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On the nature of the oxygen effect on X-ray-induced DNA single-strand breaks in mammalian cells

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The initial yield of DNA single-strand breaks in X-irradiated Chinese hamster ovary cells was about 4-fold higher in aerated samples than in those under extreme hypoxia (< 10 p.p.m. O_2), with DNA breakage yields of $3{\cdot}6 \times 10^{-9}$ and $0{\cdot}85 \times 10^{-9}$ dalton^{-1} krad^{-1} for air and N_2 irradiations, respectively. In cells in which metabolism had been inhibited by heating before irradiation, a maximal DNA break yield of about 8×10^{-9} dalton^{-1} krad^{-1} was found for samples irradiated in air or under moderate hypoxia (200 p.p.m. O_2), while extreme hypoxia (< 10 p.p.m. O_2) gave a yield of $4{\cdot}3 \times 10^{-9}$ breaks dalton^{-1} krad^{-1}. When cysteamine was added to heated cells irradiated under extreme hypoxia, the DNA break yield was close to that obtained for viable cells under extreme hypoxia. Similarly, when the reducing agent, $Na_2S_2O_4$, was added before irradiation to remove O_2 chemically, the breakage yield for heated cells was close to that obtained for viable cells under extreme hypoxia. A 10-fold difference in the radiation effect was observed between maximal sensitivity (8×10^{-9} dalton^{-1} krad^{-1}) seen in heated cells under air or 200 p.p.m. O_2 and the near to minimal sensitivity ($0{\cdot}85 \times 10^{-9}$ dalton^{-1} krad^{-1}) seen in viable cells under < 10 p.p.m. O_2. A reasonable interpretation of these findings is that at radiation-induced free radical sites, fast chemical reactions occur which result in the fixation or the restoration of the damage. Thus, an oxygen-enhancement effect exists in mammalian cells for the X-ray-induction of DNA single-strand breaks; this effect appears to depend mainly upon radiation chemical events.

1. Introduction

The presence of oxygen during irradiation increases the initial yield of DNA single-strand breaks about 3- to 4-fold in mammalian cells in tissue culture (Palcic and Skarsgard 1972, Lennartz, Coquerelle and Hagen 1973), bacterial cells (Dean, Ormerod, Serianni and Alexander 1969, Ho and Ho 1972, Town, Smith and Kaplan 1972) and intracellular λ phage (Johansen 1974).

In contrast, several groups have observed that certain treatments before irradiation will result in about the same DNA break yield under air and N_2. Dean *et al.* (1969) reported that 0·02 M EDTA added to *M. radiodurans* cells treated with the R1 enzyme fraction from *S. albus* resulted in an equal number of breaks under O_2 and N_2 irradiation. This was interpreted as being due to inhibition by EDTA of an enzymic repair function occurring preferentially in N_2-treated cells. Other reports with the same interpretation are those by Ho and Ho (1972), who observed an annulment of the initial air/N_2 DNA breaks ratio with p-chloromercuribenzoate (PCMB) added to *E. coli* 15'T$^-$ cells, and by Town *et al.* (1972), who showed that the air/N_2 breaks ratio of 3·2 seen in X-irradiated *E. coli* K-12 cells was greatly diminished in heat-inactivated or cold-shocked cells. In the case of the EDTA effect, it was postulated (Alexander,

Dean, Lehmann, Ormerod, Feldschreiber and Serianni 1970) that enzyme inactivation, perhaps of the ligase-mediated sealing of the 5′-phosphate, 3′-OH bond, was brought about by removal of divalent ions (cofactors). Ho and Ho (1972) suggested functional enzymic SH-groups were inactivated by PCMB; and Town *et al.* (1972) attributed the effect to enzyme inactivation by heat or cold-shock.

However, recently Johansen (1974) and Johansen, Boye and Brustad (1973) have measured DNA breaks in λ phage DNA irradiated inside a bacterial cell with an electron pulse under O_2 or N_2 and observed that an O_2/N_2 single-strand breaks ratio of about 4 is also found within 100 msec after irradiation. They attributed this oxygen effect to chemical restoration of free radicals by hydrogen-donating (HD) species in anoxic systems. This type of restoration is diminished in aerobic systems owing to competition with the HD species by oxygen.

Further investigations in our laboratory of the oxygen-nitrogen effect on DNA single-strand break production in Chinese hamster ovary cells have resulted in data which are best interpreted in terms of fast chemical reactions occurring at the radiation produced target sites which can account for at least the major portion of the initial differential in DNA break yields. (A preliminary report of these studies was presented at the 5th International Congress for Radiation Research; Roots and Smith 1974.)

2. Materials and methods

2.1. *Cell-line, culture conditions and radioactive labelling*

Near diploid (2N = 21) Chinese hamster ovary (CHO) cells were grown on glass discs (diameter = 22 mm) placed in 10×35 mm plastic Petri dishes with 2 ml Eagle's minimum essential medium (MEM) containing 15 per cent foetal calf serum (both from Grand Island Biological Company) plus the antibiotics, penicillin (170 units/ml) and streptomycin (170 μg/ml). The population doubling-time was approximately 15 hours. Incubations were done at 37°C in a 95 per cent air-5 per cent CO_2 incubator.

Cells in exponential growth were used in all experiments. Forty-eight hours in advance of the X-ray treatment, 2 ml cultures of about 7×10^4 cells/ml were set up as described above with the addition of 0·05 μCi/ml thymidine-2-^{14}C (56·5 Ci/mol; New England Nuclear). The survival under these labelling conditions was about 73 per cent relative to a 100 per cent survival of unlabelled cells.

2.2. *Irradiation procedures*

At the end of the 48-hour labelling period, the cells were washed with culture medium, the glass disc transferred to another 10×35 mm Petri dish and covered with 1·25 ml Dulbecco's (Dulbecco and Vogt 1954) phosphate-buffered salt solution (PBS) at pH 7·3. A small amount of liquid covering the cells was desirable to facilitate distribution of dissolved compounds added before irradiation. The Petri dish was then placed in a closed irradiation chamber made of nylon (Rockwell and Kallman 1973). Air or N_2 (< 10 p.p.m. O_2) was fed into the chamber through glass and copper pipes. The effluent gas could be monitored for O_2 concentration with a Hersch cell (Engelhard Industries Ltd., Sutton, England). To ensure extreme hypoxia, a gassing time of 50 min was

needed. Clonal assays of dose–response curves under these conditions gave an oxygen-enhancement ratio of 3·04. Irradiations were done with a 250 kVp X-ray unit at 15 mA with external filtration of 0·25 mm Cu plus 1·0 mm Al. Ferrous sulphate dosimetry gave a dose-rate of 377 rads/min. However, a comparison of the D_0 values from dose–response curves of cells irradiated aerobically attached to either plastic or glass indicated that the absorbed dose for the cells attached to glass was about 1·3 times higher than for those attached to plastic. Consequently, the effective dose-rate was estimated to be 490 rads/min. Irradiations were done with the nylon chamber placed on crushed ice. At the side of the chamber a small needle opening blocked by a replaceable rubber stopper (2 mm diameter) provided the means, through which a solution could be added to the cells through a syringe without interrupting the gassing.

2.3. *Pre-X-ray treatments*

When cells were heated before being irradiated, the glass disc with the cells was placed in a 10×35 mm plastic Petri dish and covered with 2 ml PBS. The dish was set on the surface of a water-bath and heated for 15 min at 70°C. The 2 ml PBS was then aspirated and 1·25 ml PBS was added before gassing.

In the experiments in which cysteamine was added before irradiation, 0·1 ml of a freshly-mixed $6{\cdot}25 \times 10^{-2}$ M cysteamine-PBS solution was injected into the solution over the cells 15 min before irradiation to give a final concentration of 5×10^{-3} M. In the hypoxic experiments, cysteamine was dissolved in N_2-bubbled PBS.

For the samples treated with sodium dithionite ($Na_2S_2O_4$) (Matheson, Coleman and Bell, Los Angeles, Ca.), 0·1 ml freshly-prepared $6{\cdot}25 \times 10^{-2}$ M $Na_2S_2O_4$-PBS solution was injected into the sample 5 min before irradiation. For N_2-gassed samples, the sodium dithionite was dissolved in N_2-bubbled PBS. Sodium dithionite is often used in anaerobic enzyme titration studies (e.g., Mayhew and Massey 1973), and to remove cellular oxygen for anoxic irradiations (Johansen and Howard-Flanders 1965).

2.4. *Treatment after X-irradiation*

Immediately after irradiation, the Petri dish was placed on ice, the irradiated solution aspirated and 2 ml of cold PBS without Ca^{++} and Mg^{++} was added. The cells were then scraped off the glass disc with a rubber policeman, dispersed while maintained on ice, counted and added to the lysing layer in 0·1 ml at 10^5 cells/ml. This procedure took less than 2 min.

2.5. *Sucrose gradient sedimentation*

Several improved variations of the alkaline sucrose gradient centrifugation technique are now used in studies on unirradiated and irradiated mammalian cellular DNA. These rely on a high salt concentration or detergent in the gradient for lipid-protein-DNA separation. Results vary depending on such variables as lysing time and temperature and the composition of the lysing layer, especially regarding the EDTA concentration and the presence of detergent. Our gradient technique is a modification of an earlier method developed by Voiculetz, Smith and Kaplan (1974).

Gradients of 4·33 ml were made in 5 ml cellulose nitrate tubes from 5 and 20 per cent sucrose (w/v) solutions. The solutions were made by dissolving the sucrose in a solvent consisting of 0·3 M NaOH, 0·25 M Na acetate, 0·5 M $NaClO_4$ and 10^{-3} M Na_2 EDTA. A 0·2 ml lysing layer (0·2 M NaOH, 0·008 per cent deoxycholic acid and 0·04 M Na_2 EDTA) was pipetted on to the gradient, followed by the addition of approximately 10^4 cells in 0·1 ml PBS as described above.

To obtain a reproduceable peak position (especially for unirradiated cells), a minimum lysing time of about 5 hours (at room temperature in the dark) was required; longer periods of lysing up to 24 hours gave the same results.

Centrifugation was done at 20°C at 30 000 r.p.m. using a SW50·1 rotor in a Beckman L2 or L2 65B centrifuge. Test runs were made at two higher speeds (35 000 and 40 000 r.p.m.) as well as at lower speeds down to 10 000 r.p.m. with non-irradiated cells and with cells irradiated with 2·45 and 7·35 krads after either air or N_2 gassing to determine the phage T2/CHO DNA molecular weight ratio at various centrifugation speeds. After centrifugation, the bottom of each tube was pierced, and about 24 fractions were collected on to filter paper discs. These were washed with 5 per cent trichloroacetic acid, followed by an ethanol rinse, dried and the acid-insoluble labelled DNA counted in 5 ml of toluene scintillation fluid [4 g Omnifluor (New England Nuclear) per litre toluene]. Customarily 95 per cent or better of the radioactivity added to the gradient was recovered in the profile.

2.6. *Calculation of molecular weights*

Mammalian DNA (about 0·1 μg/gradient) and T2 phage DNA (less than 10 μg/gradient) were shown to sediment at constant velocity through the gradient. Since the sedimentation constant of each fraction, S_i, is proportional to the average molecular weight, M_i, in that fraction (Studier 1965)

$$S_i \propto M_i^{0.4} \tag{1}$$

and also proportional to the average distance, d_i, travelled by M_i (Burgi and Hershey 1963)

$$S_i \propto d_i/\omega^2 t \tag{2}$$

a combination of the two equations gives

$$M_i = K(d_i/\omega^2 t)^{2.63} \tag{3}$$

where ω is the angular velocity and t the centrifugation time and where the exponent in the Studier equation has been replaced by 0·38 (Freifelder 1970). The value of the gradient calibration constant, K, was found from sedimentation runs with freshly ^{3}H-labelled T2 phage DNA. A molecular weight of $5{\cdot}5 \times 10^7$ daltons was used for the average molecular weight of the T2 phage single-stranded DNA genome (Freifelder 1970) at the average sedimentation distance, d_i.

The weight average molecular weight, M_w, can be calculated from the net radioactivity counts in each fraction, c_i, as

$$M_w = \frac{\sum c_i M_i}{\sum c_i} \tag{4}$$

where c_i is the net counts in the ith fraction from the top. Substituting equation (3) into equation (4), we obtain

$$M_w = \frac{K}{(\omega^2 t)^{2\cdot 63}} \frac{\sum c_i (d_i)^{2\cdot 63}}{\sum c_i} . \tag{5}$$

Similarly, the number average molecular weight, M_n, may be calculated by substituting equation (3) into

$$M_n = \frac{\sum c_i}{\sum c_i / M_i} \tag{6}$$

giving

$$M_n = \frac{K}{(\omega^2 t)^{2\cdot 63}} \frac{\sum c_i}{\sum c_i / d_i^{2\cdot 63}} . \tag{7}$$

Profile shapes have been plotted as percent radioactivity per fraction against the distance sedimented. Since the number of DNA breaks is inversely proportional to the number average molecular weight, M_n, the yield of breaks has been expressed as the slope of the function $1/M_n$ versus X-ray dose.

Using a computer programme, M_n values were calculated from equation (7) above. In the M_n calculations, only the fractions within the profile limits were used to calculate M_n. Cut-off limits were usually at the first fraction on each side of the peak, which had 1 per cent or for profiles closer to the top of the gradient about 2 per cent, of the total profile counts. This always eliminated at least the top 4 fractions which, if included in the M_n calculation above, give large variations in M_n values. Further, the weight average molecular weight, M_w, was also calculated using the same profile limits as those set for the M_n calculations to obtain an estimate of the M_w/M_n ratio. These ratios and further considerations of molecular calculations are presented in § 4.

3. Results

3.1. *Technical considerations*

We found no significant difference in the molecular weight calculations for lysing periods of 1 to 22 hours for either air or N_2 irradiated DNA; however, as mentioned in § 2, a minimum lysing period of about 5 hours was necessary to give a reproduceable peak position for unirradiated cells. We are aware that denaturation of irradiated cellular DNA by means other than the use of alkali has been reported to give a lower break yield for both oxygen and N_2, but a higher O_2/N_2 single-strand breaks ratio (Lennartz *et al.* 1973) evidently due to the preferential formation of alkali labile sites in DNA irradiated under N_2. According to our data, such preferential hydrolysis must occur within 1 hour in alkali.

In the speed dependency studies, duplicate or triplicate samples of unirradiated cells, as well as cells irradiated under air or N_2 with 2·45 and 7·35 krads, were centrifuged at various speeds in two different experiments giving a minimum of 4 samples per speed. For unirradiated cells, a stable molecular weight of approximately $M_w = 2{\cdot}5 \times 10^8$ daltons was obtained from 10 000 to 20 000 r.p.m., while 30 000 r.p.m. gave an apparent molecular weight

33 per cent smaller ($M_w = 1{\cdot}6 \times 10^8$ daltons). The X-irradiated samples in air showed no significant speed dependence from 10 000 to 35 000 r.p.m. For the hypoxic irradiations, speed dependence was discernible for 2·45 krads but absent at 7·35 krads. Consequently, 7·35 krads was the lowest X-ray dose used in

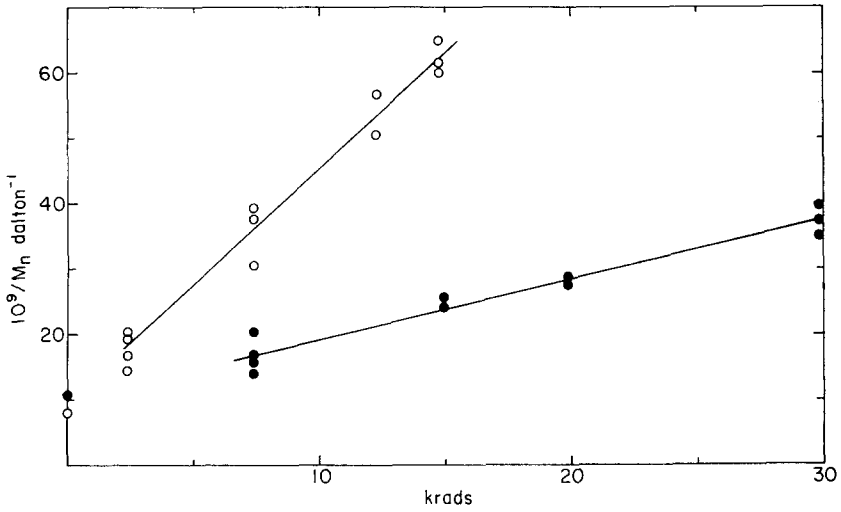

Figure 1. DNA single-strand breaks after aerobic (○) or anoxic (●) irradiations. The yields are $3{\cdot}6 \times 10^{-9}$ and $0{\cdot}85 \times 10^{-9}$ breaks per dalton per krad for air and anoxic conditions, respectively.

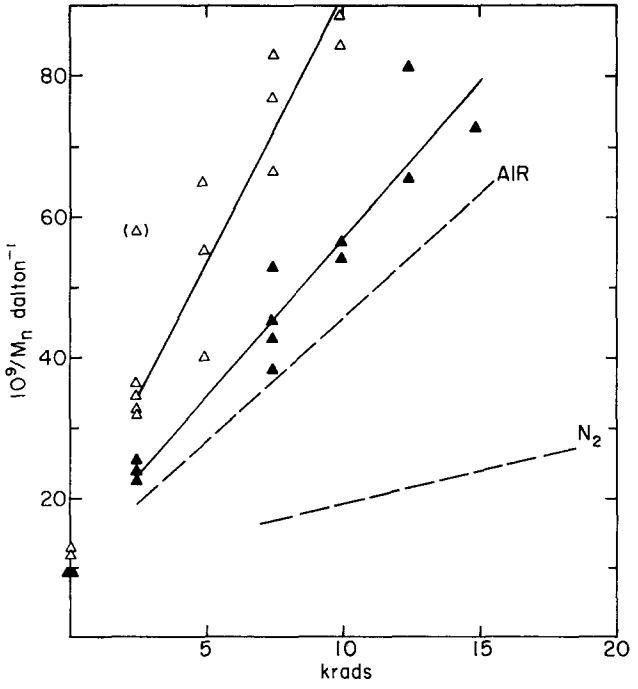

Figure 2. DNA single-strand break yields of heated cells irradiated under air (△) or N_2 (▲) equilibration. The dashed lines represent the initial break yields for aerobic and anoxic irradiations shown in figure 1. The yields for heated cells are $8{\cdot}1 \times 10^{-9}$ and $4{\cdot}3 \times 10^{-9}$ breaks per dalton per krad for air and N_2 equilibrations, respectively. The data point in parenthesis was not used in the regression analysis.

DNA break yield determinations after N_2 irradiation unless heated cells were used. Probably data points which lie below about $1/M_n = 15 \times 10^{-9}$ dalton^{-1} are subject to the speed dependence error when spun at 30 000 r.p.m. under our conditions. Unirradiated control samples were always run to detect heat-, cysteamine- or $Na_2S_2O_4$-induced changes in DNA, but these unirradiated samples were excluded from the determination of slope values in figures 1 and 2.

3.2. *Initial DNA break yields*

The initial yields of DNA single-strand breaks in air and N_2 were found from the slope values in figure 1 which were derived by linear regression analysis (the values for unirradiated cells were omitted). These yields are $3{\cdot}6 \times 10^{-9}$ and $0{\cdot}85 \times 10^{-9}$ breaks per dalton per krad for air and N_2 irradiations, respectively. A breakage efficiency for these conditions of 29 eV/break was calculated for air according to

$$\frac{6{\cdot}25 \times 10^{13} \dfrac{\text{eV}}{\text{rad-g}}}{3{\cdot}6 \times 10^{-12} \dfrac{\text{breaks}}{\text{rad-dalton}} \dfrac{6{\cdot}02 \times 10^{12} \text{ dalton/cell}}{10^{-11} \text{ g/cell}}}.$$

Similarly, for irradiations under N_2 the slope value of $0{\cdot}85 \times 10^{-9}$ dalton^{-1} krad^{-1} gives 122 eV/break. This gives an air/N_2 strand-breaks ratio of approximately 4.

3.3. *The yield of DNA breaks after heat treatment*

When the yield of DNA breaks was measured after heating the cells at 70°C for 15 min, an increase in the number of DNA breaks was observed, especially for N_2-irradiated samples. These results are shown in figure 2 with respective air and N_2 slope values of $8{\cdot}1 \times 10^{-9}$ dalton^{-1} krad^{-1} and $4{\cdot}3 \times 10^{-9}$ dalton^{-1} krad^{-1} obtained by linear regression analysis (the zero dose points were omitted). The dashed lines represent the original break yields shown in figure 1. Originally, the aim of the heat treatment was to inactivate repair enzymes (e.g., ligase); however, heat may also cause membrane changes, possibly resulting in leaky cells. Preliminary experiments with 15 min heating at various temperatures gave very little change in the number of DNA breaks for 45°C and 55°C, some effect at 65°C and a noticeably larger increase at 70°C, especially for the N_2-irradiated samples. Fifteen min heating at 75°C gave results that were within the 95 per cent confidence limits of the regression lines for the 70°C heat treatments shown in figure 2.

Examples of the gradient profiles used to evaluate the molecular weights for figure 2 are shown in figure 3. A comparison of the unirradiated profiles of unheated and heated cells does indicate some differences; but although some DNA breakdown may occur, there is no evidence of extensive alteration in the average size of non-irradiated DNA. Heat and unheated unirradiated controls included in the experiment were centrifuged along with the other samples at 30 000 r.p.m.; however, in a separate experiment, such controls were centrifuged at a more appropriate speed for this size DNA (20 000 r.p.m.) in order to detect heat-induced changes in the DNA. These profiles showed essentially the same

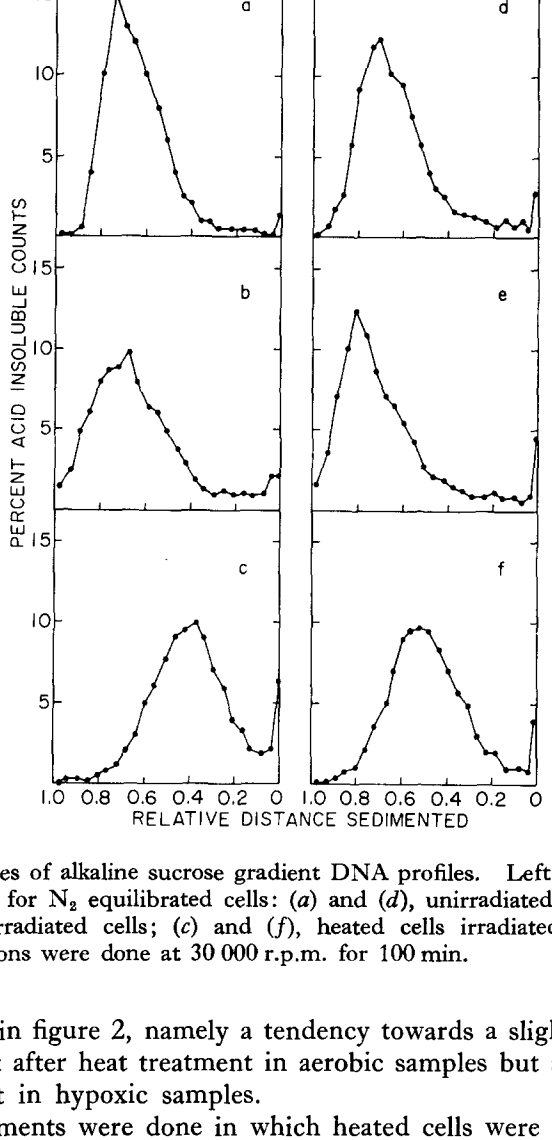

Figure 3. Examples of alkaline sucrose gradient DNA profiles. Left panels for air and right panels for N_2 equilibrated cells: (*a*) and (*d*), unirradiated cells; (*b*) and (*e*), heated, unirradiated cells; (*c*) and (*f*), heated cells irradiated with 2·45 krads. Centrifugations were done at 30 000 r.p.m. for 100 min.

result expressed in figure 2, namely a tendency towards a slightly lower DNA molecular weight after heat treatment in aerobic samples but a slightly higher molecular weight in hypoxic samples.

A few experiments were done in which heated cells were irradiated under N_2 containing 200 ± 10 p.p.m. O_2 (Liquid Carbonic Co., San Carlos, Ca.). If protective compounds (e.g., sulphydryls) leak out due to a damaged membrane caused by the heating, so that quite low O_2 concentrations become important, the break yield under the relatively poor hypoxia should be higher than under the <10 p.p.m. O_2 customarily used. We observed that irradiations of heated cells in air and 200 p.p.m. O_2 gave numerical answers that were very close; i.e., DNA breakage yields of $8{\cdot}1 \times 10^{-9}$ dalton^{-1} krad^{-1} (figure 2) and $7{\cdot}8 \times 10^{-9}$ dalton^{-1} krad^{-1} for air and 200 p.p.m. O_2, respectively, while heated cells irradiated under <10 p.p.m. O_2 gave a breakage yield of $4{\cdot}3 \times 10^{-9}$ dalton^{-1} krad^{-1} (figure 2). The breakage yield for the 200 p.p.m. O_2 irradiations was calculated from triplicate samples at three different X-ray doses (data not shown); and heated samples irradiated under <10 p.p.m. O_2 were included in the

experiment for comparison. The DNA break yield observed for unheated cells irradiated in the presence of 200 p.p.m. O_2 was increased at the most by 1·5 times that found for non-heated cells irradiated in the presence of < 10 p.p.m. O_2.

3.4. *The yield of DNA breaks after addition of cysteamine prior to irradiation of heated cells*

If hydrogen donating (HD) species leak out of the cell after heating, the addition of cysteamine to the extracellular environment should permit restoration of the endogenous concentration of HD species. Preliminary experiments were done in which cysteamine was added to non-heated cells at 10^{-2} to 10^{-4} M 15 min before irradiation in air at 7·35 krads to determine the highest concentration which did not change the DNA break yield. On the basis of these tests, 5×10^{-3} M cysteamine was routinely used.

The results of the addition of cysteamine before irradiation are illustrated in figure 4. The original yield of breaks obtained in air and N_2 for unheated cells are indicated by the two dashed lines (results of figure 1) while the two solid lines represent the yields found in air and N_2 after heating the cells (results

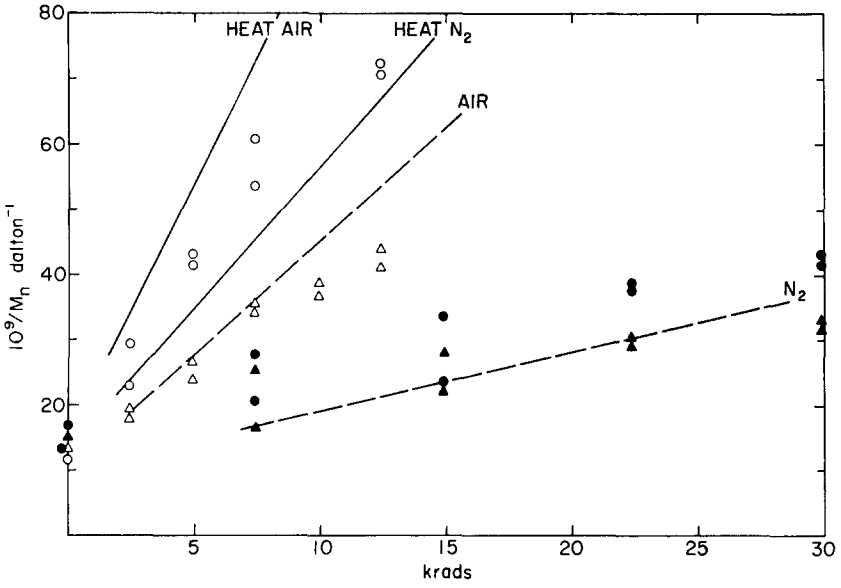

Figure 4. Yields of DNA single-strand breaks for cells irradiated in the presence of 5×10^{-3} M cysteamine. The solid lines represent the break yields under air and N_2 after heating as shown in figure 2 and the dashed lines represent the initial break yields under air and N_2 shown in figure 1. Cysteamine added to unheated cells irradiated in air (△) and in N_2 (▲). Cystamine added to heated cells irradiated in air (○) and in N_2 (●).

of figure 2). The addition of cysteamine to unheated cells did not alter the break yield very much in the aerobic or hypoxic system; however, for heated cells there was some reduction of the break yield in air and a much larger reduction under N_2, yielding a molecular weight close to that for the unheated samples irradiated under N_2. This result shows that the effect of the heat treatment on the DNA single-strand break yield can be largely reversed by

chemical treatment. Cysteamine is oxidized in air, and this might be one reason why the values for heated cells in air are not as close to the original break yield in air as is the case for the hypoxic condition.

In one experiment, cysteamine was added to heated cells immediately after irradiation under N_2 while maintaining the cells on ice under hypoxic conditions for 5 min past irradiation. The result was identical to that obtained for heat treated cells shown in figure 2, i.e., there was no reversal of the heat treatment effect if the cysteamine was added after irradiation.

3.5. *The yield of DNA breaks after addition of $Na_2S_2O_4$ before irradiation of heated cells*

To test the hypothesis that it is primarily the O_2/HD ratio which controls the fixation or chemical restoration of radiation produced target free radicals, we performed some experiments in which the reducing agent sodium dithionite, $Na_2S_2O_4$, was added at 5×10^{-3} M to heated cells. If cellular loss of hydrogen-donating species occurs after heating, the O_2/HD ratio will be changed drastically, although under severe hypoxia the O_2 content will also be low. Therefore, under highly hypoxic conditions (< 10 p.p.m. O_2) the O_2/HD ratio in heated cells is expected to be markedly different from that of hypoxic viable cells. Residual O_2, however, may still be present in the cell so that if HD compounds leak out after heat treatment, the residual O_2 level may then be large enough to cause an increase in the break yield approaching that normally found for air irradiations as illustrated in figure 2. In irradiations under N_2, addition

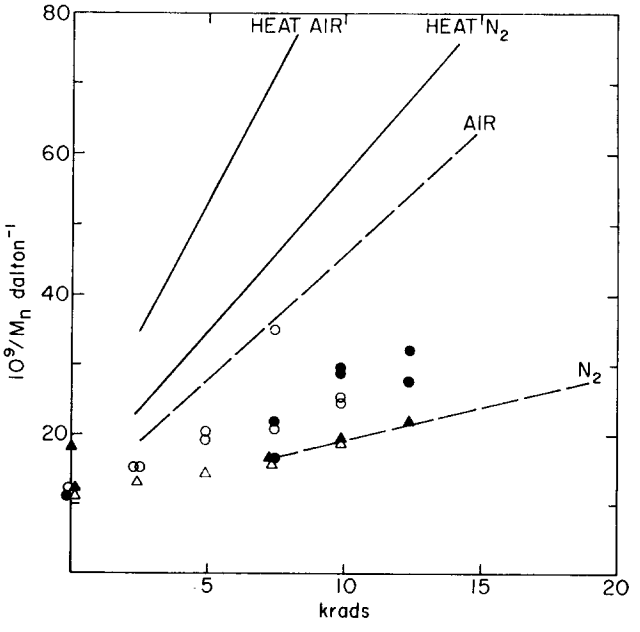

Figure 5. DNA break yield of cells irradiated in the presence of 5×10^{-3} M $Na_2S_2O_4$. The solid lines represent the break yields under air and N_2 after heating as shown in figure 2 and the dashed lines represent the initial break yields under air and N_2 shown in figure 1. Sodium dithionite added to unheated cells irradiated in air (△) and in N_2 (▲). Sodium dithionite added to heated cells irradiated in air (○) and in N_2 (●).

of $Na_2S_2O_4$ should remove residual oxygen from the cells and should maintain anoxia. In irradiations in air, the addition of $Na_2S_2O_4$ should create temporary hypoxia (or anoxia). In both cases, a drastic reduction of the break yield for heated cells under N_2 and for heated and unheated cells under air is expected. No change is expected for the unheated cells under N_2, since metabolizing cells under highly hypoxic conditions should be in a practically anoxic state.

As may be seen from figure 5, adding $Na_2S_2O_4$ to cells 5 min before irradiation under either air or N_2 resulted in a low breakage efficiency close to that found for unheated cells irradiated under N_2 if the irradiation times in air were kept short so that oxygen diffusion did not deplete the $Na_2S_2O_4$. In early experiments, however, where air was bubbled into the PBS covering the cells, the addition of $Na_2S_2O_4$ did not maintain the temporary hypoxia observed in figure 5.

4. Discussion

4.1. *Considerations pertaining to the technique*

Ratios of the calculated M_w/M_n values were evaluated routinely. In all cases the gradient profiles for irradiated cells indicated a close to random DNA molecular weight distribution, except for occasional aberrant profiles (e.g., for irradiations under N_2 shown in figure 1, a range of 1·77 to 2·08 was found).

As expressed above in § 3.1, data points which reflect speed dependence errors probably lie below 15×10^{-9} dalton^{-1} in figures 1, 2, 4 and 5. Only the data shown in figures 1 and 2 have been evaluated for DNA breakage yields. Unirradiated samples treated with heat, cysteamine or sodium dithionite, routinely included in the experiments, gave approximately the same apparent DNA molecular weight as can be seen in the figures, which at 30 000 r.p.m. has a value approximately one-third lower than the actual weight (see § 3.1 above). In a few experiments, dithionite-treated controls exhibited some DNA breakdown.

4.2. *The air/N_2 single-strand breaks ratio*

The initial number of DNA breaks measured for irradiations under N_2 depends obviously on the degree of hypoxia achieved. Apart from a concern over the quality of the gas, the irradiation vessel and adequate gas equilibration time, attachment of cells to a glass surface rather than to plastic is an important factor, as discussed fully by Moore, Pritchard and Smith (1972).

Under the conditions used in these studies the breakage efficiency in air was 29 eV/break or 19·6 breaks per cell per rad.

By manipulating the chemical environment within the cell, it was possible to show that the ratio of oxidizing to reducing species greatly affects the number of radiation-damaged molecules measured as DNA single-strand breaks. After heating the cells, the number of breaks under N_2 increased about 5-fold for extreme hypoxia (< 10 p.p.m. O_2) but increased about 10-fold for moderate hypoxia (200 p.p.m. O_2). A maximal O_2 effect was thus achieved with heated cells irradiated in air ($\sim 200\,000$ p.p.m. O_2) or in 200 p.p.m. O_2 but not in < 10 p.p.m. O_2. If heat treatment removes HD species due to a leaky

membrane, the oxygen fixation hypothesis provides a reasonable explanation for the above results, i.e., radiation-produced free radicals may be restored by H-donation or damage fixation may occur by oxygen addition leading to a strand break. The data shown in figure 4 are in accord with this hypothesis since cysteamine greatly diminished the oxygen effect in heat-treated nitrogen equilibrated samples where the O_2 content was low, and reduced the effect for aerobic irradiation of heat treated cells; while at the same time little change was caused by cysteamine at this molarity in non-heated cells. Chemical competition reactions of cysteamine and O_2 have been reported by Howard-Flanders and Alper (1957) and by Johansen and Howard-Flanders (1965) for mercaptoethanol and oxygen in *E. coli*. Pulse radiolysis experiments by Adams, McNaughton and Michael (1968) showed that free radical repair by hydrogen transfer from cysteamine can take place in dilute aqueous sugar or alcohol solutions.

The addition of the reducing agent $Na_2S_2O_4$ should result in chemical removal of oxygen; that is, complete removal of oxygen in N_2 equilibrated systems in heat-treated cells where cellular consumption of oxygen does not occur and a temporary removal or reduction of the O_2 content in air equilibrated samples. Our data show (figure 5) that in heat-inactivated cells and viable cells irradiated under air, the addition of $Na_2S_2O_4$ before irradiation yielded results that approached those obtained for viable cells under extreme hypoxia (anoxia). Assuming that the major relevant chemical reaction of $Na_2S_2O_4$ is to remove O_2, thus either removal of oxygen by sodium dithionite or the addition of HD species to heated cells containing residual oxygen should give a DNA-break yield characteristic of low target radical damage fixation. The difference between the maximal damage fixation seen for heat treated cells in air or 200 p.p.m. O_2 and the low yield observed for viable cells in N_2 is approximately 10-fold. In this regard it is interesting that survival studies on Chinese hamster fibroblast cells showed a 10-fold difference in radiation sensitivity for X-irradiation under close to maximum protection (that is, N_2 equilibration plus addition of a reducing species), and under near maximum sensitization (that is, air equilibration plus removal of reducing species by diamide) (Chapman, Borsa and Greenstock 1973). This result is not cited to correlate DNA single-strand break damage with radiosensitivity, since, obviously, it is not the initial X-ray injury so much which is decisive but the outcome after cellular repair has occurred, and since DNA single-strand breaks are just one among several types of potentially injurious events.

It seems very probable, therefore, that initial radiation injury to DNA may either be averted by radical scavenging protection or, if produced, may undergo chemical damage fixation by peroxide or hydroperoxide formation or undergo chemical restoration by reduction (i.e., by some HD species). Although most of the oxygen effect of the yield of DNA single-strand breaks appears to be the result of fast chemical reactions, our data do not exclude the existence of a very rapid repair process (e.g., one requiring only ligase activity) for the rejoining of single-strand breaks. Our results would suggest, however, that the amount of such repair must be considerably less than hypothesized to occur in bacteria based upon somewhat similar heat inactivation studies (Town *et al.* 1972).

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La production initiale de ruptures de chaîne simple d'ADN dans les cellules ovariennes d'hamster chinois, est environ 4 fois plus élevée pour les cellules irradiées en présence d'air plutôt que sous hypoxie poussée (<10 p.p.m. O_2). Le nombre de ruptures d'ADN étant respectivement de $3{\cdot}6 \times 10^{-9}$ en présence d'air, contre $0{\cdot}85 \times 10^{-9}$ dalton^{-1} krad^{-1} en présence de nitrogène.

Lorsque les cellules sont chauffées avant l'irradiation, ce qui inhibe leur métabolisme, un maximum de ruptures d'ADN d'environ 8×10^{-9} dalton^{-1} krad^{-1} est atteint pour les échantillons irradiés en présence d'air ou sous hypoxie modérée (200 p.p.m. O_2). Par contre, une hypoxie extrême (<10 p.p.m. O_2) produit seulement $4{\cdot}3 \times 10^{-9}$ ruptures/dalton par krad. Lorsque l'on ajoute de la cystéamine aux cellules chauffées et irradiées sous hypoxie extrême, le nombre de ruptures de chaîne d'ADN se rapproche fortement de celui trouvé pour les cellules viables dans les mêmes conditions d'hypoxie. Il en est de même lorsqu'un agent réducteur, comme le $Na_2S_2O_4$, est ajouté aux cellules, avant leur irradiation, afin d'absorber chimiquement l'oxygène. Dans ce cas le nombre de ruptures, pour les cellules chauffées, est proche de celui obtenu dans le cas de cellules viables sous hypoxie extrême. Lorsque les cellules sont chauffées et irradiées, sous air ou sous oxygène, à 200 p.p.m., on observe une radiosensibilité maximale de 8×10^{-9} dalton^{-1} krad^{-1}, c'est-à-dire 10 fois plus que dans le cas de cellules viables irradiées en présence de nitrogène, dans ce cas une radiosensibilité minimale de $0{\cdot}85 \times 10^{-9}$ dalton^{-1} krad^{-1} est observée. Une interprétation raisonable de ces observations est qu'au niveau des sites de ' radicaux libres ' induits par radiation, des réactions chimiques rapides se produisent, résultant en une fixation ou une restauration des dommages produits. En conclusion, l'on peut dire qu'un effet oxygène existe pour les cellules mammifères en ce qui concerne l'induction de ruptures de chaînes uniques d'ADN. Cet effet semble dépendre, en grande partie, d'évènements chimiques liés à l'irradiation.

In Ovarien-Zellen chinesischer Hamster war die Anzahl an DNS-Einzelstrangbrüchen nach Röntgenbestrahlung ca. viermal höher in Proben, die der Luft ausgesetzt waren ($3{,}6 \times 10^{-9}$ dalton^{-1} krad^{-1}), als in solchen, die unter Sauerstoffmangel (<10 p.p.m. O_2) in Stickstoffatmosphäre bestrahlt wurden ($0{,}85 \times 10^{-9}$ dalton^{-1} krad^{-1}).

Wurde der Zellstoffwechsel vor der Bestrahlung durch Wärmebehandlung inhibiert, dann wurden höhere Werte für die Anzahl der Strangbrüche, nämlich 8×10^{-9} dalton^{-1} krad^{-1} in Luft oder unter mäßigem Sauerstoffmangel (200 p.p.m. O_2) und $4{,}3 \times 10^{-9}$ dalton^{-1} krad^{-1} unter extremem Sauerstoffmangel (<10 p.p.m. O_2) gefunden. Anwesenheit von Cysteamin während der Bestrahlung in 10 p.p.m. O_2 von wärmebehandelten Zellen ergab Werte, welche denen von unbehandelten Zellen unter extremem Sauerstoffmangel vergleichbar waren.

Wurde vor der Bestrahlung das Reduktionsmittel Natriumdithionit, $Na_2S_2O_4$, zugefügt, um Sauerstoff chemisch zu binden, dann wurde das Ausmaß an Strangbrüchen in wärmebehandelten Zellen ebenfalls auf Werte von gleicher Größe wie von unbehandelten Zellen unter extremem Sauerstoffmangel vermindert. Wärmebehandelte Zellen, bestrahlt in Luft oder bei 200 p.p.m. O_2 zeigen um den Faktor 10 höhere Empfindlichkeit (8×10^{-9} dalton^{-1} krad^{-1}) gegenüber Röntgenstrahlen als unbehandelte, unter Sauerstoffausschluß bestrahlte Zellen ($0{,}85 \times 10^{-9}$ dalton^{-1} krad^{-1}). Zur Erklärung dieser Resultate wird angenommen, daß an Stellen, wo durch Strahleneinwirkung freie Radikale gebildet wurden, zwei Arten von chemischen Reaktionen stattfinden können: durch die eine wird die Schädigung irreparabel (O_2-Reaktion), durch die andere wird die Schädigung dagegen aufgehoben. Sauerstoff erhöht daher in tierischen Zellen die Wirksamkeit von Röntgenstrahlen bei der Erzeugung von Einzelstrangbrüchen in DNS. Dieser Effekt scheint hauptsächlich von der Natur der strahlenchemischen Ergebnisse abhängig zu sein.

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