclei. Black triangles show the β-araA potentiation effect for each of these fractions. At 0 μM BSO this effect is always above that without β-araA and is about equal at 500 μM BSO.

Cysteine Addition and Liquid Holding Recovery

The reversion of potentiation in EATC (Figs. 6 and 7) might have been caused by accumulation of cysteine within the cell, since BSO does not completely block the γ-glutamyl cycle and thus may lead to an increase of the substrate of the γ-glutamyl-cysteine synthetase. The addition of cysteine might therefore simulate the reversion effect if given to cell cultures pretreated with BSO at lower concentrations. To correct for the known toxicity to cysteine, L-cysteine was added at various concentrations in C medium immediately after X irradiation. Cells pretreated with BSO at 0, 250, 500, 750, and 1000 μM were exposed to this liquid holding medium for 3 h. After exposure to cysteine the usual treatment required for evaluating chromosome damage was per-

formed. Control experiments without irradiation verified the nontoxicity of cysteine addition up to 5 m*M*, since the cells were able to divide when cysteine was removed from the growth medium.

The NPSH values noted in Table I indicate that 2 m*M* cysteine in liquid holding medium raised non-protein bound SH more than twofold, from 0.37 to 0.85 μM per 10^8 cells in plateau-phase EATC (no BSO pretreatment) and from 0.26 to 0.49 μM per 10^8 cells after 500 μM BSO pretreatment. Cysteine added to the nutrient-depleted medium was able to enter the cell and contribute significantly to the NPSH pool, a prerequisite of the cysteine experiment.

Figure 9 shows the results of the cysteine experiment. A dose of 3 Gy caused 0.54 micronuclei/cell in the mean, when samples were not treated with BSO or cysteine. The addition of L-cysteine to liquid holding medium led to an increase in mean micronuclei per cell to 0.69 ± 0.06 at 5 m*M* (no BSO pretreatment). If cysteine was added to cell cultures pretreated with BSO, the cysteine effect depended on BSO concentration. At 500 μM BSO without cysteine, the micronuclei level was 0.92 ± 0.08 and with 5 m*M* cysteine 0.47 ± 0.04. Cysteine addition also lowered the micronucleus count in cells pretreated with BSO at concentrations higher than 500 μM, but to a smaller extent. In general, these data indicate that L-cysteine could reduce the chromosome damage induced under conditions of low GSH. It was less effective at higher BSO concentrations, in accordance with the hypothesis that some buildup of protective NPSH components during prolonged BSO treatment occurred which could act like the lost GSH during liquid holding recovery.

DISCUSSION

Application of BSO to mammalian cells reduces internal GSH levels by inhibiting *de novo* GSH synthesis (*2, 4*). This was confirmed in the present work using two different mammalian cell lines. In contrast to most previous investigations, prolonged

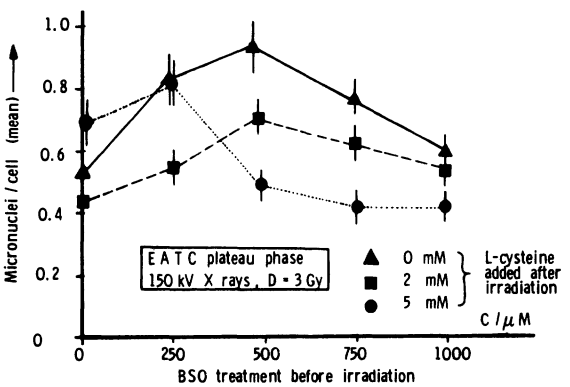

FIG. 9. The mean number of micronuclei/cell in EATC pretreated with BSO for 72 h at various concentrations, X-irradiated with D = 3 Gy, and exposed to L-cysteine at either 2 or 5 m*M* in conditioned medium during the liquid holding period. A control (no cysteine addition, black triangles) is included in this graph.

drug treatment was chosen to deplete GSH content as much as possible. This treatment did not result in serious side effects on cellular metabolism, as judged from the ability to grow and divide when BSO was removed. Similar experience with the nontoxicity of BSO has been reported. For example, Dethmers and Meister incubated three different human cell lines for up to 50 h in medium with 1 m*M* BSO and found 68% of CEM cells viable, as judged by the trypan blue exclusion test (*28*). EAT cells used in the present work were even more resistant to BSO cytotoxicity, since a 2 m*M* BSO exposure over 72 h resulted in only 5% damaged cells. CHO-K1 cells, however, were more sensitive to a long-term BSO exposure, and as a consequence, BSO concentration was limited to 0.5 m*M* instead of 2 m*M*.

In such a comparison of different cell lines, one must consider whether exposure to BSO was with a resting cell culture or with a culture in exponential growth, i.e., with sufficient supply of nutrients. In a resting culture, only GSH turnover determines its depletion when BSO is applied. In a dividing culture, depletion also occurs due to the distribution of remaining internal GSH to daughter cells. In addition to these effects, one must consider the different initial GSH content. In an exponentially growing culture, this content may be up to threefold higher, as for EATC (*31*) and other cell lines (*34*), when compared to a culture in plateau phase. Thus, even though the time course of BSO transport and binding would be similar in all these cell lines, the cytotoxicity of long-term exposure would vary considerably.

An important aim of the present work was to deplete intracellular GSH levels as much as possible by a combination of time and concentration in BSO exposure. A shorter pretreatment to BSO, for example over 24 h, as was carried out in many of the previous studies with other mammalian cell lines (*8, 10*), would have resulted in an incomplete GSH reduction, at least in EATC (Schorn and Bertsche, to be published). This may be accounted for by increasing the BSO concentration; however, to avoid biased interpretation of results, a long treatment time was chosen and the concentration range was restricted to about 2 m*M*. Due to this protocol most of the concentrations of BSO resulted in a plateau response of internal GSH, the "terminal depletion." Such a terminal depletion was considered essential for studying the impact of GSH on liquid holding recovery.

In contrast to some other reported data, the terminal GSH depletion did not result in GSH levels of less than 5% of controls. Levels of less than 1% were reported for CH V79 following exposure to 1 m*M* BSO for 24 h when the cell density was 5×10^6 cells/dish, but when the cell density increased to 9×10^6 cells/dish in plateau phase, the GSH level was 6% (*29*). This cell density effect could account also in the case of EATC or CHO-K1, since in HeLa cells with a different cell density the GSH level reached 1% of controls, as measured by the same method which has been applied in the present work (*30*). The human lymphoid cell line CEM showed depletion of GSH to less than 1% after long-term exposure to 1 m*M* BSO under growth conditions (*28*). In the work of Clark *et al.* a reduction to less than 10% in GSH after 24 h with a residual NPSH of about 20% of controls is indicated (*35*).

The NPSH pool consists largely of GSH in mammalian cells (*35, 36*). This was also confirmed for EATC in the present work (see Table I) and is likely for CHO too. Consequently, with depletion of GSH the NPSH (which is measured separately) should

decrease in a similar way. However, in EATC as well as in CHO-K1 the NPSH fraction could not be reduced to levels below 40% of untreated controls (Fig. 5). Other data reported in the literature indicate a similar phenomenon, i.e., a rather high terminal NPSH despite the strong reduction in GSH (*10, 35*). This effect must be specific for a variety of cell lines and experimental conditions during BSO exposure, since also the contrary, the concomitant decrease of NPSH to rather low levels, was found (*37*). As detailed before, the residual NPSH appearing at higher BSO concentrations and long pretreatment times could have resulted from the buildup of one or several components within the NPSH. BSO inhibits only one enzyme rather than a whole chain of reactions in GSH synthesis. By its inhibitory action, it could lead to an altered γ-glutamyl cycle and consequently to an altered assembly of NPSH serving as substrates within this cycle. Clarity on this point can be brought only when quantitative analysis of thiols at high resolution is performed, a lack of the majority of work involving BSO applications.

In both cell lines tested, the DNA histograms provided evidence of some accumulation of low DNA mass particles in BSO-treated cells. In the case of EATC, an increase in micronuclei over that present in exponential cultures could account at least in part for this accumulation. In CHO-K1, however, a rather high level of micronuclei appeared and stayed constant up to 500 μM BSO (Fig. 4). It is not clear whether this high level in CHO might have resulted from a lack of catalase activity, as was reported for EATC (*33*). As a ubiquitous molecule within the cell, GSH is known to protect against oxygen radicals, leading to increased sensitization to reactive oxygen intermediates when depleted intracellularly (*38*). These intermediates have been suspected to damage nuclear DNA and to cause chromosome breakage, as evidenced by the protective role of superoxide dismutase and catalase on micronucleus formation (*39*).

The effect of BSO treatment on final cell density (Fig. 1) is in line with the observation that prolonged exposure leads to partial blockage of S-phase cells (Fig. 3). Such a blockage affects only 10% of cells at higher BSO amounts and thus cannot fully account for the nearly twofold reduction in final cell number. To account for the entire effect, one must assume that the cells stop transit through the cell cycle at all cycle stages when they have passed through either the third or fourth mitosis after the initial setup. Since this effect appeared at all BSO concentrations and increased in EAT cultures at higher concentrations (Fig. 1), whereas the GSH or NPSH content reached fairly constant residual values, a primary influence of GSH on growth reduction cannot be deduced from these data.

The main object of this study was to assess liquid holding recovery from chromosome damage in GSH-depleted cells. The term "liquid holding recovery" has been derived from observations on the unlimited reproductive capacity (macrocolony formation) after treatment with ionizing radiation. It was found that this capacity could be regained to some degree when the irradiated cells were left in a liquid holding medium without nutrients like BSS or C medium for some hours before exposing them to optimal growth conditions. Such a recovery can be described theoretically by repair of repairable lesions, shifting the distribution of these lesions in the cell culture to lower mean values (*40*). The same effect was observed on the chromosome damage level, using micronuclei

as evidence for breakage of chromosomes or chromatids due to the radiation treatment.[2] In EATC as well as in other mammalian cell lines, a causal relationship may exist between micronuclei formation and loss of unlimited reproductive capacity (*41*).

Although there is no doubt that liquid holding recovery includes repair events on the DNA level, no details are known with respect to mammalian cells. It is not even clear what kind of damage is repaired. The final outcome is either a healthy cell or a cell bearing chromosome aberrations. Since acentric fragmentation and exchanges can be induced by certain restriction enzymes which cut through the DNA (*42*), the repair of double-strand breaks possibly contributes to liquid holding recovery.

In the present work an impact of BSO pretreatment on liquid holding recovery of lesions leading to micronuclei was found. The impact was stronger in CHO cells but evident also in EATC (Figs. 6 and 8). It seems likely that this lack of recovery was caused by the low GSH content, since only the GSH but not the NPSH or protein-bound thiols was reduced to fairly low levels. This does not exclude the possibility that other thiols, likely to be within the NPSH fraction, contribute to liquid holding recovery, such as cysteine/cystine. Additional experiments with addition of cysteine to the liquid holding medium have shown that cysteine is able to influence the outcome of recovery with respect to micronuclei (Fig. 9).

A number of previous reports are in line with the observations relating to L-cysteine. For example, the dose–effect relationship for dicentrics and excess acentrics of human G_0 lymphocytes was quite different in the presence of 60 m*M* cysteine (*43*). Although the drug was added before irradiation, a strong effect on the quadratic component of the dose–response relationship points to some repair involvement.

As was shown here for EATC and CHO-K1, BSO application is able to sensitize cell cultures to aerobic irradiation. Aerobic sensitization was also observed in V79 and A549 cells, as indicated by a reduction in the shoulder of the survival curve (*44*). Aerobic sensitization could be detrimental to the important aim of radiation therapy of sensitizing hypoxic parts of a tumor with SH-depleting drugs, since it diminishes the therapeutic gain in OER reduction by these drugs. Moreover, the data presented here, as well as other data, indicate that GSH or NPSH content must be lowered to a few percentage of controls to sensitize completely (*10*). A better approach to this problem may combine BSO and some electrophilic drugs like misonidazole to achieve full thiol depletion in the tumor without exceeding the dose limits of human neurotoxicity. A number of recently published studies (*8, 45*) indicate renewed hope for such an approach.

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