Biochemical Pharmacology, Vol. 40, No. 3, pp. 621–627, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

A COMPARISON OF THE TECHNIQUES OF ALKALINE FILTER ELUTION AND ALKALINE SUCROSE SEDIMENTATION USED TO ASSESS DNA DAMAGE INDUCED BY 2-NITROIMIDAZOLES

PAUL W. CRUMP, E. MARTIN FIELDEN, TERRY J. JENNER and PETER O'NEILL*
MRC Radiobiology Unit, Chilton, Didcot, Oxfordshire OX11 0RD, U.K.

(*Received* 18 *January* 1990; *accepted* 16 *March* 1990)

Abstract—The induction of DNA single-strand breaks (DNA-SSB) in Chinese hamster V79-379A lung fibroblasts by misonidazole or RSU-1069 under both aerobic and hypoxic conditions was examined following incubations for up to 4 hr at 310°K using the technique of alkaline filter elution. Incubation with RSU-1069 induces DNA-SSB under both hypoxic and aerobic conditions, whereas incubation with misonidazole induces DNA-SSB only under hypoxia. The yield of breaks is dependent on both agent concentration and contact time. Following identical treatments with these agents, the yield of DNA-SSB (expressed in radiation dose equivalents) determined by alkaline filter elution is about one order of magnitude less than that previously determined by alkaline sucrose gradient sedimentation. In contrast to radiation induced DNA-SSB, alkaline elution is less sensitive than alkaline sucrose gradient sedimentation when determining DNA-SSB induced by RSU-1069 and misonidazole. During the filter elution assay, either increasing cell lysis from 2 to 4 hr, the pH of the lysing buffer from pH 8.7 to 12.5 or the elution buffer from pH 12.2 to 12.5 does not significantly effect the yield of DNA-SSB. Increasing the pH of the lysing or elution buffers to >pH 13 however results in considerable degradation of the DNA, whereby 50–85% of the total DNA passes through the filter with the lysing solution. This effect was similar for DNA from both control and chemically insulted cells. In conclusion, it is apparent that incubation with these agents results in the induction of DNA damage which is expressed as a DNA-SSB only after prolonged treatment under alkaline conditions. Further, the use of alkaline elution to study DNA-SSB damage induced chemically must be treated with caution in the light of these findings.

Misonidazole (1-(2-nitro-1-imidazoyl)-3-methoxy-2-propanol) and RSU-1069 (1-(2-nitro-1-imidazoyl)-3-(1-aziridinyl)-2-propanol) are preferentially cytotoxic towards hypoxic mammalian cells [1–3]. This is believed to be due to the interaction of reactive species, formed on bioreduction of the agents, with DNA under hypoxic conditions [4–6]. Under aerobic conditions, oxygen is thought to compete or interfere with bioreductive activation of these agents via futile cycling [7]. Misonidazole has been shown to induce DNA single strand breaks (DNA-SSB) in mammalian cells under hypoxic but not aerobic conditions [8–9]. It has been demonstrated that RSU-1069 induces both DNA single and double strand-breaks (DNA-SSB or DNA-DSB) under aerobic or hypoxic conditions [9] and also DNA cross-links under hypoxia [10]. The extent of DNA damage induced by RSU-1069 is greater under hypoxic conditions than that produced by misonidazole on a concentration basis [9]. The difference in the ability of these two agents to induce DNA damage, especially with RSU-1069 in air, is related to substitution of the 3-methoxy group of misonidazole with an alkylating aziridine group to give RSU-1069 [11, 12].

In this investigation the concentration dependence of DNA-SSB under conditions equivalent to those required for the onset of cytotoxicity was determined using the technique of alkaline filter elution. The filter elution assay was chosen since it is possible to observe that misonidazole induces DNA-SSB at concentrations of 5 mmol/dm^3 for a contact of 2 hr under hypoxia [13]. Whereas observation of DNA-SSB using sucrose sedimentation requires concentrations of misonidazole of at least 50 mmol/dm^3 for an equivalent contact period of 2 hr under hypoxia [9]. During the course of these studies it was found that for identical treatments with either misonidazole or RSU-1069 the yield of DNA-SSB (expressed in radiation equivalents) determined by alkaline filter elution is about an order of magnitude less than that previously determined by alkaline sucrose gradient sedimentation [9]. To resolve this discrepancy and gain more information on the type of DNA lesion, the conditions used for alkaline filter elution (e.g. the duration of cell lysis, the pH of the lysing and elution buffers) were modified to those conditions used during alkaline sucrose gradient sedimentation [9].

MATERIALS AND METHODS

Chemicals. RSU-1069 was synthesized in these laboratories by P. Webb [14] and misonidazole was supplied by Dr C. E. Smithen (Roche Products Ltd, Welwyn, U.K.). Sarkosyl, proteinase K and tetraethylammonium hydroxide (TEAH) were purchased from the Sigma Chemical Co. (Poole, U.K.). Scintillation liquid was purchased from Beckman. All other reagents were of AnalaR grade and used as supplied. Solutions of misonidazole and RSU-1069

* To whom correspondence should be addressed.

were freshly prepared for each experiment in order to minimize their degradation in aqueous media [12].

Cell cultures. Chinese hamster V79-379A cells were maintained as monolayer cultures (1–2 × 10^7 cells/mL) in 25 mL Falcon flasks containing growth medium [Eagle's minimal essential medium, fetal calf serum (10%), penicillin (50 I.U./mL), streptomycin (50 I.U./mL) and *l*-glutamine (2 mmol/dm)]. Suspension cultures were formed from monolayers. The cells were washed with Earle's balanced salt solution and then detached using trypsin/EDTA (0.5 and 0.2%, respectively) before resuspension in growth medium [Eagle's minimal essential medium for suspension cultures, fetal calf serum (10%), penicillin (50 I.U./mL), streptomycin (50 I.U./mL) and *l*-glutamine (2 mmol/dm^3)].

Radioactive labelling of cellular DNA. Cells in monolayer were incubated in growth medium containing 0.5 μCi/mL [^{3}H]thymidine (25 mCi/mmol, Amersham International, Bucks, U.K.) or 0.1 μCi/mL [^{14}C]thymidine (50 μCi/mmol, Amersham International) for 24 hr at 310° K. Active medium was then replaced with fresh medium containing cold thymidine (0.1 mg/mL final concentration) and the cells incubated for a further 2 hr at 310°K.

Cell irradiation. Labelled cells in suspension were irradiated on ice at a dose rate of 0.75 Gy/min in air using ^{60}Co γ-irradiation and then kept on ice, until required, to minimize repair.

Drug incubation. Cells in suspension were incubated with fixed concentrations of drug for varying periods. The concentrations of misonidazole or RSU-1069 used were 5–50 mmol/dm^3 and 0.1–1 mmol/dm^3, respectively. Briefly, all solutions of drug or cell suspensions were equilibrated with N_2 + 5% CO_2 (<10 ppm O_2) or maintained aerobically (air + 5% CO_2) for 1 hr at 310°K, before they were mixed to give 5 × 10^6 cells/mL and then incubated for fixed times (0–4 hr) under the appropriate gassing conditions at 310°K. Aliquots of the agent and cell suspensions were kept on ice until analysed for DNA-SSB by either assay.

Alkaline filter elution. An alkaline elution assay incorporating dual-labelling was used [15]. Dual-labelling is thought to increase the precision of the assay by basing DNA-SSB measurement on actual DNA elution rates, relative to the rate of elution of a reference (irradiated DNA). This method allows the DNA-SSB to be expressed in radiation dose equivalents. Chemically insulted or irradiated [^{14}C]thymidine labelled cells were mixed with an equal number of [^{3}H]thymidine labelled cells (which had been irradiated with a known dose of γ-radiation and used as an internal standard) and placed onto polycarbonate filters (2 μm pore size, 25 mm diameter, Nucleopore) to give a total of 2 × 10^5 cells/filter. The cells were then washed twice with ice-cold PBS/EDTA (1 mM) to remove any residual agent, lysed (2–4 hr at 293°K) with various lyse solutions then followed by a further wash with a solution of EDTA (0.02 mol/dm^3). Either a solution of sarkosyl (2%), proteinase K (0.5 mg/mL), NaCl (2 mol/dm^3) in Tris (1 mol/dm^3)/EDTA (20 mmol/dm^3) buffer at pH 8.7 ± 0.1 or solutions of sarkosyl (1%), EDTA (10 mmol/dm^3) and varying concentrations of NaOH (0.05, 0.1 and 0.5 mol/dm^3, pH 12.5, 12.9 and 13.4, respectively) were used to lyse the cells. DNA retained on the filter was eluted off with a solution (30 mL) of TEAH (0.1 mol/dm^3) and EDTA (20 mmol/dm^3) of pH 12.2, 12.5 or 13.0 (adjusted with NaOH) with a flow rate of 2 mL/hr. Each fraction was added to scintillant (10 mL), the number of counts determined and a quench, dual-label, cpm to dpm correction performed for spill-over. An elution curve was obtained from a log–log plot of per cent DNA retained on filter (agent or radiation insulted cells) against the per cent DNA retained on the filter from irradiated internal standard cells. The relative rate of DNA elution was determined from a linear least squares analysis of the slope of each elution curve. The yield of DNA-SSB (expressed in radiation equivalents) was determined by comparing a plot of relative rate of DNA elution from irradiated cells as a function of absorbed dose with a plot of relative rate of DNA elution from chemically insulted cells as a function of concentration of the agent or contact time (where the same absorbed dose of radiation was given as an internal standard). Dose equivalent data were only calculated from the results of experiments where irradiated and chemically insulted cells had been given the same dose of γ-radiation as an internal standard, since the rate of elution of the DNA from the insulted cells shows a dependency on the dose of radiation given to the internal standard cells (P. W. Crump, unpublished finding).

To determine the molecular weight of DNA passing through the filter during cell lysis at pH 13.4, the cells were lysed (0.25 mL of lysis solution) on the filter for 2 hr. The lysate (containing DNA) was eluted directly onto the top of a sucrose gradient and the gradients subsequently handled as described below.

Alkaline sucrose gradient sedimentation. Irradiated [^{3}H]thymidine cells were harvested/washed by centrifugation and resuspended in 0.2 mL Tris–HCl (10 mmol/dm^3) EDTA (1 mmol/dm^3), NaCl (0.15 mol/dm^3) buffer, pH 7.5. A sample (30 μL) of cell suspension was gently placed in a lysing layer (NaOH, 0.5 mol/dm^3; EDTA, 10 mmol/dm^3, sarkosyl, 1%; pH 13.5) on top of a 5–20% sucrose gradient (NaOH, 0.1 mol/dm^3, NaCl, 1 mol/dm^3; EDTA, 1 mmol/dm^3, sarkosyl, 0.1%; pH 11.5). The gradients were left to stand for 4 hr, at 293°K before centrifugation at 10,000 rpm for 14 hr and 293°K, in a 6 × 5.5 mL swing-out SW55 rotor (Beckman). The gradients were collected, washed and the resultant activity measured by liquid scintillation counting. The data were analysed as previously described [9, 16, 17].

RESULTS

The elution profiles of cellular DNA after incubation of V79 cells with misonidazole or RSU-1069 (the concentration of agent chosen represents that required to reduce survival to approximately 0.1%) under various experimental conditions are shown in Fig. 1a and b, respectively. Following either γ-irradiation or incubation with misonidazole the elution curves are linear (Fig. 1a), whereas the elution

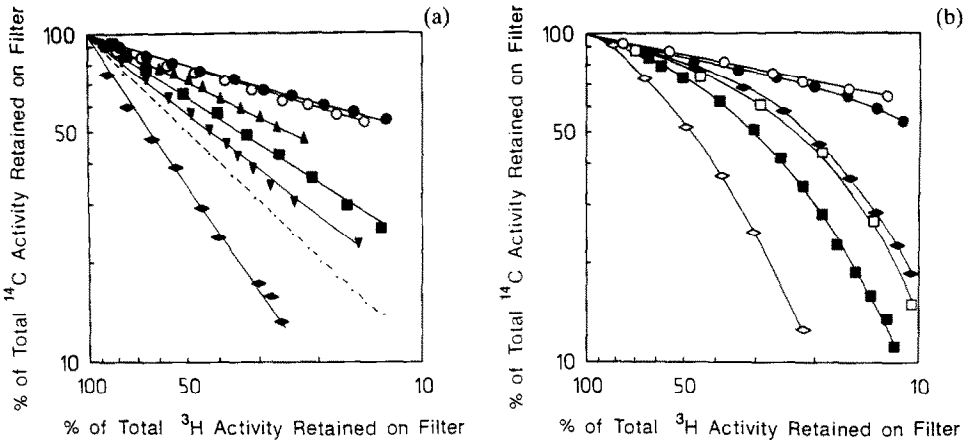

Fig. 1(a). Elution of DNA from V79 cells following incubation with misonidazole under aerobic and hypoxic conditions (relative to a 3 Gy γ-irradiated internal standard). (●), (▲), (■) and (▼) incubation with misonidazole (5 mmol/dm^3) 0, 2, 3 and 4 hr, respectively, under hypoxia; (◆) miso (50 mmol/dm^3) 2 hr under hypoxia; (○) 4 hr incubation with misonidazole (5 mmol/dm^3) under aerobic conditions; (-----) radiation only (3 Gy). (b) Elution of DNA from V79 cells following incubation with RSU-1069 under aerobic and hypoxic conditions (relative to γ-irradiated (5 Gy) internal standard). (○) and (□) incubation with RSU-1069 (100 μmol/dm^3) under aerobic conditions 0 and 2 hr, respectively; (◇) 2 hr (500 μmol/dm^3). (●), (◆) and (■) incubation with RSU-1069 (200 μmol/dm^3) under hypoxia 0, 2, and 3 hr, respectively.

profiles following incubation with RSU-1069 are non-linear (Fig. 1b). As can be seen in Fig. 1a, the induction of DNA-SSB after incubation with misonidazole occurs only under hypoxia. On the other hand, DNA-SSB are induced by RSU-1069 (Fig. 1b) under both hypoxic and aerobic conditions. In contrast to previously published observations whereby the yield of DNA-SSB is increased by a factor of two on going from aerobic to hypoxic conditions [9], incubation with RSU-1069 induces approximately similar yields of DNA-SSB under both gassing conditions. This observation may be a reflection of crosslink formation under hypoxia [10] whereby the rate of elution is modified.

As shown in Fig. 1a, the induction of DNA-SSB was observed even at concentrations of misonidazole of 5 mmol/dm^3. It was therefore possible to measure misonidazole induced breaks with the alkaline filter elution technique at similar concentrations to those used in cytotoxicity studies [18, 19]. Of the two agents used, RSU-1069 is the more efficient at inducing DNA-SSB. RSU-1069 induces DNA-SSB under hypoxia at concentrations of <0.2 mmol/dm^3; whereas greater concentrations of misonidazole are required to observe DNA degradation. Increasing either the contact time (0–4 hr) or the concentration of misonidazole and RSU-1069 was found to increase the yield of DNA-SSB. The dependence of the relative rate of DNA elution on contact time with misonidazole (5 mmol/dm^3 under hypoxia at 310°K) was found to be approximately linear (Fig. 2). Also shown is the equivalent dependence on radiation dose. From Fig. 2, the yield of chemically-induced DNA-SSB can therefore be expressed in terms of radiation dose equivalents. The yield of DNA-SSB (expressed in radiation dose equivalents) after incubation with 5 mmol/dm^3 misonidazole for 2 or 4 hr under hypoxia and at 310°K was estimated to be 1 Gy and 2.3 Gy, respectively. These results are comparable to the amount of DNA-SSB detected by alkaline filter elution after incubation of CHO cells with misonidazole under similar experimental conditions [13]. With 50 mmol/dm^3 misonidazole (for 2 hr under hypoxia at 310°K) the yield of DNA-SSB is equivalent to a radiation dose of 9 Gy using alkaline filter elution. This value is about one order of magnitude less than the radiation dose equivalent

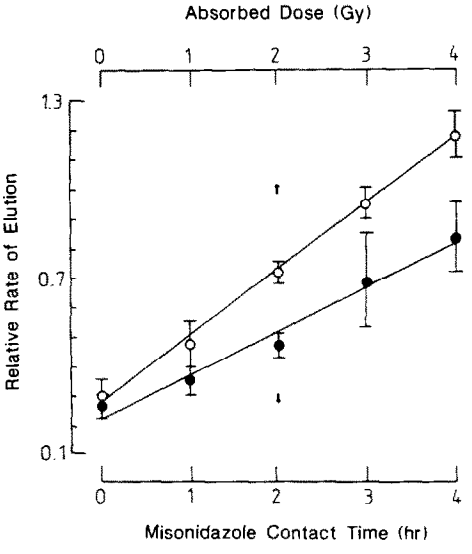

Fig. 2. Relative rate of DNA elution following either incubation with misonidazole (5 mmol/dm^3) under hypoxia (●) or γ-irradiation (○) [relative to a γ-irradiated (3 Gy) internal standard] as a function of contact time or absorbed radiation dose, respectively.

Table 1. Comparison of the yield of DNA-SSB induced by incubation of V79 cells with 2-nitroimidazoles (2 hr) as determined by alkaline filter elution or alkaline sucrose gradient sedimentation assays

Drug treatment	Yield of DNA-SSB expressed in radiation dose equivalents (Gy)	
	Filter elution*	Sucrose sedimentation†
Miso (50 mmol/dm^3) under hypoxia	9	110
RSU-1069 (200 μmol/dm^3) under hypoxia	5	110
RSU-1069 (300 μm/dm^3) under aerobic conditions	11	110

* Internal standard (10 Gy) used throughout the alkaline elution assay.
† Published data [9], drug treatments, selected to give equivalent amounts of DNA damage.

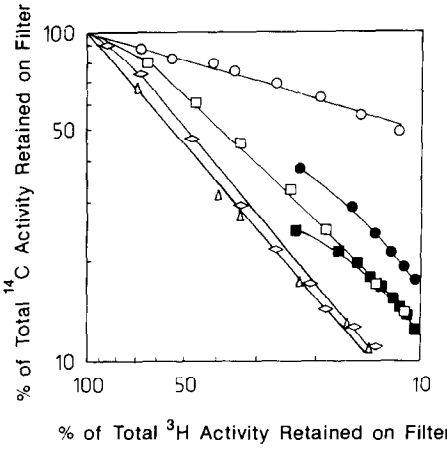

Fig. 3. Effect of different lysing conditions, on the elution of DNA from V79 cells following incubation with RSU-1069 (300 μmol/dm^3) under aerobic conditions [relative to a γ-irradiated (10 Gy) internal standard]. (○) and (□) 0 and 2 hr RSU-1069 incubation, respectively, 2 hr proteinase-K + detergent, pH 8.7 lyse. (◇) 2 hr RSU-1069 incubation, 4 hr proteinase K + detergent, pH 8.7 lyse. (△) 2 hr RSU-1069 incubation, NaOH (50 mmol/dm^3), pH 12.5 lyse. (●) and (■) 0 hr and 2 hr RSU-1069 incubation, respectively, 2 hr NaOH (0.5 mol/dm^3), pH 13.4 lyse.

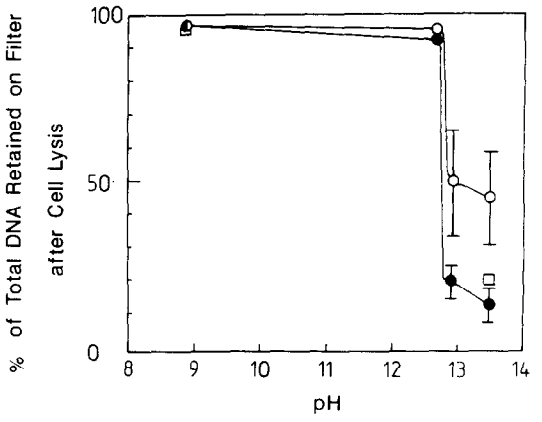

Fig. 4. Effect of pH of the lysing solution on the per cent DNA retained on the filter from V79 cells (control) or following incubation with RSU-1069 (300 μmol/dm^3) under aerobic conditions (10 Gy internal standard), prior to DNA elution. (□) V79 control cells. (○) and (●) 0 and 2 hr RSU-1069 insulted cells.

of 110 Gy estimated for V79 cells treated under the same experimental conditions but using alkaline sucrose gradient sedimentation to assess the DNA damage [9]. In Table 1 the yields of DNA-SSB (expressed in radiation dose equivalents) determined by alkaline elution for V79 cells are compared to previously measured yields obtained using alkaline sucrose gradient sedimentation [9]. From these results it is suggested that approximately 10 to 20 times fewer DNA-SSB were detected by alkaline filter elution than by the sucrose gradient assay following equivalent drug treatments.

These discrepancies in the yield of DNA-SSB were investigated further since different lysing conditions (e.g. duration of cell lysis and the pH of the lysing solution) are used in the two techniques. The effect of modifying either the lysing conditions or lysing solutions on the rate of elution of DNA after incubation with RSU-1069 under aerobic conditions is shown in Fig. 3. These conditions were chosen to avoid any potential problems associated with hypoxia, for example the formation of DNA cross-links [10]. Increasing the duration of the proteinase-K cell lysis from 2 to 4 hr or using a lysing solution of NaOH (pH 12.5) does not have any significant effect on the rate of DNA elution (Fig. 3). If lysis solutions of NaOH were used at pH $\geq$ 12.9, the rate of elution of DNA becomes independent of the experimental conditions and similar to the rate of elution of DNA from control cells (Fig. 3). The dependence of the percentage of DNA retained on the filter after cell lysis as a function of pH is shown in Fig. 4. The percentage DNA retained is independent of pH up to approximately pH 12.8 whereas above this pH the DNA is eluted through the filter together with the cell lysing solution. From Fig. 4 it is apparent that the percentage DNA retained on the filter after cell lysis decreases from 97% to about 15% on increasing the pH of the lysis solution. The molecular weight of DNA from control cells passing through the filters during the cell lysis stage of the filter elution, was subsequently determined by alkaline sucrose gradient sedimentation. Under certain lysing conditions, for example, proteinase-K, pH 8.7 and NaOH (pH 12.5 and 12.9) it was not possible to determine a molecular weight since insufficient DNA passes through the filter. The relative molecular

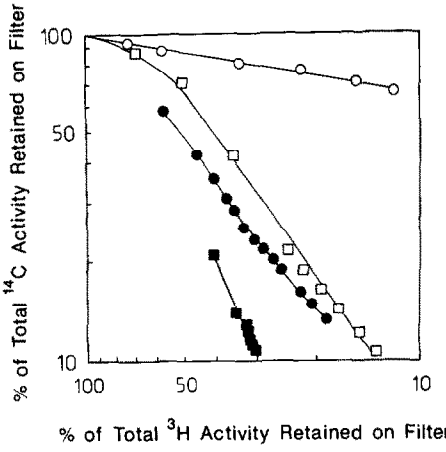

Fig. 5. Effect of a 15 hr delay prior to elution of DNA from V79 cells after incubation with RSU-1069 (300 μmol/dm^3) under aerobic conditions (10 Gy internal standard). (○) and (□) 0 and 2 hr, respectively, no-delay in DNA elution. (●) and (■) 0 and 2 hr, respectively, DNA elution delayed for 15 hr.

weight of DNA passing through the filter during cell lysis at pH 13.4 was found to be 1.75×10^8 daltons, compared to 4.4×10^8 daltons for the DNA of control cells lysed directly on top of the sucrose gradients. A comparable difference in the molecular weight of DNA from cells lysed before layering onto a sucrose gradient and DNA from cells lysed directly on a sucrose gradient has been previously observed [16]. Pretreatment of the filters with a solution of NaOH (pH 13.4) did not affect the rate of elution or the per cent DNA retained on the filter (data not shown). It is evident that the increased rate of elution is not due to alkaline-induced changes of the filters. Similarly, increasing the pH of the elution buffer from 12.2 to 12.5 does not affect the rate of elution of DNA. If, however, there is a delay of 12 to 24 hr before eluting the buffers (pH 12.2) it was not possible to differentiate between the rate of DNA elution from control or insulted cells since more than 50% of the total DNA elutes through in the first fraction (Fig. 5). Increasing the pH of the elution buffer to pH 13 was found to have a similar effect.

DISCUSSION

The induction of DNA-SSB in V79 cells by misonidazole or RSU-1069 as determined by alkaline filter elution was found to be qualitatively consistent with previous findings [13]. For example, the induction of DNA-SSB occurs only under hypoxia with misonidazole, whereas RSU-1069 induces DNA-SSB under both hypoxic and aerobic conditions.

Although the relative effects of RSU-1069 and misonidazole are qualitatively similar to those previously reported [9], it is apparent that quantitative differences are observed in the extent of DNA degradation induced by these agents when comparing the elution and sedimentation techniques. From a comparison of the data obtained, it is clear that: (i) with the elution technique, DNA damage may be detected at much lower concentrations of agent than with sedimentation; and (ii) using a concentration whereby DNA damage may be assessed with the two techniques (e.g. 50 mmol/dm^3 misonidazole), the sedimentation technique is about an order of magnitude more sensitive at detecting SSB. Whereas the first point is related to the high background level of damage in the controls with sedimentation, thereby limiting the observation of DNA damage at low concentrations of agent. The second point is, in part, a reflection of the different pH conditions and therefore the stability of potential alkali-labile sites (see later).

Modification of the filter elution assay to incorporate pH conditions similar to those used during the alkaline sucrose gradient sedimentation assay proved to be unsuccessful. Where lysing solutions of pH < 12.5 are used with the filter elution assay, the rate of DNA elution is suggested to be dependent on the number of DNA-SSB, whereas at pH > 12.9 the rate of DNA elution is independent of the number of DNA-SSB. This pH effect upon elution is in agreement with previous findings from alkaline filter elution assay [20] and sets a limit on the lysing conditions which may be used. It was not possible therefore to make a comparison of DNA-SSB induced by these agents under the same denaturing conditions.

The yield of damage induced by RSU-1069 has also been determined using the DNA precipitation assay recently developed by Olive *et al.* [21] and compared with the yield of DNA damage measured using alkaline unwinding method. A similar discrepancy in the yield of chemically-induced DNA damage was observed after an identical treatment with RSU-1069, whereby more DNA-SSB are observed using the DNA precipitation than with alkaline unwinding method. Further, with the unwinding technique, it has been inferred that DNA-SSB are not produced after incubation with misonidazole (5 mmol/dm^3) for 2 hr under hypoxia [22]. The cell lysing and alkaline denaturing conditions used during alkaline unwinding or filter elution assays are less forcing than those used during the DNA precipitation or alkaline sucrose sedimentation assays [9, 21, 23]. For example the cell lysing conditions used during the DNA precipitation and alkaline sucrose gradient sedimentation assays respectively involve a heat treatment step [21] or lysing at pH > 13 [9].

The elution profiles following incubation with RSU-1069 were found to be curved (see Results). From these elution profiles, it is apparent that the rate of DNA elution is indicative of the induction of an alkali-labile, DNA lesion by RSU-1069 [15]. Such lesions are progressively expressed as a DNA-SSB on incubation under alkaline conditions [24]. It has previously been shown that the formation of DNA-SSB in plasmid DNA by reduced 2-nitroimidazole only occurs after heating in alkali at pH > 12.5 [25]. These results and the observations from molecular studies [6, 10–12, 25] suggest that incubation with misonidazole or RSU-1069 induces an alkali-labile DNA lesion. It is suggested that the denaturing conditions used during alkaline sucrose gradient sedimentation are sufficiently forcing compared to those

used during the alkaline filter elution assay, to convert these alkali-labile DNA lesions into DNA-SSB. The formation of labile DNA base adducts which yield SSB is a possible explanation for the order of magnitude difference in the yield of DNA-SSB determined by the filter elution compared to the sucrose gradient assay. It is inferred that only 10% of the total DNA-SSB induced by these agents are observed using the technique of alkaline filter elution and therefore that the major damage induced by these agents are base adducts which are converted to SSB under forcing alkaline conditions.

These results raise the question of the quality of the misonidazole induced DNA-SSB (under hypoxia) compared to the radiation induced DNA-SSB (under aerobic conditions), in terms of their effect on cell survival. The assumption is made that DNA is the primary cellular target for the cytocidal effects of these drugs or radiation and other possible cellular targets or effects can be ignored. Incubation with misonidazole (5 mmol/dm^3 for 2 or 4 hr under hypoxia at 310°K) reduces the survival of V79 cells to approximately 75 and 0.1%, respectively [26], whereas 1 or 2.3 Gy of γ-radiation, (the radiation doses in air giving similar yields of DNA-SSB, see Results) reduces the survival of V79 cells to approximately 80 and 60%, respectively [14]. It should be noted that radiation induced DNA-SSB are readily rejoined [27], whereas there is evidence that misonidazole induced DNA-SSB are not all readily repaired [8, 13]. Such differences in repairability of the lesions may contribute to the difference in cell toxicity of similar amounts of radiation and misonidazole induced DNA-SSB which are qualitatively different types of lesions. However, as discussed above, misonidazole treatment will also induce a greater level of alkali-labile sites.

CONCLUSION

It is concluded that more information of chemically induced DNA damage may be obtained by using different techniques, in combination, to assess cellular DNA damage. In agreement with molecular studies, the major DNA lesion induced upon bioreduction of these 2-nitroimidazoles is an adduct(s) which is readily labile at pH $>$ 12.5. Therefore, identification of these alkali-labile DNA lesions induced by these agents remains a major priority. With alkali-elution the question arises as to the property of DNA which influences the elution rates and is it a reflection of the yield of SSB? Indeed the use of only one method to assess chemically induced DNA damage may be misleading due to their differing sensitivities. The use of two or more assays is recommended and should reveal significant information about cellular DNA damage induced by different agents.

Acknowledgements—We are grateful to Dr O. Sapora for helpful discussions.

REFERENCES

1. Stratford IJ and Adams GE, The toxicity of the radiosensitizer misonidazole towards hypoxic cells in vitro: a model for mouse and man. *Br J Radiol* **51**: 745–746, 1978.
2. Brown JM, Cytotoxic effects of the hypoxic cell radiosensitizer Ro 7-0582 to tumour cells *in vivo*. *Radiat Res* **72**: 469–486, 1977.
3. Stratford IJ, Walling JM and Silver ARJ, The differential cytotoxicity of RSU-1069: cell survival studies indicating interaction with DNA as a possible mode of action. *Br J Cancer* **53**: 339–344, 1986.
4. Varghese AJ and Whitmore GF, Detection of a reactive metabolite of misonidazole in hypoxic mammalian cells. *Radiat Res* **97**: 262–271, 1984.
5. Stratford IJ, O'Neill P, Sheldon PW, Silver ARJ, Walling JM and Adams GE, RSU-1069, a nitroimidazole containing an aziridine group. *Biochem Pharmacol* **35**: 105–109, 1986.
6. Silver ARJ, O'Neill P, Jenkins TC and McNeil SS, The phosphate-group of DNA as a potential target for RSU-1069, a nitroimidazole-aziridine radiosensitizer. *Int J Radiat Onc Biol Phys* **12**: 1203–1206, 1986.
7. Biaglow JE, Cellular electron transfer and radical mechanisms for drug metabolism. *Radiat Res* **86**: 212–242, 1981.
8. Palcic B and Skarsgard LD, Cytotoxicity of misonidazole and DNA damage in hypoxic mammalian cells. *Br J Cancer* **37** (Suppl. III): 54–59, 1978.
9. Jenner TJ, Sapora O, O'Neill P and Fielden EM, Enhancement of DNA damage in mammalian cells upon bioreduction of the nitroimidazole-aziridines RSU-1069 and RSU-1131. *Biochem Pharmacol* **37**: 3837–3842, 1988.
10. O'Neill P, McNeil SS and Jenkins TC, Induction of DNA crosslinks *in vitro* upon reduction of the nitroimidazole-aziridines RSU-1069 and RSU-1131. *Biochem Pharmacol* **36**: 1787–1792, 1987.
11. Silver ARJ and O'Neill P, Interaction of the aziridine moiety of RSU-1069 with nucleotides and inorganic phosphate. *Biochem Pharmacol* **35**: 1107–1112, 1986.
12. O'Neill P, Jenkins TC, Stratford IJ, Silver ARJ, Ahmed I, McNeil SS, Fielden EM and Adams GE, Mechanism of action of some bioreducible 2-nitroimidazoles: comparison of *in vitro* cytotoxicity and ability to induce DNA strand breakage. *Anti-Cancer Drug Design* **1**: 271–280, 1987.
13. Taylor YC, Evans JW and Brown JM, Radiosensitization by hypoxic pretreatment with misonidazole: an interaction of damage at the DNA level. *Radiat Res* **109**: 364–373, 1987.
14. Adams GE, Ahmed I, Sheldon PW and Stratford IJ, RSU-1069, a compound more efficient than misonidazole *in vitro* and *in vivo*. *Br J Cancer* **49**: 571–577, 1984.
15. Kohn KW, Ewig RAG, Erickson LC and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. In: *DNA Repair. A Laboratory Manual of Research Procedures* (Eds. Friedberg E and Hanawalt P), pp. 379–401. Marcel Dekker, New York, 1981.
16. Millar BC, Sapora O, Fielden EM and Loverock PS, The application of rapid-lysis techniques in radiobiology. *Radiat Res* **86**: 506–514, 1981.
17. Blöcher D, DNA double strand breaks in ehrlich ascites tumour cells at low doses of X-rays. I. Determination of induced breaks by centrifugation at reduced speed. *Int J Radiat Biol* **42**: 317–328, 1982.
18. Whitmore GF, Gulyas S and Varghese AJ, Sensitising and toxicity properties of misonidazole and its derivatives. *Br J Cancer* **37** (Suppl. III): 115–119, 1978.
19. Walling JM, Stratford IJ, Adams GE, Silver ARJ, Ahmed I, Jenkins TC and Fielden EM, Studies on the mechanisms of the radiosensitizing and cytotoxic properties of RSU-1069 and its analogues. *Int J Radiat Oncol Biol Phys* **12**: 1083–1086, 1986.

20. Kohn KW, Erickson LC, Ewig RAG and Friedman CA, Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**: 4629–4637, 1976.
21. Olive PL, Chan APS and Cu CS, Comparison between the DNA precipitation and alkali unwinding assays for detecting DNA strand breaks and cross-links. *Cancer Res* **48**: 6444–6449, 1988.
22. Rajaratham S, Stratford IJ and Adams GE, Pre-incubation with electron affinic radiosensitizers followed by hyperthermia or X-rays. *Int J Radiat Oncol Biol Phys* **8**: 767–770, 1982.
23. Ahnström G, Techniques to measure DNA single-strand breaks in cells: a review. *Int J Radiat Biol* **54**: 695–707, 1988.
24. Lafleur MVM, Woldhuis J and Loman H, Alkali-labile sites in biologically active DNA: comparison of radiation induced potential breaks and apurinic sites. *Int J Radiat Biol* **39**: 113–118, 1981.
25. Silver ARJ, McNeil SS, O'Neill P, Jenkins TC and Ahmed I, Induction of DNA strand breaks by reduced nitroimidazoles. *Biochem Pharmacol* **35**: 3923–3928, 1986.
26. Stratford IJ and Gray P, Some factors affecting the specific toxicity of misonidazole towards hypoxic mammalian cells. *Br J Cancer* **37**: 129–131, 1978.
27. Elkind MM and Kamper C, Two forms of repair of DNA in mammalian cells following irradiation. *Biophys J* **10**: 237–245, 1970.