Technical Communication

R.J. Hodgkiss[a]; B. Vojnovic[a]; M. Woodcock[a]; B.D. Michael[a]

[a] Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, U.K.

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A single-shot rapid-mixing device for radiobiological studies with mammalian cells

R. J. HODGKISS†, B. VOJNOVIC, M. WOODCOCK
and B. D. MICHAEL

Gray Laboratory of the Cancer Research Campaign, P.O. Box 100,
Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, U.K.

A single-shot rapid-mixing device is described for the rapid addition of solutions of radiation-modifying agents, to cell suspensions, at well-defined times relative to a pulse of radiation. The liquid injection system could be used to initiate or quench a wide range of chemical or biochemical reactions. The rapid-mixing device is based on a syringe driven by a stepper motor and can inject up to $2\,cm^3$ liquid in $<100\,ms$. The radiation source, a 4 MV Van de Graaff accelerator, provides an electron beam which is deflected from the beam dump on to the sample in two stages, providing a 10 ms radiation pulse. A digital delay circuit defines the interval between mixing and irradiation. The apparatus has been designed to study the kinetics of processes that occur over a time range extending from about 0·1 s to some minutes. It bridges the gap between the ranges available with conventional fast-mixing and those using standard X- or γ-irradiation methods. The time resolution of the technique has been examined by following the timecourse of radiosensitization by oxygen in mammalian cells. The timecourse of radioprotection of aerobic mammalian cells by dithiothreitol has been measured using the technique.

1. Introduction

The lethal effects of radiation on living cells are thought to be mediated by free-radical lesions in critical target molecules; such free-radical lesions may then be fixed to lethal damage or repaired to non-lethal configurations by reaction with dose-modifying chemicals (e.g. Alexander and Charlesby 1955, Howard-Flanders and Alper 1957, Koch and Howell 1981, Willson 1983, Hodgkiss and Middleton 1983, Hodgkiss and Stratford 1988). Time-resolved experiments carried out in bacteria and mammalian cells have demonstrated that the lifetime of the initial free-radical damage is limited to several milliseconds following irradiation (e.g. Michael and Harrop 1980), although we have recently reported evidence for a longer-lived component lasting for at least several tens of milliseconds (Hodgkiss *et al.* 1987).

A number of fast-mixing techniques have been used to study the interaction between radiation and radiation-modifying agents in cells. Rapid movement of bacterial cells between two gaseous environments has been used to show that the lifetime of oxygen-modifiable radiation damage is less than 10 ms (Howard-Flanders and Moore 1958). An alternative method of modifying the gaseous environment is to expose cell monolayers to an explosion of a dose-modifying gas,

†Correspondence and reprint requests to Dr R. J. Hodgkiss.

usually oxygen, shortly before or after a brief (5 ns) pulse of radiation (e.g. Michael *et al.* 1973, 1978, Watts *et al.* 1978, Michael and Harrop 1980, Michael *et al.* 1986). This technique allows a wide range of intervals between irradiation and exposure to gas to be explored with a time resolution of about $100\,\mu s$; however, with this system it is not possible to rapidly mix non-gaseous dose-modifying agents with cells. Similar experiments have also been carried out where two radiation pulses are used, the first of which radiochemically depletes the intracellular oxygen (Ling *et al.* 1978). A comparison of the cellular response to single radiation pulses with that for two pulses separated by a defined interval, enabled the diffusion kinetics of oxygen into cells to be inferred.

In other experiments a cell suspension is made to flow rapidly through narrow silica or glass tubing and mixed rapidly with a solution of radiation-modifying agent at a junction, before or after passing through a continuous beam of radiation (e.g. Adams *et al.* 1968, Shenoy *et al.* 1975, Whillans and Hunt 1978, Whillans 1982, Whillans and Hunt 1982, Watts *et al.* 1983, Hodgkiss *et al.* 1984, Kandaiya 1986, Hodgkiss *et al.* 1987). A similar apparatus was used to rapidly lyse suspensions of *E. coli* at short times after irradiation (Fox *et al.* 1976). Although continuous-flow rapid mixing allows any water-soluble substance to be mixed with the cells, the time-scale of mixing limits the resolution of the experiments to about 2 ms. A second problem with this technique is that the maximum time between mixing and irradiation that can be examined is limited by the maximum length of glass tubing that can be conveniently accommodated between the radiation source and the mixing point. Although this could be made quite long, in principle, by the inclusion of loops or spirals of tubing, the volume of cell suspension required to fill the tubing, and therefore the volume required to produce each sample, increases in proportion to the length of tubing. Although the time range can be extended by slowing down the liquid flow, the extension so obtained is limited by the requirement to maintain a certain minimum velocity to ensure turbulent rather than laminar flow.

Other rapid-mixing methods which have been applied to radiobiology have included injection of one solution from a syringe, driven by a falling weight or by gas pressure (Dewey and Michael 1965, Eccleston *et al.* 1980). Although fast mixing times (0·4 and 50 ms respectively) have been reported, these have been with rather small volumes ($0·02–0·05\,cm^3$). Similarly, an electrically operated solenoid has been used to drive a syringe, but only $0·15–0·25\,cm^3$ was delivered in 150–250 ms (Boye *et al.* 1974, Sapora *et al.* 1975, Stratford *et al.* 1977).

In this paper we describe a single-shot rapid-mixing device that can mix up to $2\,cm^3$ of liquid with cell suspensions in <100 ms. The apparatus is able to cover the time range from *ca.* 100 ms to many minutes between irradiation and mixing. With this apparatus the timescale of interaction between dose-modifying agents and radiation can be explored over longer timescales than is possible using conventional liquid-flow rapid-mixing systems. This should, for example, allow the study of radioprotection by charged thiols, which enter cells slowly (Whillans and Hunt 1978). The time scale of mixing is illustrated using oxygen, and an example of a time-course after mixing is given regarding thiol radioprotection.

2. **Materials and methods**

2.1. *Electron pulse generation*

The pulsed radiation source for these experiments was the Gray Laboratory's 4 MV Van de Graaff accelerator. The vertical accelerating tube is coupled to a 90°

Penner type achromatic magnetic deflector (Penner 1961), resulting in a horizontal beam. Electrons are generated by a Pierce type of gun structure which can be biased to inject either a DC electron beam (1–2 mA current) or a pulsed beam. The present application required electron pulses of about $10\,\mu C$ charge, which was beyond the normal pulse capability of the Van de Graaff (about $2\,\mu C$). Pulses of $10\,\mu C$ were generated by operating the accelerator in the continuous beam (DC) mode with the beam resting on a water-cooled dump (figure 1) and then being deflected momentarily by the magnet system into the mixing apparatus. Limitations in the existing beam deflection magnets (hysteresis, inductance) and in their associated power supplies (maximum rate of current rise, control switching transients) prevented production of pulses with well-defined rise and fall times; however, a low-inductance $5°$ deflection coil placed upstream of the $90°$ deflectors could be readily driven to produce 'pulses' of $<200\,\mu s$ rise time by sweeping the beam across the entrance slit of the $90°$ magnet chamber. A two-step deflection sequence was employed, to keep the power deposited by the beam (several kilowatts) onto the chamber entrance, within acceptable limits. At the start of the sequence the $5°$ deflector is energized, shortly followed by the energization of the $90°$ deflector. The
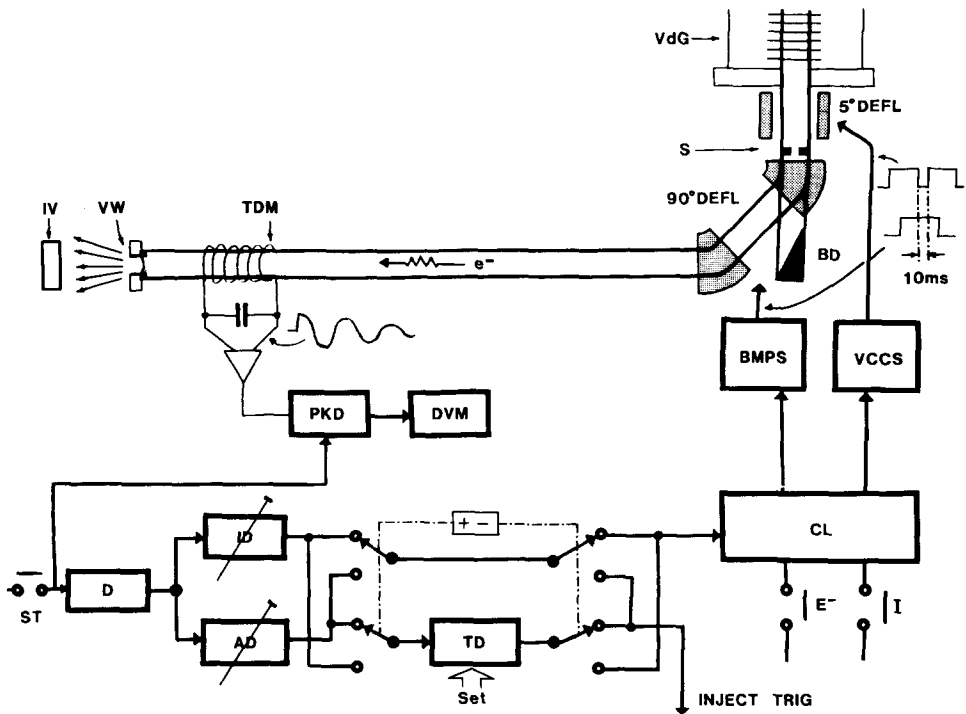

Figure 1. Arrangement used to generate and monitor electron pulses produced by magnetically deflecting a continuous electron beam generated by a Van de Graaff generator. AD, accelerator delay; BD, beam dump; BMPS, bending magnet power supply; CL, control logic; D, reset delay; DVM, digital voltmeter; E^-, manual trigger of electron pulse; I, manual trigger of injection; ID, injection delay; IV, irradiation vessel; PKD, peak detector; S, magnet chamber entrance slit; ST, sequence trigger; TD, 0–9999·99 s delay; $+ -$, e^- before/after injection; TDM, toroidal dose monitor; VCCS, voltage-controlled current source; VdG, Van de Graaff accelerator; VW, vacuum window.

5° deflector is then de-energized for *ca.* 10 ms, allowing the beam on to the sample. After the radiation pulse the 90° deflector and then the 5° deflector are switched off, returning the beam to the dump. The beam is thus present for <0·5 s on the magnet chamber entrance and a clean pulse is obtained, with negligible 'dark' current (<1 nC per pulse). The electron pulse thus produced is passed through a vacuum window (4 μm thick tantalum) and is used to irradiate the irradiation vessel.

The charge per pulse is monitored using an inductive monitor (Vojnovic 1987), placed close to the beam line. This type of monitor is largely insensitive to pulse shape or duration as the electron pulse triggers a damped oscillation in a resonant circuit. The peak amplitude of this waveform is measured using a precision-rectifier peak-detector, coupled to a digital voltmeter. A logic signal control sequence ensures that the measurement system is reset before each pulse, and that the peak detector output is digitally held for an indefinite period in the voltmeter. A $4\frac{1}{2}$ digit instrument is employed, providing a resolution of 1 nC for charge measurements up to 20 μC. Alternative methods of beam monitoring such as a secondary emission chamber could also be used. A secondary emission monitor would need to be moved out of the beam line to prevent damage when the accelerator used in this work delivers high continuous beam currents, required for other applications.

2.2. *Sequence generator*

A simple digital sequence generator, shown in figure 1, controls the timing of events within the instruments. At the start of the sequence all the delays are reset, and two compensating delay circuits are triggered, one of which compensates for the delay introduced by the electron pulse generation, the other for the delay introduced by the injection system. Appropriate adjustment of these delays ensures that the electron pulse occurs half-way along the liquid injection ramp when a nominally zero time delay is selected. A programmable, six-decade time delay circuit can be switched in so that the injection occurs either before or after the electron pulse, with time intervals up to 10^4 s in 10 ms increments. In addition, either the electron pulse or the injection shot can be triggered manually and independently of the sequence. Two 2-decade counters keep track of the number of electron pulse and injection shot trigger events occurring during an experiment.

The modifications to the accelerator consist only of the addition of a small 5° deflection coil on the accelerator beam line, and timing circuits to energize this coil at the correct time relative to energization of the main deflection coils. When not energized the 5° coil has no effect on the operation or performance of the accelerator for other purposes.

2.3. *Injection and mixing system*

Various types of injection system were considered, including hydraulic (e.g. Whillans 1982) and pneumatic, which could inject a large solution volume in a short time. Although compressed air and hydraulic devices are available to do this, they generally do not offer the flexibility provided by electrically operated drivers. A high power stepper motor drive (Unimatic Engineering Ltd) was available for use with a conventional rapid-mix instrument and we adapted this for the present purpose.

The liquid injection apparatus consists of a sturdy framework which holds a Luer-tipped Pyrex glass syringe driven by a low inertia ram. This ram is activated by a stepper motor driven low friction ball screw arrangement as shown in figure 2.
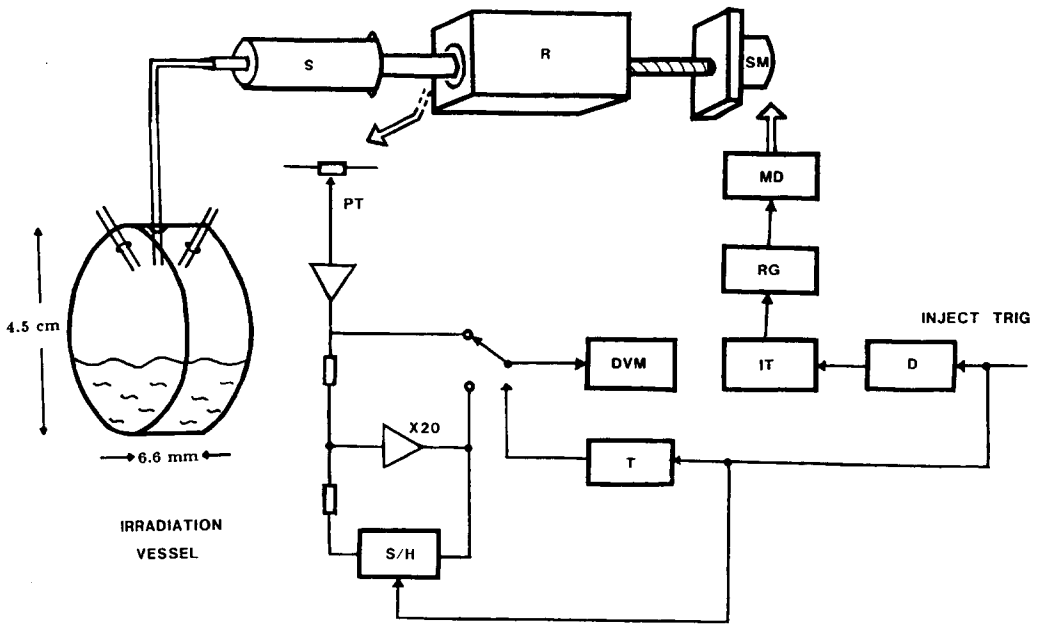
Figure 2. Arrangement used to inject liquid shot into the irradiation vessel from a stepper motor-driven syringe. The syringe volume and the volume changes are monitored on a digital voltmeter using a resistive position transducer. D, 500 ms delay; DVM, digital voltmeter; IT, injection time set; MD, motor driver; PT, position transducer; R, ram; RG, ramp generator; S, syringe; S/H, sample and hold; SM, four-phase stepper motor; T, 3 s timer; X20, injected volume amplifier.

A fairly wide-bore syringe is employed (50 cm^3 volume, 2·5 cm diameter) (Rocket of London, Watford) to minimize the linear travel required for injection. Although a small-diameter, long-travel syringe could have been employed to minimize the forces required during injection, this would have required a high rotation speed of the motor. Stepper motors tend to provide high pull-in torques at relatively low rotational speeds, and their torque drops sharply above the maximum design stepping rate which corresponds to speeds of the order of 200–300 rpm. A long-barrelled syringe would thus have required a coarser ball-screw and consequently greater problems with backlash. In addition, a wide-bore syringe can be loaded with a greater volume of solution. The syringe employed is thus a reasonable compromise between ease of availability and use of standard components on the one hand, and the required level and flexibility of performance on the other. About 2 cm^3 of solution is injected, corresponding to 3·5 mm travel, in *ca*. 80 ms.

The stepper motor employed is a four-phase, 200 steps/revolution unit (Unimatic Engineering Ltd). For this application it was deemed acceptable to overdrive the motor by *ca*. 300 per cent to generate the required torque, as the duty cycle is very low; the motor windings are only energized for a short time before and after the injection cycle, thus minimizing power dissipation. The motor velocity is increased from zero to maximum during the 80 ms injection time, in order to prevent unsynchronized operation. The motor current, acceleration and loading were empirically adjusted for optimum settling of the syringe barrel after the shot.

The position of the barrel, and hence solution volume, was sensed by a resistive position transducer, the output of which is applied to a digital voltmeter. In addition to displaying the solution volume it was considered desirable to monitor the actual volume delivered to the irradiation cell. This is achieved by an auto-zero circuit which is activated a few hundred milliseconds before the injection phase, and which provides a display for some 3 s of the 'new' syringe position to × 10 higher resolution than the quiescent display. This arrangement is shown in the lower half of figure 2.

The irradiation vessel is a cylindrical silica container 4·5 cm in diameter, 8 cm^3 volume, with a 0·5 mm thick irradiation window facing the beam (Plastic Laminated Glassware, London). The mean internal depth of the vessels at 90° to the radiation beam was 6·6 mm. As shown in figure 2 the mixing vessel has three holes on the cylindrical surface. Injection is via the central hole and proceeds with some force, so that the liquid content at the bottom of the mixing vessel is violently driven up into its entire volume, thus ensuring efficient mixing.

2.4. *Sample preparation*

V79 379A Chinese hamster cells were maintained as exponentially growing suspension cultures in Eagle's minimal essential medium (MEM) with 7·5 per cent fetal calf serum (fcs) (Flow Labs). For irradiation experiments, cells were centrifuged, washed by resuspending the pellet in Earle's salts solution (ESS) and centrifuging, before finally resuspending the cells in ESS at 2×10^6 cm^{-3}. The cell suspension was stirred under air + 5 per cent CO_2 or nitrogen + 5 per cent CO_2 (< 10 ppm O_2, British Oxygen Company) at 20°C in a conical flask, with a side-arm and fitted with a drechsel head, until required. No loss of cell viability was seen for at least 2 h under these conditions. A glass syringe, purged with the appropriate gas, was used to transfer aliquots of the cell suspension (2 cm^3) to a pre-gassed silica irradiation vessel. With this volume of cell suspension the maximum sample depth was 1 cm. The cell suspension was then further equilibrated with air or deoxygenated, by passing air + 5 per cent CO_2 or nitrogen + 5 per cent CO_2 as appropriate via a 16-gauge needle ∼0·5 cm from the surface at ∼1 l min^{-1} over the surface of the cell suspension, for > 7 min. The gas flow was set at a level sufficient to disturb the surface of the liquid, thereby stirring it and ensuring equilibration with the gas phase. The solution to be mixed with the cells was aerated or deoxygenated by bubbling vigorously with the appropriate gas in a conical flask, with a side-arm and fitted with a drechsel head, for > 20 min before being drawn up into a glass syringe which was purged with the appropriate gas. Following irradiation, the cells were centrifuged, resuspended in fresh medium, and known numbers plated on plastic 5 cm Petri dishes in MEM + 10 per cent fcs for a 7-day colony-forming assay.

2.5. *Dosimetry*

At the start of each experiment the Van de Graaff beam current and toroidal dose monitor were calibrated by Fricke dosimetry carried out in the silica irradiation vessels to be used for irradiating cell suspensions. The radiation dose delivered to the vessels was carried by adjusting the beam current. The depth–dose curve was measured using a thin sandwich-type ionization chamber (Model 631, D. A. Pitman Ltd, Weybridge) and layers of absorbing material. The dose distribution across the diameter of the irradiation vessel was monitored by irradiation of glass slides placed in the appropriate position. The resultant darkening of the

slide, corresponding to the distribution of radiation dose, was measured using a Joyce–Loebl scanning densitometer.

3. Results

In these experiments electrons of 3·5 MeV energy are normally used, with about 20 per cent variation in the dose deposited over the depth of liquid within the vessel. The thickness of the electron entrance window of the vessel and the depth of solution were arranged such that the cell suspension was entirely in the build-up region of the depth–dose curve, the dose reaching a maximum at the back of the vessel and ensuring adequate penetration of electrons into the corners of the vessel. Depth–dose measurements showed a maximum at about 75 mm equivalent depth in water for 3·5 MeV electrons in broad field geometry. The vessel is placed *ca.* 60 cm from the vacuum window; the radiation field, after suitable focusing of the beam and its inevitable scattering in air, is uniform to within 5 per cent over the diameter of the irradiation vessel.

The timecourse of mixing a hypoxic cell suspension with aerated ESS is shown in figure 3. As oxygen penetrates the cells they become more sensitive to radiation and therefore the surviving fraction obtained for a fixed dose (22 Gy) decreases. Most of the resultant change in surviving fraction is complete by 100 ms after the start of mixing. In some experiments (data not shown) the liquid ejected from the mixing syringe was divided into two streams by a T-piece outlet nozzle. This did not significantly change the timecourse of radiosensitization of hypoxic cells by the aerated medium.
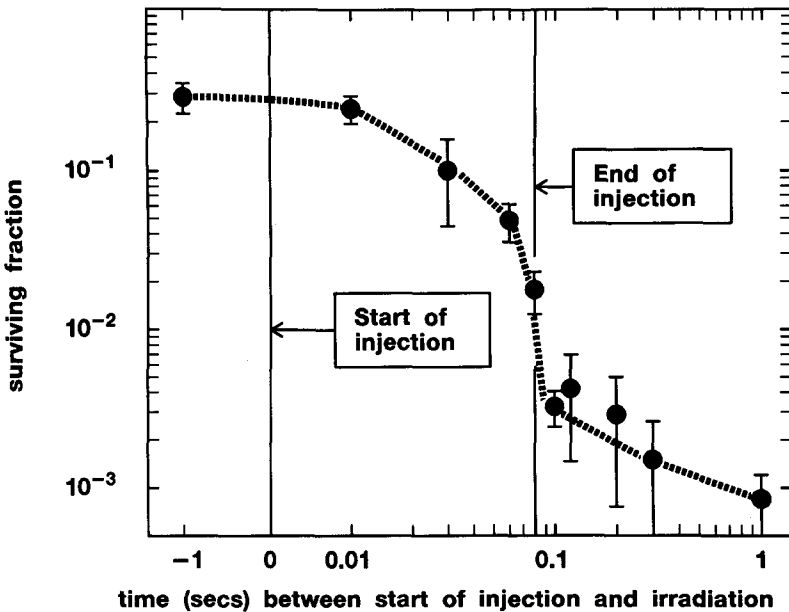
Figure 3.   Timecourse of radiosensitization of hypoxic cells, irradiated with 22 Gy 3·5 MeV electrons, by oxygen. Vertical lines indicate the start (0 s) and end (0·08 s) of the liquid injection. Each point represents the mean and standard error of three replicate experiments.

Technical communication

In figure 4 the timecourse of radioprotection of aerobic cells by the thiol radioprotector dithiothreitol (DTT) is shown for doses of 22 Gy. Auto-oxidation of the DTT in the mixing syringe was prevented by making the solution hypoxic before loading the syringe. The effect of addition of the DTT (20 mmol dm^{-3} after mixing) is substantially complete by 1 s after the start of mixing. Measurements of oxygen concentrations with an oxygen electrode show that auto-oxidation, with 20 mmol dm^{-3} DTT in ESS at 20°C, is too slow to make a significant reduction in the amount of oxygen in the irradiation vessel over the timecourse of these experiments (data not shown).

4. Discussion

In these experiments irradiation and mixing were carried out in ESS rather than full growth medium with serum. Despite the absence of serum, small amounts of protein carried over with the cells did lead, on occasion, to foaming as a result of the vigorous liquid injection. However, although use of serum would probably exacerbate this problem, it should be possible to use MEM without serum. ESS was used in these experiments because of our interest in depleting cellular thiols in later experiments, and to enable comparison with published work on the liquid-flow rapid-mix system; the presence of cysteine in MEM could reduce the efficiency of our protocols for depletion of cellular thiols.

Oxygen (130 µmol dm^{-3} after mixing) has been shown to penetrate and fully sensitize mammalian cells to radiation within 2 ms of contact (Watts *et al.* 1978, Whillans and Hunt 1982, Hodgkiss *et al.* 1984). Using a low concentration of oxygen, Ling *et al.* also found that a significant amount of oxygen diffused to the
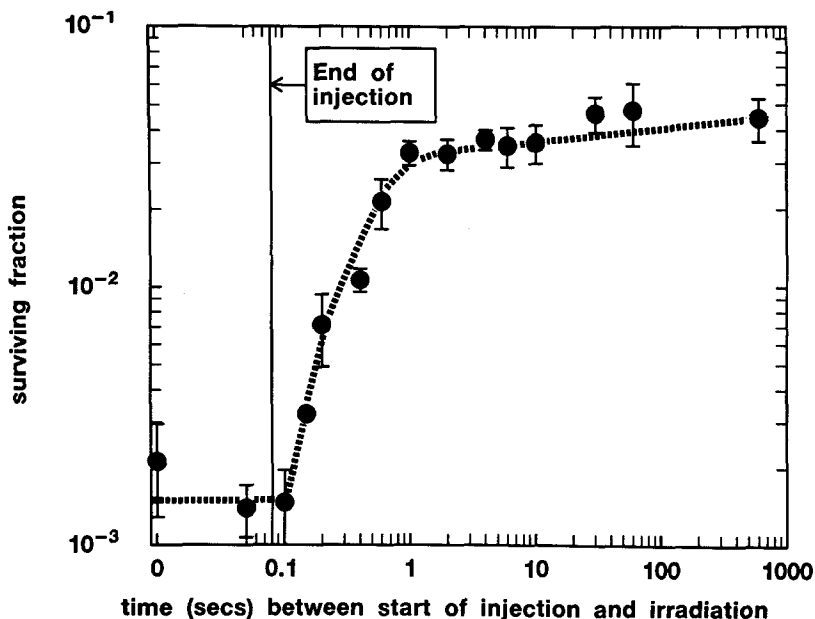
Figure 4.   Timecourse of radioprotection of aerobic cells, irradiated with 22 Gy 3·5 MeV electrons, by DTT. A vertical line at 0·08 s indicates the end of the liquid injection. Each point represents the mean and standard error of three replicate experiments.

critical target sites in mammalian cells within 3 ms. In the present work the timecourse of radiosensitization by addition of aerated ESS therefore mainly reflects the timecourse of mixing rather than that of penetration of oxygen into the cells. It can be seen that the timecourse of mixing is mainly determined by the timecourse of injection of the aerated ESS. The radiation pulse is also relatively short compared with the 80 ms required to inject 2 cm^3 liquid into the irradiation vessel. Nevertheless, a compromise injection time of this order is reasonable to prevent excessive frothing and spillage of the liquid in the irradiation vessel, as well as potential damage to the system in case of barrel stiction. The time range below 100 ms is accessible with 'conventional' liquid-flow rapid-mixing techniques. An attempt to improve the efficiency of mixing within the irradiation vessel by using two jets did not change the timecourse of radiosensitization. However, it seems likely that better time resolution could be achieved, if required, by reducing the volume of liquid injected (e.g. 1 cm^3 could be injected in 40 ms).

The timecourse of radioprotection by DTT is rather slower than the timecourse of radiosensitization by oxygen, with full radioprotection developing over 1 s after mixing. This may reflect the increased molecular size and probably reduced lipophilicity of DTT compared with oxygen. Lipophilicity has been shown to have a major effect on the rate of penetration of neutral nitroimidazole radiosensitizers into cells (Watts *et al.* 1983). Although mixing hypoxic DTT with aerobic cells will initially reduce the oxygen content of the cell suspension from *ca.* $260 \, \mu\text{mol dm}^{-3}$ to $130 \, \mu\text{mol dm}^{-3}$ until restored by the gas stream, there is no detectable change in radiosensitivity of the cells as a result of this. However, with the lower amount of oxygen the radioprotective effect of DTT may be enhanced (e.g. Denekamp *et al.* 1988).

The data in figure 4 illustrate the use of the present technique for studying the time scale of uptake of modifying agents. Ongoing studies at our laboratory use this method to compare the penetration kinetics of various radioprotectors.

Acknowledgements

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