Biochemical Pharmacology, Vol. 36, No. 11, pp. 1787–1792, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

INDUCTION OF DNA CROSSLINKS *IN VITRO* UPON REDUCTION OF THE NITROIMIDAZOLE-AZIRIDINES RSU-1069 AND RSU-1131

PETER O'NEILL,* SHONA S. MCNEIL and TERENCE C. JENKINS†

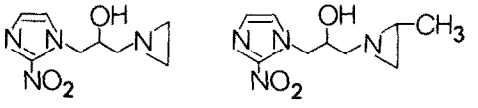
Division of Molecular Processes, Medical Research Council, Radiobiology Unit, Chilton, Didcot, Oxon., OX11 0RD, U.K.

(*Received* 31 *October* 1986; *accepted* 17 *December* 1986)

Abstract—The interaction of the nitroimidazole-aziridines RSU-1069 and RSU-1131, as parent or radiation-reduced species, with plasmid DNA in aqueous solution at pH 7 results in strand breakage. The yields of DNA single strand breaks (ssb), "alkali-labile" damage and DNA crosslinks induced by these alkylating agents have been assessed. It is shown that DNA crosslinks are induced only by the *reduced* nitro-compounds. RSU-1069, as parent or reduced compound, is more efficient at producing these effects than the equivalent form of RSU-1131. Further, RSU-1069 is about 2× more susceptible to nucleophilic attack by inorganic phosphate and deoxynucleotides than RSU-1131. RSU-1069 also shows greater selectivity for reaction with the nucleotide base moiety than does the less-reactive monomethyl analogue, RSU-1131. The yields of ssb and "alkali-labile" damaged sites induced by the two agents reflect their respective chemical reactivities and appear largely to determine their *aerobic* cytotoxicities. In contrast, the yield of DNA crosslinks induced by the reduced compounds appears to correspond rather better with the observed *hypoxic* cytotoxicities. From these findings it is suggested that the induction of DNA crosslinks by these agents may play a major role in their effectiveness as hypoxia-selective cytotoxins.

The compound RSU-1069 [NSC 347503, 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol—structure in Fig. 1] is a more efficient hypoxic cell radiosensitiser and chemopotentiator than misonidazole [1–4]. Additionally, RSU-1069 is, on a concentration basis, ~100-fold more toxic towards hypoxic relative to aerobic cells *in vitro* [5–7]. Progressive methyl-substitution of the aziridine moiety of RSU-1069 results in decreased cytotoxicity [8, 9] even though the radiosensitising efficiencies of the compounds remain similar [8].

Cellular and molecular studies indicate that under aerobic conditions, RSU-1069 and its methyl-substituted aziridine analogues act as *monofunctional* alkylating agents whereas upon reduction they become *bifunctional* in character [5, 6, 9–12]. Studies with plasmid DNA have shown [9] that the efficiency of strand break formation decreases with increasing methyl-substitution of the aziridine function for both the parent and radiation-reduced compounds. Further, such studies have emphasised that RSU-1069 alkylates DNA at both the phosphate and purine base sites via the aziridine group [10–12], a process that leads to strand breakage and the formation of "alkali-labile" damaged sites. These latter sites are indicative of DNA base damage [11, 13]. Preliminary studies have indicated that reduced RSU-1069 may function as a crosslinking agent [11].



RSU-1069 RSU-1131

Fig. 1. Structures of RSU-1069 and -1131.

The present study was undertaken to investigate the ability of parent and reduced RSU-1069 and RSU-1131 (the *mono*methyl-substituted aziridine analogue of RSU-1069, Fig. 1) to induce "alkali-labile" sites and crosslinks upon interaction with plasmid DNA. Further, differences in the distribution of attack at the various sites upon DNA by the two agents may be assessed. The interaction of RSU-1131 with inorganic phosphate and deoxynucleotides was also examined to establish its chemical reactivity in comparison with that of RSU-1069. Elucidation of the molecular mechanisms of alkylating action by the parent and reduced forms of these 2-nitroimidazoles (and the subsequent expression as DNA damage) will assist in identifying those molecular features required for such compounds to act as hypoxia-selective cytotoxins.

MATERIALS AND METHODS

Compounds. RSU-1069 and RSU-1131 [1-(2-nitro-1-imidazolyl)-3-(2-methyl-1-aziridinyl)-2-propanol] were prepared as previously described [1, 8, 9]. The two compounds were recrystallised in

* To whom correspondence should be addressed.
† Present address: CRC Biomolecular Structure Unit, Institute of Cancer Research, Block F, Clifton Ave, Belmont, Sutton, Surrey, SM2 5PX.

the form of pale-yellow prisms from ethanol containing 5% v/v triethylamine to inhibit acid-catalysed polymerisation. RSU-1069 had m.p. 393.5–394 K (lit.: 392–394 K [1, 8]); RSU-1131 had m.p. 383.5–384 K (lit.: 382–384 K [8]).

Other chemicals. All other reagents were of "AnalaR" grade (BDH) except the deoxynucleotides which were obtained from Sigma and used as supplied.

Determination of strand breakage in pBR322 plasmid DNA. The plasmid pBR322 was maintained in *E. coli* HB101 and extracted using conventional methods [14]. DNA concentrations were determined spectrophotometrically.

Radiation-reduced compounds were generated by irradiation of N_2-saturated, aqueous solutions of the compounds (2–4 mmol dm^{-3}) containing sodium formate (0.1 mol dm^{-3}), buffered at pH 7.0 with phosphate (40 mmol dm^{-3}), using a linear accelerator as detailed previously [10]. In all experiments the solutions were irradiated to effect ≥85% conversion of the 2-nitroimidazoles to reduced product(s) within 5 min (assessed from the loss of optical absorption at 325 nm using a Beckman DU-8B spectrophotometer).

Solutions of parent or reduced compounds (2–4 mmol dm^{-3}) were diluted with solutions of plasmid DNA (final concentration about 180 $\mu g\ cm^{-3}$) and incubated at 310 K under aerobic conditions as previously described [9, 10, 15]. Following exposure for given times, the treated plasmid DNA was assayed for strand breakage either immediately or following a post-incubation treatment with NaOH at pH 12.5 [11, 15]. This latter treatment does not result in degradation of unmodified type-I (closed-circular) DNA. Strand breakage of DNA [type-I → type-II (open-circular)] was assayed using agarose (0.8%) gel electrophoresis and densitometry as previously detailed [10].

Assessment of crosslinking in pBR322 plasmid DNA. Solutions of parent and radiation-reduced compounds were diluted with a solution of plasmid DNA and incubated at 310 K under aerobic conditions. The final concentrations of DNA and the parent or reduced 2-nitroimidazoles were 180 $\mu g\ cm^{-3}$ and 2–4 mmol dm^{-3}, respectively. Following incubation for given time intervals, the DNA was assayed for crosslinks based upon the stability of type-II DNA following a post-incubation treatment with NaOH at pH 13.0 for 10 min. This treatment results in degradation of both type-I and -II DNA in the absence of the compounds whereas the type-II DNA is stabilised when re-adjusted to pH 7.0–8.0 with buffer if crosslinks are present. The stability of the type-II DNA in the presence of either parent or reduced forms of the compounds was assayed using electrophoresis as outlined above.

Interaction of 2-nitroimidazoles with 2'-deoxynucleotides (dNMP). The interaction of RSU-1131 with inorganic phosphate and 2'-deoxynucleoside 5'-monophosphates (dNMP) was followed using a Beckman 344 liquid chromatography system for HPLC analysis as previously described [9, 12] [5 μm Spherisorb-CN column, Hichrom Ltd., Reading, U.K.; isocratic elution with a mobile phase of methanol (11.5% v/v) in KH_2PO_4 (10 mmol dm^{-3}), pH 3.0, at a flow rate of 1 $cm^3\ min^{-1}$]. The detection of RSU-1131 and its metabolites was performed at 310 nm and dNMP products detected at 270 nm.

Table 1. Relative rate constants* for interaction of RSU-1069 and RSU-1131 with dNMP at pH 7

dNMP	RSU-1131†	RSU-1069‡
dGMP	1.6	2.8
dAMP	1.2	1.6
dCMP	0.95	1.3
dTMP	1.0	1.0

* Relative rate constants taking k(compound + dTMP) = 1.0.
† k(RSU-1069 + dTMP)/k(RSU-1131 + dTMP) ≈ 2.
‡ Data taken from ref. 12.

RESULTS

Interaction of RSU-1131 with deoxynucleotides

The reactivity of RSU-1131 (8.0 mmol dm^{-3}) with dGMP, dAMP and dTMP at pH 7.0 was determined from the yield of RSU-1131-P (the phosphorylated product resulting from reaction with inorganic phosphate) formed under competition conditions in aqueous phosphate solution (500 mmol dm^{-3}) in the absence and presence of known concentrations of the dNMPs (80–500 mmol dm^{-3}). Initial experiments with phosphate alone (500 mmol dm^{-3}, pH 7.0) indicated that the formation of RSU-1131-P is complete within 48 hr incubation at 310 K. Based upon competition kinetics (see ref. 12), the relative reactivities determined for RSU-1131 with the dNMPs are presented in Table 1. Data previously determined for RSU-1069 are also shown in Table 1. The rate constant for interaction of RSU-1131 with inorganic phosphate (50–500 mmol dm^{-3}) is estimated to be $5 \times 10^{-4}\ dm^3\ mol^{-1}\ sec^{-1}$; RSU-1069 is thus about 2× more reactive towards phosphate than RSU-1131.

The rate constant for hydrolysis of RSU-1131 to give the ring-opened aziridine product [9] is estimated to be $<2 \times 10^{-5}\ sec^{-1}$ from the dependence of the first-order rate of formation of RSU-1131-P upon phosphate concentration. If it is assumed that the interaction of RSU-1131 with dTMP represents reaction at the phosphate function for the deoxynucleotides used, then it is inferred from the rate constants that RSU-1131 also reacts with the purine base moiety of dGMP and, to a lesser extent, that of dAMP. The reaction(s) products formed following incubation of RSU-1131 with dNMP (400 mmol dm^{-3}) for 48 hr were identified as single, homogeneous peaks under the HPLC conditions employed and were not resolved into separate peaks attributable to isomeric products resulting from nucleophilic attack at the two aziridine ring carbon positions. The product distributions determined are shown in Table 2 together with values previously determined for RSU-1069. In support of the above assumption, dTMP produces only one major product following reaction with RSU-1131; this product is assumed to result from reaction with the phosphate moiety. Only with dGMP (Table 2) are at least

Table 2. Yields of products formed* on interaction of RSU-1131 with dNMP at pH 7

dNMP	Percentage of total identified products†		
	A	B	C
dTMP	100 (100)	—	—
dCMP	>90 (93)	<10 (<10)	—
dAMP	<10 (28)	>90 (72)	—
dGMP	51 (48)	32 (18)	11 (34)

* Values in parentheses represent data for RSU-1069 from ref. 12.

† Based upon optical absorption at 310 nm; only products >5% of total by HPLC are included.

two major (>5% of total) products formed. The increased reactivity of RSU-1131 towards dGMP (Table 1) is, however, less marked than that witnessed with RSU-1069 [12]. Based upon the relative reactivities of the two compounds with dGMP and dTMP, the percentage attack at the nucleoside base moiety of dGMP by RSU-1069 and RSU-1131 is estimated to be 64% and 36% respectively.

Interaction of RSU-1131 and RSU-1069 with plasmid DNA

(i) *Alkali-treatment at pH* 12.5. Both *parent* RSU-1069 [9–11] and RSU-1131 [9] induce single strand breaks (ssb) in plasmid DNA at pH 7.0 with RSU-1069 being more efficient as shown in Table 3. Post-incubation treatment with NaOH at pH 12.5 results in a greater loss of type-I DNA with time for both compounds, as shown for RSU-1131 (Fig. 2); the enhancement in ssb yield (due to alkali-labile sites) produced by RSU-1069 and RSU-1131 is 56% and 50% respectively, based upon the times required to effect 50% degradation of the initial type-I DNA with and without a post-incubation treatment. Such an analysis of the data "normalises" for the differences in overall reactivity shown by the two compounds. A comparison of the relative effects of parent (unreduced) RSU-1069 and RSU-1131, based upon reactivity towards phosphate and DNA together with their aerobic *in vitro* cytotoxicities [9], is presented in Table 3.

The time-courses for formation of ssb in DNA by *radiation reduced* RSU-1069 and RSU-1131 are

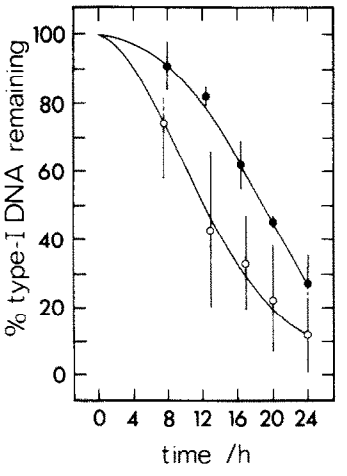


Fig. 2. The timecourse for DNA strand breakage by 2 mmol dm^{-3} *parent* RSU-1131 at pH 7 with (○) and without (●) a post-incubation treatment with NaOH at pH 12.5.

presented in Figs 3A and 3B, respectively, for the conditions with and without an NaOH treatment. The presence of alkali-labile sites induced by both reduced compounds is apparent from the increased yields of ssb after a post-incubation alkali treatment. Based upon the incubation times required to effect 50% degradation of the initial type-I DNA under both conditions (i.e. normalisation for differences in reactivities), the enhancement of ssb yield following NaOH treatment is >140% and 44% for reduced RSU-1069 and RSU-1131, respectively. Whereas the yields of "alkali-labile" sites induced by both parent compounds are similar once the alteration in reactivity is taken into account, *reduced* RSU-1069 clearly produces ≥3× more such sites than *reduced* RSU-1131 for a comparable level of DNA ssb (determined at pH 7.0). If the altered reactivity is taken into account, reduced RSU-1069 becomes >6.5× more efficient at producing "alkali-labile" sites than RSU-1131, from comparison of the time required for DNA damage represented by 50% loss of type-I DNA. A comparison of the relative effects of reduced RSU-1069 and RSU-1131, based upon their abilities to induce direct and "alkali-labile" ssb together with the hypoxic cytotoxicities of the two compounds *in vitro* [9], is given in Table 3.

Table 3. The relative effects of the parent and radiation-reduced forms of RSU-1069 and RSU-1131 in their interaction with plasmid DNA at pH 7

Property of compound	Relative effect (RSU-1069: RSU-1131)	
	parent/oxic	reduced/hypoxic
Cytotoxicity*	2.3	~20
ssb, pH 7.0*	3.4	2.3
Alkali-labile sites	~3.0	>6.5
Crosslinks	not observed	~15–20
Phosphate reactivity	~2.0	n.d.
dNMP reactivity	≈2.0	n.d.

* Data from ref. 9.

n.d. = not determined.

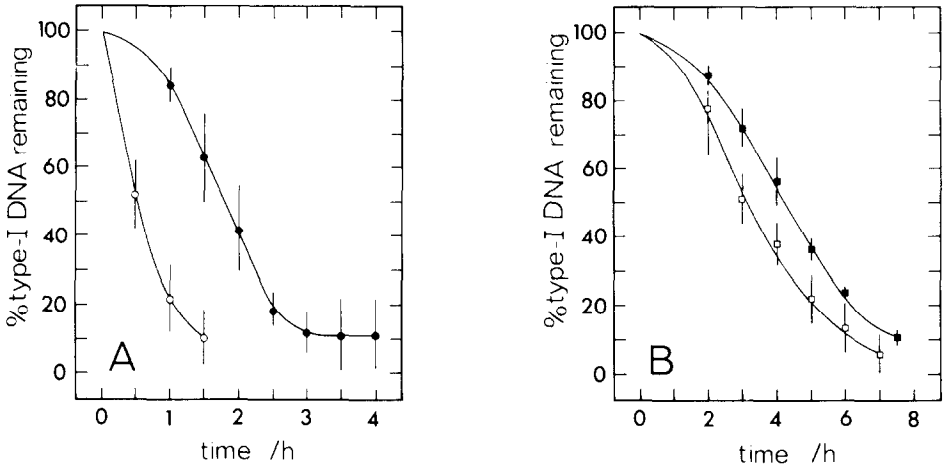


Fig. 3. The timecourse for DNA strand breakage by *radiation-reduced* compounds (2 mmol dm^{-3}). (A) RSU-1069 and (B) RSU-1131, with (○, □) and without (●, ■) a post-incubation treatment with NaOH at pH 12.5.

(ii) *Alkali-treatment at pH* 13.0. Under the conditions of alkali-treatment at pH 13.0, type-I and -II plasmid DNA are unstable and as a result show greater electrophoretic mobility than untreated DNA [16]. This condition was chosen since a post-incubation treatment of DNA after exposure to the *reduced* compounds for various times results in a stabilisation of the type-II DNA to alkali. This stabilisation has previously been demonstrated to represent DNA crosslinks which may then act as foci for renaturation of the DNA under neutral conditions [16–18]. Assuming that stabilisation of type-II DNA is indicative of crosslinks which are stable at pH 13.0, the dependence of the yield of type-II DNA (crosslinks within type-II DNA) with time following incubation with reduced RSU-1069 and RSU-1131 and a post treatment at pH 13.0 is shown in Fig. 4. Stabilisation of the type-II DNA was not observed following exposure to parent compounds. From Fig. 4, the maximal yield of crosslinks within type-II DNA were observed after approximately 1 hr and 3–4 hr incubation with reduced RSU-1069 and RSU-1131, respectively. The relative crosslinking ability of type-II DNA by these reduced 2-nitroimidazoles goes through a maximum (Fig. 4) due to continued degradation of the DNA under these conditions. Based upon the yield of type-II DNA crosslinks at about 1 hr, reduced RSU-1069 is about 15–20× more efficient than reduced RSU-1131. This increased efficiency takes into account differences in both reactivity and distribution of attack upon the DNA. The percentage loss of type-I DNA, in the absence of alkali, at these times is 15% and 27%, respectively, for RSU-1069 and RSU-1131.

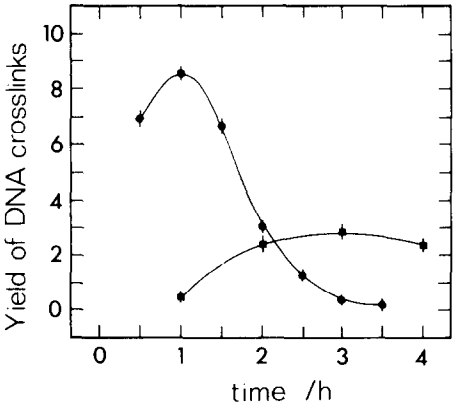


Fig. 4. The timecourse for formation of DNA crosslinks by *radiation-reduced* RSU-1069 (●) and RSU-1131 (■), each 2 mmol dm^{-3}, following a post-incubation treatment with NaOH at pH 13.0. The yield of intact type-II DNA under these conditions represents the yield of crosslinks.

In order to gain an estimate of the relative efficiencies (normalised for reactivity) of reduced RSU-1069 and RSU-1131 to effect type-II DNA crosslinking, incubation times with the two compounds were chosen to yield equivalent levels of ssb damage under neutral conditions (35% degradation of type-I DNA). The relative crosslink yields and losses of type-I DNA at both pH 7.0 (ssb) and pH 12.5 ("alkali-labile" sites) induced by the two compounds are presented in Table 3. *Reduced* RSU-1069 is about 3–4 times more efficient per ssb (pH 7.0) at producing crosslinks than *reduced* RSU-1131.

DISCUSSION

Previous studies of the *in vitro* cytotoxicity [9] have shown that the large (about 100:1) differential hypoxic:aerobic cytotoxicity ratio shown using RSU-1069 (for a 10% level of survival following 3 hr incubation at 310 K) is reduced to about 12:1 with RSU-1131. This differential toxicity is comparable to that observed with the 2-nitroimidazole radiosensitiser, misonidazole [6, 9]. RSU-1131 is, however, more cytotoxic under both aerobic and hypoxic conditions, on a concentration basis, than misonidazole

[8, 9]. Comparison of the interactions of *parent* RSU-1069 and RSU-1131 with inorganic phosphate, deoxynucleotides and plasmid DNA leads to the following facts:

(1) the yields of induced "alkali-labile" sites are similar when related to a fixed amount of ssb damage at pH 7;
(2) crosslinking of DNA is not observed;
(3) RSU-1069 is more reactive than RSU-1131 towards both phosphate and dNMP; and
(4) RSU-1069 shows an increased selectivity for alkylation at the purine base moieties compared to RSU-1131.

The difference in efficiency of ssb formation is presumably a reflection of their different reactivities towards DNA, as emphasised in the approximately 2-fold difference in reactivites with inorganic phosphate. Since RSU-1069 is only about 2.3× more cytotoxic than RSU-1131 under *aerobic* conditions, this increased effect of RSU-1069 is consistent with that for DNA damage and probably is a reflection of the different reactivities of the aziridine moieties of the two compounds. It should be noted that neither misonidazole nor RSU-1137 (the aziridine ring-opened hydrolysis product of RSU-1069) induce ssb at pH 7.0 [10, 15]. In the cellular situation, the repairability and rate of repair of the different lesions is also a consideration which, as yet, cannot be assessed.

In contrast to the aerobic situation, the *hypoxic* cytotoxicity of RSU-1069 is about 20× that of RSU-1131 [9]. It is evident that the enhanced differential toxicity of RSU-1069 is dominated by its hypoxic toxicity. From the interaction of *radiation-reduced* RSU-1069 and -1131 with plasmid DNA the following points are apparent:

(1) "alkali-labile" sites are formed, with reduced RSU-1069 showing the greater yield (Table 3), and
(2) DNA crosslinking is important, with RSU-1069 being about 15–20× more effective, on a concentration basis, than RSU-1131.

The formation of DNA crosslinks upon interaction of the reduced nitro-compounds with plasmid DNA is consistent with the *bifunctional* character proposed for the reduced product(s). Whereas the relative efficiencies of reduced RSU-1069 and RSU-1131 to induce ssb are similar to those with the parent compounds, *reduced* RSU-1069 is much more efficient than *reduced* RSU-1131 at producing DNA crosslinks and "alkali-labile" sites (even when similar yields of DNA ssb are produced at pH 7). Indeed, the increased hypoxic cytotoxicity of RSU-1069 relative to RSU-1131 (Table 3) is in agreement with the greater efficiency with which reduced RSU-1069 produces crosslinks (15–20×) and alkali-labile sites (>6.5×), for equi-concentrations of the two compounds. The possible formation of "alkali-labile" sites due to the generation of glyoxal or other metabolites upon reduction [15] may be neglected on the timescales reported in this study.

From the observed binding of reduced RSU-1069 to calf thymus DNA *in vitro* [10] and the observations in this study, it is inferred that the DNA crosslinks result from initial binding of a reactive nitro-reduction product(s) followed by an interstrand reaction of the aziridine function with either sugar-phosphate or purine sites. These bound sites would have to be stable to alkali (pH 13.0) to be detected by our assay. Whether this subsequent interaction preferentially occurs at the DNA base sites is not known. The increased yield of "alkali-labile" sites induced by reduced RSU-1069 compared to reduced RSU-1131 suggest, however, that the DNA bases are the favoured sites of attack. It is tentatively suggested that the crosslinkage formed involves base-to-base coupling. This suggestion is supported by the fact that the phosphotriesters produced following reaction of aziridines with the DNA phosphodiester back-bone are expected to be unstable at pH 7.0 due to the proximity of the amino-group in such a product [$-NHCH_2CH_2OP(O)(O\text{-sugar})_2$] to the phosphorus centre. This instability is comparable with that of phosphotriesters of RNA where decomposition is facilitated by the β-hydroxyl group. Cleavage of the formed DNA phosphotriester at pH 7.0 should be similarly facile without the requirement for alkaline conditions [19, 20].

CONCLUSIONS

From this study it is evident that the *aerobic* cytotoxicity of these agents is largely governed by the chemical reactivity of the aziridine *monofunctional* alkylating function. Conversely, it is clear that this feature does not solely influence the cytotoxicity in the hypoxic situation. Of the chemical end-points examined, only the yield of DNA crosslinks induced by the *reduced* compounds appears to correspond with the cytotoxic behaviour witnessed in hypoxia (Table 3). Preliminary findings of DNA damage produced within mammalian cells by RSU-1069 (Sapora, Jenner, O'Neill and Jenkins, unpublished data) are consistent with the classes of DNA damage reported in this study. The induction of DNA crosslinks by agents which are converted upon bioreduction to metabolites with potential *bifunctional* alkylating character may therefore play a major role in determining their effectiveness as hypoxia-selective cytotoxins.

Acknowledgements—The authors wish to thank Prof. G. E. Adams and Drs I. J. Stratford and E. M. Fielden of this Unit for helpful comments.

REFERENCES

1. G. E. Adams, I. Ahmed, P. W. Sheldon and I. J. Stratford, *Br. J. Cancer* **49**, 571 (1984).
2. G. E. Adams, I. Ahmed, P. W. Sheldon and I. J. Stratford, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1653 (1984).
3. D. W. Siemann, K. Maddison and K. Wolf, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1657 (1984).
4. D. J. Chaplin, R. E. Durand, I. J. Stratford and T. C. Jenkins, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1091 (1986).
5. I. J. Stratford, P. O'Neill, P. W. Sheldon, A. R. J. Silver, J. M. Walling and G. E. Adams, *Biochem. Pharmac.* **35**, 105 (1986).
6. I. J. Stratford, J. M. Walling and A. R. J. Silver, *Br. J. Cancer* **53**, 339 (1986).

7. R. P. Hill, S. Gulyas and G. F. Whitmore, *Br. J. Cancer* **53**, 743 (1986).
8. I. Ahmed, T. C. Jenkins, J. M. Walling, I. J. Stratford, P. W. Sheldon, G. E. Adams and E. M. Fielden, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1079 (1986).
9. P. O'Neill, T. C. Jenkins, I. J. Stratford, A. R. J. Silver, I. Ahmed, S. S. McNeil, E. M. Fielden and G. E. Adams, *Anti-Cancer Drug Design* in press.
10. A. R. J. Silver, P. O'Neill and T. C. Jenkins, *Biochem. Pharmac.* **34**, 3537 (1985).
11. A. R. J. Silver, P. O'Neill, T. C. Jenkins and S. S. McNeil, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1203 (1986).
12. A. R. J. Silver and P. O'Neill, *Biochem. Pharmac.* **35**, 1107 (1986).
13. M. Osborne and K. Merrifield, *Chem. Biol. Interact.* **53**, 183 (1985).
14. D. B. Clewell and D. R. Helinski, *Biochemistry* **9**, 4428 (1970).
15. A. R. J. Silver, S. S. McNeil, P. O'Neill, T. C. Jenkins and I. Ahmed, *Biochem. Pharmac.* **35**, 3923 (1986).
16. D. M. Freifelder, *Physical Biochemistry*, pp. 411–419. Freeman, San Francisco (1982).
17. P. H. Pouwels, C. M. Knijnenburg, J. van Rotterdam, J. A. Cohen and H. S. Jansz, *J. molec. Biol.* **32**, 169 (1968).
18. P. H. Pouwels, J. van Rotterdam and J. A. Cohen, *J. molec. Biol.* **40**, 379 (1969).
19. A. Holy and K. H. Scheit, *Biochim. biophys. Acta* **138**, 230 (1967).
20. T. V. McCarthy and T. Lindahl, *Nucleic Acids Res.* **13**, 2683 (1985).