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CORRESPONDENCE

Rejoining of DNA single-strand breaks in mammalian cells incubated in buffer or in medium after aerobic or anaerobic X-irradiation

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1. Introduction

The rejoining of DNA single-strand breaks in mammalian cellular DNA has been varified for cells in culture (e.g., Alexander, Dean, Lehmann, Ormerod, Feldschreiber and Serianni 1970, Donlon and Norman 1971) as well as for ascites tumour cells in mice (Matsudaira, Nakagawa and Hishizawa 1969), and mouse testis (Ono and Okada 1974) and dog neurons (Wheeler and Lett 1972) irradiated *in vivo*. It seems likely that the same number of DNA single-strand breaks is produced in all phases of the cell-cycle (Humphrey, Steward and Sedita 1968), although the rejoining rate may vary slightly in the different phases (Lohman 1968). The problem of X-ray-induced breaks produced in the alkali-labile regions of the DNA molecule (normally not measurable, since alkaline treatment ruptures these so-called linker sites) and the repair of such breaks, which may be S-phase dependent, has been discussed by Lett, Sun and Wheeler (1972).

We have investigated the extent to which DNA single-strand breaks produced by X-irradiation under air or N_2 are rejoined after aerobic incubation in either a phosphate (PBS) or a carbonate (CBS) buffered salt solution or complete growth-medium (MEM) in order to detect any differences in the DNA rejoining process(es) of aerobically and anaerobically irradiated mammalian cells.

2. Materials and methods

Exponentially growing Chinese hamster ovary (CHO) cells were used in all experiments. Our conditions for cell culture and ^{14}C-thymidine (TdR)-labelling of cellular DNA have been reported (Roots and Smith 1974). At the end of the 48 hour labelling period, the cells were washed once with culture medium and reincubated in 2 ml MEM for 1 hour before use. Unless the cells were reincubated after the removal of ^{14}C-TdR, the DNA sedimentation profiles sometimes had two peaks. Just before irradiation, the cells attached to a 22 mm diameter glass disc were transferred to another 10×35 mm plastic Petri dish and covered either with 1 ml Dulbecco's (Dulbecco and Vogt 1954) phosphate-buffered salt solution (PBS) at pH 7·3, or in one set of experiments (see table) with 1 ml phosphate-buffered complete culture medium at pH 6·8. The uncovered plastic Petri dish was then placed in a Nylon X-ray chamber (Rockwell and Kallman 1973) resting on ice to prevent rejoining of DNA breaks during irradiation. For most of the experiments (see figure), the atmosphere above the cells was

Gassing conditions	Irradiation in phosphate buffered medium (pH 6·7)			Irradiation in PBS (pH 7·3)		
	$\frac{10^9}{M_n}$ dalton^{-1}		Percentage rejoined by 10 min†	$\frac{10^9}{M_n}$ dalton^{-1}		Percentage rejoined by 10 min
	Without incubation	10 min incubation		Without incubation	10 min incubation	
N_2	36·5	27·2		39·4	29·9	
	36·0	23·0		38·2	29·1	
	39·0	23·8		38·4	26·9	
	37·8	27·1				
	Avg. 37·3	Avg. 25·3	38·4 ± 7·0	Avg. 38·7	Avg. 28·6	30·8 ± 4·3
Air	35·3	21·1		29·8	16·7	
	34·7	17·0		32·5	16·8	
	31·7	20·4		29·1	21·2	
	37·2	19·5				
	Avg. 34·7	Avg. 19·5	53·0 ± 6·4	Avg. 30·5	Avg. 18·2	50·0 ± 8·5

$\frac{10^9}{M_n}$ dalton^{-1}-values for single-stranded DNA of Chinese hamster ovary cells X-irradiated under aerobic or anaerobic gas equilibration in either phosphate-buffered MEM or PBS with or without 10 min post irradiation incubation in MEM

† The percent of DNA single-strand breaks rejoined at the end of a 10 min incubation was calculated based on $1/M_n = 6 \times 10^{-9}$ dalton^{-1} for non-irradiated cells as shown in the figure.

equilibrated with air or N_2 (35 ± 15 p.p.m. O_2) for 50 min before the irradiation; and, for cells under N_2 the effluent gas was monitored for oxygen-content with a Hersch cell (Engelhard Industries Ltd., Sutton, England). However, in another set of experiments (see table), gas equilibration with N_2 was for 50 min before the 40 min X-ray exposure under N_2 as before, but the aerobic samples were equilibrated for 80 min instead of 50 min before the 10 min X-irradiation to give an equal amount of time for the combined gassing-irradiation treatment for both systems. Irradiations were done with a 250 kVp X-ray unit at 15 mA and 0·25 mm Cu plus 1·0 mm Al external filtration. Under these experimental conditions, ferrous sulphate dosimetry gave an average absorbed dose-rate of 362 rad/min; however, the effective dose-rate to the attached cells was determined to be approximately 1·3 times higher, i.e., 470 rad/min due to electron back-scattering from the glass (Roots and Smith 1974). In all cases, aerobic samples were irradiated for 10 min (4·7 krad), whereas anaerobic samples were irradiated for 40 min (18·8 krad) to give approximately the same initial yield of DNA single-strand breaks (estimated from figure 1 in Roots and Smith 1974).

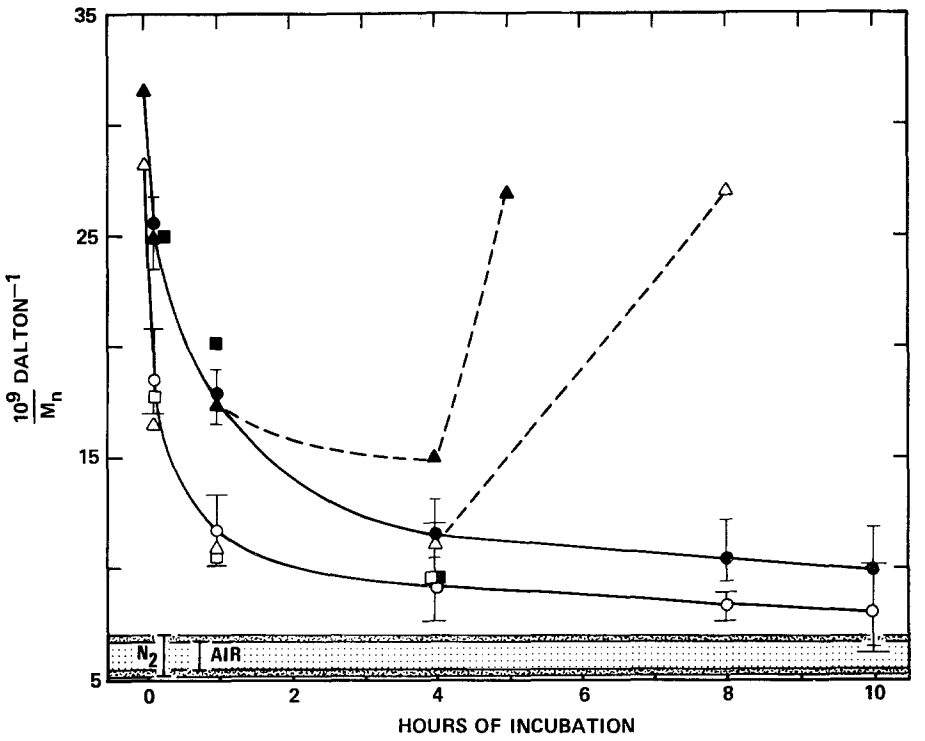
To investigate the capacity of cells to repair DNA strand breaks, samples were incubated in 2 ml of MEM or in PBS, which had a phosphate concentration of $\sim$0·01 M, or sodium-bicarbonate-buffered salt (CBS) solution with salt concentrations identical to those in MEM (i.e., 0·001 M phosphate). Unirradiated control cells and irradiated samples were incubated at 37°C in an air-CO_2 incubator for the desired length of time. For a full description of the alkaline sucrose-gradient centrifugation technique and the method of calculating DNA molecular weights, see Roots and Smith (1974).

3. Results

3.1. *Initial rate of rejoining of DNA single-strand breaks*

In the figure, the reciprocal of M_n, which is proportional to the number of DNA single-strand breaks, has been plotted against various periods of aerobic incubation after X-irradiation in air or N_2. The rejoining process appears to consist of a fast and a slow component. At the end of 10 min of incubation in MEM, $\sim$42 per cent of the DNA strand breaks were rejoined in aerobically irradiated cells as opposed to only $\sim$24 per cent for cells irradiated under N_2, based on an unirradiated control value for $1/M_n$ of 6×10^{-9} dalton^{-1}. Although the initial rejoining kinetics appeared to differ depending on the gassing conditions, the initial rejoining rates were essentially the same for cells incubated in buffer or in complete medium within the two gassing systems.

The effect of PBS or CBS incubations on cell viability (unlabelled, unirradiated cells) was examined for the conditions used in the analysis of strand breaks, i.e., a 50 min period on ice in PBS followed by incubations at 37°C in either MEM, PBS or CBS. Samples of about 200 cells were plated in 15×60 mm plastic Petri dishes. Three hours were allowed for cellular attachment before starting the experiment. The results of two separate experiments showed a reduction in the survival of cells incubated at 37°C in buffer versus MEM. For example, relative to 100 per cent survival in MEM there was a reduction in survival in PBS of about 7, 45 and 90 per cent after 1, 4 and 8 hours, respectively. This means that irreversible cell damage occurred after a 1 hour incubation at



Rejoining rate of DNA single-strand breaks after MEM (air, o; N_2, ●), PBS (air, △; N_2, ▲) or CBS (air, □; N_2, ■) incubations at 37°C in an air-CO_2 incubator for various lengths of time after aerobic (4·7 krad) or anaerobic (18·8 krad) X-irradiations in PBS. A gas equilibration time of 50 min was used, followed by the 40 or 10 min irradiation for hypoxic and aerobic samples, respectively. Each point is the average of two or more experiments. The error bars represent the range in data for MEM incubations. The number of DNA breaks is directly proportional to $1/M_n$ which is plotted *versus* incubation time. The solid lines represent rejoining kinetics of MEM incubated cells while the dashed lines represent PBS incubated samples. The shaded area represents the range of $1/M_n$-values for unirradiated MEM incubated cells.

37°C in PBS (a total exposure to PBS of 1 hour 50 min) even though buffer-induced DNA damage was not apparent under these conditions, even for irradiated cells (see figure). Similar results were obtained for CBS. On further incubation, however, buffer-induced DNA breakdown did become apparent for both irradiated (see figure) and unirradiated cells (data not shown).

We were concerned that these apparent differences in the initial rate of repair for cells irradiated in air versus N_2 might be the result of a selective detrimental effect of holding and/or irradiating cells in buffer under N_2. To evaluate these parameters, the aerobic samples were equilibrated for 80 min before the 10 min irradiation to give a combined gassing and irradiation time of 90 min for both air and N_2. One set of samples was equilibrated, irradiated and incubated for 10 min in growth medium while the other set was equilibrated and irradiated in PBS before the 10 min incubation in MEM. The reciprocal values of M_n calculated from these experiments are given in the table. From these values, the extent of rejoining after 10 min incubation in MEM was calculated to be

50–53 per cent for aerobic cells and that for cells irradiated under N_2 was 31–38 per cent, as shown in the table. These results indicate that the rejoining kinetics are somewhat slower for cells irradiated under N_2 versus air, whether irradiated in buffer or MEM.

3.2. *Final rate of rejoining of DNA single-strand breaks*

The figure shows that rejoining continues at a slow rate in MEM incubated cells up to at least 10 hours after irradiation. By 10 hours, about 90 per cent of the initial yield of DNA single-strand breaks were repaired by the cells that had been irradiated in air. The final and slower rate of rejoining evident in MEM incubated cells could not be followed in buffer-incubated cells owing to the deleterious effect on DNA (i.e., a decrease in sedimentation coefficient) of prolonged incubations in buffer which appeared earlier for hypoxic irradiations (by 4 to 5 hours) than for aerobic irradiations (by ~ 8 hours) (dashed lines in the figure).

4. Discussion

4.1. *Rejoining of DNA single-strand breaks after X-irradiation in air*

The extent of rejoining within the first 10 min of incubation was about 50 per cent for aerobic irradiations in phosphate-buffered MEM or PBS (see table). The early, rapid rejoining process of DNA single-strand breaks in human lymphocytes has been reported by Donlon and Norman (1971) to be linear with incubation time, showing that the rejoining rate is constant initially. From their data we have calculated that about 27 per cent of the breaks were repaired in 10 min at 37°C for irradiations below 40 krad based on the authors' estimated break yield of 10 breaks per cell per rad.

Prolonged incubations in MEM resulted in additional rejoining at a much slower rate. After 10 hours of incubation under our conditions, the extent of rejoining was ~ 90 per cent (see figure). The existence of a slow process for the repair of DNA lesions in mammalian cells after ionizing radiation has been suggested by McBurney, Graham and Whitmore (1972) from their alkaline sucrose-gradient studies on DNA single-strand breaks, and by others (Hill 1967, Spiegler and Norman 1970 a, Clarkson and Evans 1972) from studies on unscheduled DNA synthesis.

4.2. *Rejoining of DNA single-strand breaks after anaerobic X-irradiation*

The extent of rejoining within the first 10 min of incubation was about 35 per cent for anaerobic irradiations in phosphate buffered MEM or PBS (see table). Thus, it appears that the repair of DNA single-strand breaks is slower in cells that were irradiated under anoxia as compared with cells irradiated in air. Johansen, Boyce and Brustad (1973) and Johansen (1975). using a λ-phage infected *E. coli* system, also found an impairment of the rejoining of λ-phage DNA single-strand breaks after anaerobic irradiations. In their case, however, anoxia was maintained during the post-irradiation incubation. Similarly, Modig, Edgren and Révész (1974) have reported a reduced rate of rejoining for hypoxically irradiated Chinese hamster cells, especially for hypoxic incubations. This slower initial rejoining rate for mammalian cells after anaerobic irradiation

could be due to energy depletion in the hypoxic cells; this depletion would be enhanced by continued incubation under hypoxia. The importance of the availability of energy for the rejoining process has been discussed by Matsudaira, Furuno and Otsuka (1970).

The slower rate of rejoining could also be due to the production of a different type of lesion in DNA by anaerobic irradiation, or one type of lesion could be produced in different amounts under aerobic and anaerobic conditions. In this connection, Setlow and Carrier (1973) have reported that for *in vitro* γ-irradiated bacterial DNA there are about 2 endonuclease sensitive sites per single-strand break for anoxic irradiations, but only about 1·2 sites per single-strand break for aerobic irradiations, which suggests that a higher yield of a particular lesion occurred in the anoxic system.

The preferential production of alkali-labile lesions in cells irradiated under N_2 versus air has been pointed out by Lennartz, Coquerelle and Hagen (1973). They reported increases of 50 and 28 per cent in the DNA single-strand break yield of thymocytes irradiated with 10 MeV electrons in N_2 and air, respectively, if the isolated DNA was denatured in alkali rather than by a heat-formaldehyde treatment. It is not known whether X-ray-induced, alkali-labile lesions disappear completely after incubation. The data from prolonged incubations in the figure might indicate that they persist to some degree after incubation.

The radiation-induced incorporation of nucleotides into non-replicating DNA observed in unscheduled DNA-synthesis studies (Hill 1967, Spiegler and Norman 1969, 1970 a, b, Clarkson and Evans 1972, Shaeffer and Merz 1971), and in repair-replication studies (Painter and Young 1972, Brent and Wheatley 1971, Fox and Fox 1973) probably reflects the same type(s) of rejoining process (es) demonstrable with alkaline sucrose-gradient analyses. It is possible that the rejoining of DNA single-strand breaks that one observes may also include the excision repair of DNA base damage.

4.3. *Are there two pathways for the repair of DNA single-strand breaks?*

In bacteria, the rejoining of X-ray-induced DNA single-strand breaks can be divided into two separate pathways both on the basis of physiological and genetical requirements. Thus, better than 90 per cent of the breaks are repaired by a system that can proceed to completion in the absence of growth medium and that requires DNA polymerase I. A second pathway which can only repair a few breaks, shows an absolute requirement for complete growth medium and the functioning of *rec* and *exr* genes (Town, Smith and Kaplan 1973).

We have attempted to determine if there are two physiologically distinct repair pathways in mammalian cells since mutant mammalian cells deficient in these two proposed pathways are not available. The data in the figure certainly suggest that most of the X-ray-induced breaks can be rejoined in the absence of growth medium.

We have been unable to demonstrate the existence of a growth medium dependent repair process in mammalian cells. In the absence of appropriate mutants deficient in this process we could only try to compare the extent of repair in buffer versus medium and in buffer plus extra incubation in medium, as had been done with bacteria. Unfortunately, such experiments were inconclusive for mammalian cells, because of the buffer-induced breakdown of DNA

(see figure) and because of the insensitivity of the gradient technique for distinguishing between small numbers of unrepaired strand breaks (see §4.4). We are presently investigating another technique for measuring DNA single-strand breaks (Ahnström and Edvardsson 1974) that may offer more sensitivity, so that the question of the presence of a growth-medium-dependent pathway may be resolved.

4.4. *General considerations and summary*

With an M_n value for the unirradiated control DNA of $\sim 1{\cdot}7 \times 10^8$ daltons (see figure), it is barely possible to detect, with the alkaline sucrose-gradient technique, a DNA profile shift after 500 rad. With a breakage efficiency of 20 single-strand breaks per cell per rad (Roots and Smith 1974) and taking the DNA content per cell to be $\sim 10^{-11}$ g, 500 rad produces $\sim 10^4$ single-strand breaks per cell or about 0·3 breaks per unit of control size DNA. This is the sensitivity of the gradient technique. The rejoining of about 90 per cent of the initial number of breaks after 4·7 krad in air, i.e., about 10^5 single-strand breaks per cell, would still leave $\sim 10^4$ single-strand breaks per cell unrepaired, which again is close to the resolution of the technique. Thus, currently the lack of sensitivity of the alkaline sucrose-gradient technique for mammalian cells requires the use of supra-lethal doses of ionizing radiation. The biological relevance of such data, therefore, must be questioned.

It is also important to remember that the disappearance of DNA single-strand breaks does not necessarily mean functional restoration of the irradiated DNA. In this connection, Schaeffer and Merz (1971) reported that there was no correlation between the amount of unscheduled DNA synthesis and the D_0 values for the mammalian cell lines they studied; and, in repair replication studies, Fox and Fox (1973) found that 3 to 4 times more nucleotides were inserted per single-strand break in an X-ray-sensitive lymphoma cell-line as compared with a more resistant cell-line. The importance of the quality of the rejoining process is further suggested by the fact that a radiation-damaged mammalian cell usually proceeds through one or more mitoses before lethality is manifested, possibly due in part to translational inability brought about by misrepair.

In summary, using the sucrose-gradient centrifugation technique, the capacity of Chinese hamster ovary cells to rejoin DNA single-strand breaks when incubated in either a buffered salt solution or culture medium has been studied after X-irradiation under air and N_2. The initial rate of rejoining was about the same for cells incubated in buffered salt solution or in growth-medium. After doses which resulted in approximately the same initial yield of breaks, i.e., 4·7 and 18·8 krad for air and N_2, respectively, the rate of rejoining of DNA single-strand breaks within the first 10 min of aerobic incubation was somewhat slower for cells irradiated under N_2 versus air. After the initial rapid rate of rejoining, a slower rejoining rate was discernible for medium-incubated cells. This slow process could not be studied for cells incubated in buffered salt solution due to buffer-induced DNA breakdown.

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