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HEAT-STIMULATED NITROREDUCTIVE BIOACTIVATION OF THE 2-NITROIMIDAZOLE BENZNIDAZOLE *IN VITRO*

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Abstract—Hyperthermia enhances nitroimidazole cytotoxicity, possibly through increased nitroreductive bioactivation. Using C3H/He mouse liver microsomes and KHT tumour homogenates, we have investigated the effects of temperature (33–44°) on the anaerobic nitroreduction of benznidazole (BENZO) to its amine metabolite *in vitro*. Microsomal nitroreductase activity was unaltered after 2 hr anaerobic incubation at 37 and 41°. However at 44° and 47°, inactivation occurred with half-lives of 68 and 17 min respectively. At 33° microsomal reduction rates were 45% lower than at 37°. Reduction rates were increased by 22% at 41 compared to 37°, and by 0–54% depending on substrate concentration at 44°. Microsomal amine formation followed Michaelis–Menten kinetics up to 41°. The 4° rise from 33 to 37° increased the apparent V_{max} by 45% (from 0.54 to 0.98 nmol min^{-1} mg^{-1} protein) with a further increase of 32% occurring at 41°. Apparent K_m values were unaltered. Deviation from Michaelis–Menten kinetics was seen for amine formation at 44°. The kinetics of parent drug disappearance exhibited deviation from the Michaelis–Menten relationship at all temperatures studied. KHT tumour BENZO amine formation rates were also markedly increased at elevated temperatures, e.g. by 26% at 37° compared to 33° and by a further 35% from 42.5 to 57.4 pmol min^{-1} mg^{-1} protein over the range 37–41°. In contrast to the microsomal results, tumour reduction rates were enhanced by an average of 54% (range, 26–79%) at 44° compared to 37° at low as well as high substrate concentrations. These results support the hypothesis that hyperthermia-enhanced nitroimidazole cytotoxicity may be a result of increased nitroreductive bioactivation.

Nitroimidazole radiosensitizers and chemosensitizers are known to be preferentially toxic towards hypoxic tumour cells, the presence of which may limit the curability of some tumours by radiotherapy [1]. Hyperthermia enhances tumour radiosensitivity and increases the cytotoxicity of the 2-nitroimidazole misonidazole (MISO) both *in vivo* and *in vitro* [2–4]. This may be a consequence of enhanced reduction of the nitro group yielding cytotoxic species such as the nitroso, hydroxylamino and other intermediates, a hypoxia-facilitated process implicated in the biological activity of various nitro compounds [5, 6]. More direct support for this hypothesis comes from *in vitro* studies using nifurtimox, a 5-nitrofuran, the hypoxic reduction and cytotoxicity of which were considerably increased by heat up to 43° [7].

Benznidazole (BENZO) is a lipophilic 2-nitroimidazole used in the treatment of Chagas' disease [8] and is currently undergoing clinical trials as a chemosensitizer in cancer chemotherapy [9]. In view of the role of bioreductive activation in nitroimidazole cytotoxicity, we have investigated the effects of temperature on the reduction of BENZO to its corresponding amine by mouse liver microsomal preparations and whole tumour homogenates *in vitro*. BENZO was used as the substrate in this study because of its recent evaluation as a chemosensitizer [9] and because its reduction to the amine can be monitored by a specific high-performance liquid chromatography (HPLC) assay developed in our laboratory [10].

Clinical hyperthermia treatment temperatures range from 41 to 45° [11]. We have been able to demonstrate clear effects of such clinically relevant increases in temperature on the stability and kinetics of enzymes catalysing the nitroreduction of BENZO. We have also examined the kinetics of this reaction at 33° as mice often experience hypothermia after nitroimidazole administration [12]. Transplantable mouse tumours also frequently grow in their normothermic hosts at temperatures considerably below 37° [13, 14], including the intramuscular KHT tumour used in the present study which normally rests at 33° (Walton and Bleehen, unpublished data).

MATERIALS AND METHODS

Chemicals. Benznidazole (Ro 07-1051; BENZO; *N*-benzyl-[2-nitroimidazoyl] acetamide) and Ro 07-0602 (1-[2-nitroimidazoyl-1-yl]-3-*n*-butoxypropan-2-ol) were provided by Roche Products, Welwyn Garden City, U.K., and benznidazole amine (Ro 11-1721; BENZO amine; *N*-benzyl-[2-aminoimidazoyl] acetamide) was supplied as the hydrochloride salt by Roche, Basle, Switzerland. NADPH and NADH were obtained from Sigma (Poole, Dorset, U.K.) and zero grade nitrogen from the British Oxygen Company (London, U.K.).

Enzyme preparations. Liver microsomes were prepared using standard techniques [15] from 13–15-

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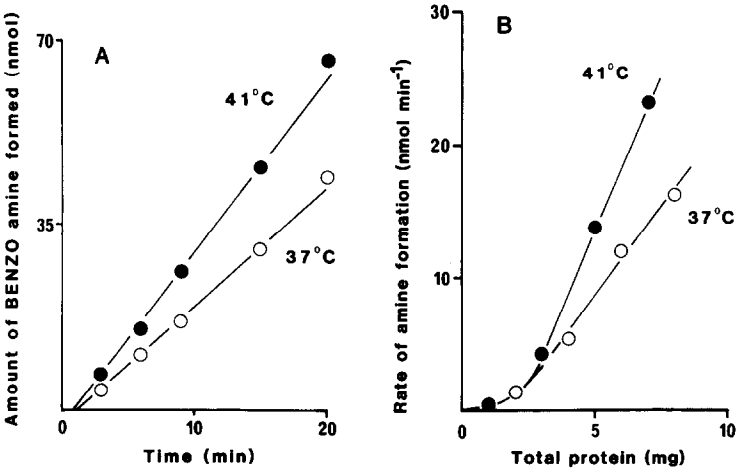

Fig. 1. (A) Typical progress curves for BENZO amine formation by mouse liver microsomes at 37° (○) and 41° (●). Incubation mixtures contained 1 mg ml^{-1} microsomal protein and 0.4 mM BENZO. (B) Effect of microsomal protein concentration and temperature on BENZO amine formation rate. Open symbols 37°, closed symbols 41°. The results shown are from two separate experiments, similar results were obtained in repeat experiments.

week-old male C3H/He mice which had been fasted overnight. After separation and recovery, microsomal pellets were washed in sodium phosphate buffer (83 mM, pH 7.4), stored at −70°, and used within 6 weeks of preparation [16]. The KHT fibrosarcoma was grown intramuscularly in the gastrocnemius muscle of the hind leg in C3H/He mice [17] and used when the mean leg diameter was 10–12 mm. KHT tumour homogenates were prepared in 83 mM sodium phosphate buffer (pH 7.4) using all-glass or Teflon–glass homogenisers. All enzyme preparations were handled on ice.

Assay procedures. Anaerobic incubations were carried out under nitrogen using specially adapted 25 ml flasks in a shaking waterbath set at the desired temperature and agitated at 100–150 strokes min^{-1}, using previously described conditions [18]. Each incubation contained 1 mg ml^{-1} microsomal protein or 800 μl of 50% (w/v) KHT whole tumour homogenate, 0.9 mM NADPH and NADH, and 83 mM sodium phosphate buffer (pH 7.4) in a final volume of 3 ml. Flasks were preincubated at the appropriate temperature and pregassed with nitrogen for 5–6 min before the reaction was started by the addition of BENZO in dimethyl sulphoxide (≤50 μl). Samples (100 or 200 μl) were removed through air-tight septa at 4–5 consecutive time points out to 20 min, and the reaction stopped by the addition of 2 vol. of methanol containing internal standard (Ro 07-0602). Analysis was by paired-ion, reverse-phase, isocratic HPLC using Waters μBondapak (C18) Rad-Pak columns (0.8 × 10 cm, 10 μm bead size; Waters Assoc., Milford, MA), with u.v. detection at 229 nm as previously described [10].

Protein was determined by the method of Lowry *et al.* [19].

Kinetic analysis. Michaelis–Menten kinetics were established using the criteria described by Henderson [20]. Apparent K_m and V_{max} values with SE limits, were determined by weighted ($W = v^2$) least-squares linear-regression analysis of $1/v$ versus $1/s$ [20] using the Generalised Linear Interactive Modelling (GLIM) programme of the Royal Statistical Society of London on an IBM mainframe computer. Half-lives were calculated from enzyme stability data using a linear regression analysis programme for a Texas instruments TI 59 desk-top calculator.

RESULTS

Characteristics of enzyme reaction

The incubation conditions used were shown to be non-limiting at 37 and 47° with respect to reduced cofactor concentration and nitrogen flow rate at 1 mM BENZO. The reaction was totally inhibited in air and there was no measurable activity using boiled microsomes or in the absence of reduced cofactors. In all cases, progress curves for the formation of BENZO amine were linear out to at least 10 and usually 20 min, following an initial short lag period (e.g. Fig. 1A). This delay may represent the time required for intermediates to accumulate before amine formation can proceed. Reaction rates were derived from linear progress curves before 50% substrate depletion occurred. The rate of amine production was linear above a microsomal protein concentration of 1 mg ml^{-1} (Fig. 1B) and enhanced at elevated temperatures (Fig. 1A and B).

Enzyme stability

Figure 2 shows the activity of liver microsomal nitroreductases catalysing BENZO amine formation after anaerobic preincubations for up to 2 hr at various temperatures. Reductase activity was very stable at 37 and 41°. There was in fact some suggestion of a small increase in activity after incubation for up to 80 min at 41°. However, at 44° and above denaturation occurred with 45% and 90% loss of activity at 44 and 47° respectively after a 1 hr incubation. Loss of activity was exponential and the half-lives were 68 and 17 min respectively.

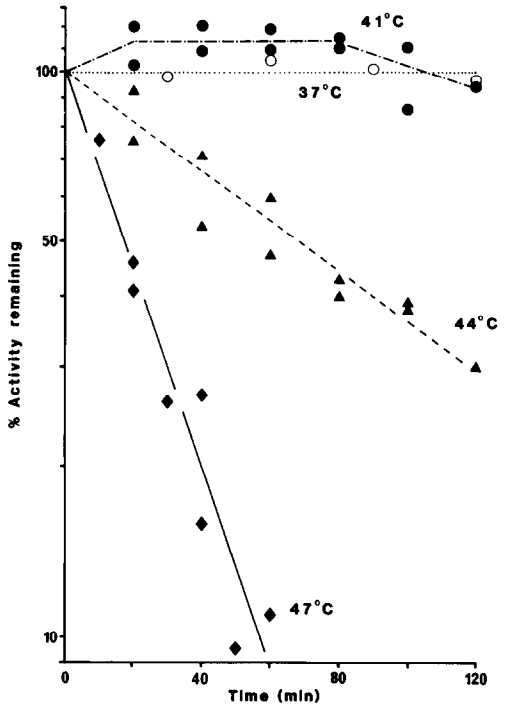

Fig. 2. Effects of anaerobic preincubation time and temperature on residual microsomal BENZO amine formation activity assayed subsequently at 37°. Symbols: ○, 37°; ●, 41°; ▲, 44°; ◆, 47°. Incubation mixtures contained 1 mg ml^{-1} microsomal protein and 1.0 mM BENZO. The preincubation protein concentration was 1 mg ml^{-1}. Pooled data from seven independent experiments.

Microsomal nitroreduction kinetics

The anaerobic generation of BENZO amine by mouse liver microsomes followed Michaelis–Menten kinetics at 33, 37 and 41° (e.g. Fig. 3A and B and Fig. 4A). Thus proportional changes in reaction rate at these different temperatures were similar at all BENZO substrate concentrations (0.005–0.4 mM). The 4° decrease in temperature from 37 to 33° resulted in substantially lower rates of BENZO amine formation with a 45% reduction in apparent V_{max} (Table 1). The 4° increment from 37 to 41° gave a similar quantitative increase in reaction rate, and the apparent V_{max} was correspondingly elevated by 32% (Table 1). The apparent K_m for BENZO amine formation was unaltered at both 33 and 41° compared to 37° (Table 1).

Table 1. Effect of temperature on the apparent K_m and apparent V_{max} for BENZO amine formation by mouse liver microsomes under anaerobic conditions *in vitro*

Microsomal preparation	Temp (°)	Apparent K_m* (μM)	Apparent V_{max}* (nmol min^{-1} mg^{-1} protein)
A	41	23.8, 21.3 (4.82) (2.85)	1.27, 1.11 (0.106) (0.058)
	37	18.4, 19.0 (3.53) (2.44)	0.89, 0.91 (0.0629) (0.0421)
B	37	20.4, 17.6 (7.07) (7.18)	1.09, 0.873 (0.105) (0.177)
	33	17.8, 21.6 (4.25) (4.76)	0.563. 0.513 (0.0656) (0.0579)

* Parameters (±SE) are shown for each of two independent determinations, with 6–7 substrate concentrations per determination. Other details are as described in the Materials and Methods.

There was a noticeable deviation from Michaelis–Menten kinetics for BENZO amine formation by liver microsomes at 44° (Fig. 4B). Thus, compared to 37°, the proportional change in reaction rate at 44° was markedly dependent on substrate concentration. For example, the rate was increased by 54% at 0.4 mM BENZO, but only by 0–15% at 0.2 mM and below.

The kinetics of microsomal BENZO disappearance under anaerobic conditions also exhibited marked deviation from the Michaelis–Menten relationship, in this case at all temperatures studied (e.g. Fig. 3C and D). Plots of s against v gave no indication of sigmoidicity at low [s] nor of any inhibition at high [s] (e.g. Fig. 3C). However, linear transformations of s/v versus s revealed two linear regions, indicative of complex kinetic behaviour, and possibly the involvement of more than one enzyme (e.g. Fig. 3D).

Stoichiometry

The stoichiometry of BENZO conversion to BENZO amine by liver microsomes was determined at two substrate concentrations for which 60–80% BENZO loss occurred after 20 min incubation (Table 2). At 37, 41 and 44° the stoichiometry was relatively constant with mean values of 2.25 and 1.85 moles of BENZO consumed for each mole of amine formed at 0.04 and 0.02 mM BENZO respectively. However, at 33° the stoichiometry of the reaction was consistently altered at both substrate concentrations such that 28–36% more BENZO was consumed for each mole of amine generated.

Tumour nitroreduction

Table 3 shows that temperature also affects the rate of amine formation by whole homogenates of KHT tumours at 1 mM BENZO concentration. A 4° temperature decrease from 37 to 33° resulted in a 21% reduction whereas an increase in temperature to 41° enhanced the rate of reaction by 35%. No further marked increase in rate ensued at 44°, but the rate was 41% faster than at 37°, and a similar 56% increase in rate occurred at 0.1 mM substrate concentration (data not shown).

The effects of temperature up to 41° are similar to those reported above for liver microsomes. However, marked differences in effect were seen at 44°. In KHT tumour preparations the amine formation rates were elevated at 44° compared to 37° to a similar extent at both 0.1 and 1 mM BENZO substrate concentrations. This increase was comparable to that occurring with liver microsomes at 0.4 mM BENZO but not at substrate concentrations <0.2 mM BENZO (see above).

DISCUSSION

Nitroreduction is implicated in many of the biological activities of nitro compounds, including the

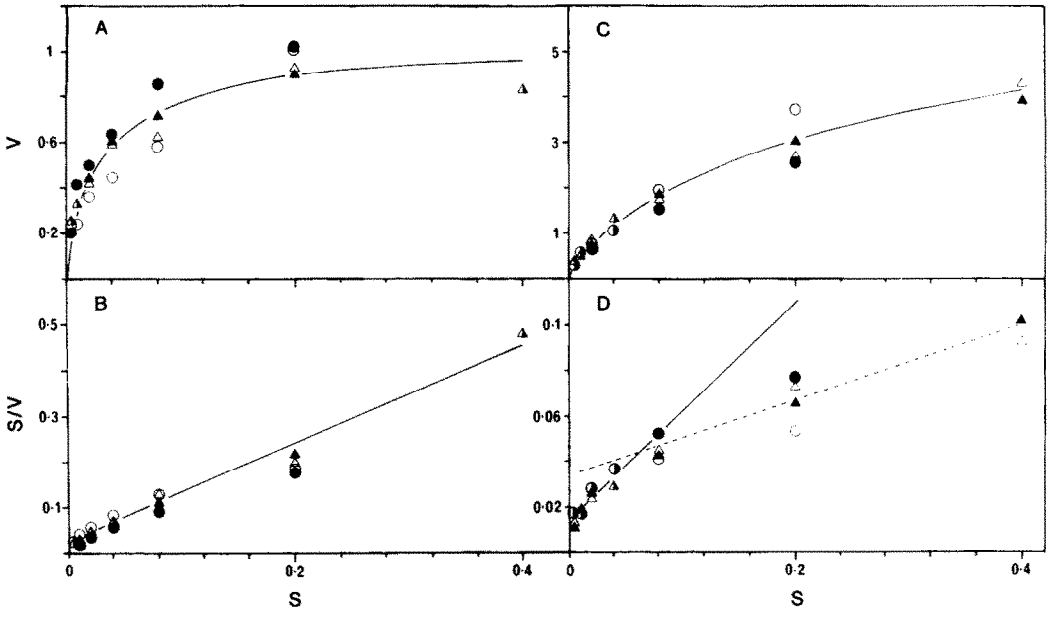

Fig. 3. Representative plots of v against s and s/v versus s for mouse liver microsomal BENZO amine formation (panel A and B respectively), and BENZO loss (panel C and D respectively) at 37°. The microsomal protein was 1 mg^{-1} ml^{-1}. Data from four independent experiments with 6–7 substrate concentrations in each. Units of v and s are nmol min^{-1} mg^{-1} protein and mM respectively. Lines were fitted to the data by eye.

preferential hypoxic cell cytotoxicity of nitroimidazole radiosensitizers [21]. Using the 2-nitroimidazole benznidazole (BENZO) currently undergoing clinical evaluation as a chemosensitizer [9], we have shown that moderate increases in temperature (33–41°) can greatly stimulate nitroreductive bioactivation by mouse liver and tumour preparations under anaerobic conditions *in vitro*. The lower temperature was investigated as transplantable mouse tumours, including the intramuscular KHT sarcoma used here, frequently grow at such hypothermic temperatures in their hosts [13, 14], and in addition because the core temperatures of mice treated with nitroimidazoles commonly fall to similarly low values [12]. The upper range was selected to cover the target temperatures currently employed in clinical hyperthermia treatments [11].

Table 2. Effect of temperature on the stoichiometry of BENZO conversion to its amine in mouse liver microsomes under anaerobic conditions *in vitro*

Substrate (mM)	Temp (°)	Stoichiometry* Rate of BENZO loss	:	Rate of amine formation
0.04	33	2.89	:	1.0
	37	2.25	:	1.0
	41	2.22	:	1.0
	44	2.27	:	1.0
0.02	33	2.52	:	1.0
	37	1.72	:	1.0
	41	1.85	:	1.0
	44	2.03	:	1.0

* Values are mean of two or four independent determinations.

Microsomal enzymes catalysing nitroreduction of BENZO to its amine were remarkably stable under anaerobic conditions at 37 and 41° but were readily inactivated at 44° and also at 47°. With other enzymes this is invariably a result of enzyme denaturation [22]. In spite of the complex, multi-step nature of the reaction, the microsomal reduction of BENZO to its amine followed Michaelis–Menten kinetics at 33, 37 and 41°. At 44° BENZO amine formation no longer followed Michaelis–Menten kinetics, precluding an estimate of the apparent K_m and V_{max}. This deviation may be a result of several factors, including enzyme denaturation and increased breakdown or covalent binding of reactive intermediates at the higher temperature. Microsomal BENZO disappearance kinetics deviated markedly from Michaelis–Menten behaviour at all four temperatures studied. Plots of s/v against s demonstrated two distinct linear regions. This type of behaviour may result from a single enzyme with unusual kinetics

Table 3. Effect of temperature on the rate of BENZO nitroreduction by KHT tumour homogenates under anaerobic conditions *in vitro*

Temp (°)	Rate of amine formation* (pmol min^{-1} mg^{-1} protein)
33	33.7 ± 5.2
37	42.5 ± 5.2
41	57.4 ± 10.3
44	59.8 ± 8.9

* Values represent mean ± SE of four independent experiments. Incubations contained 1 mM BENZO substrate.

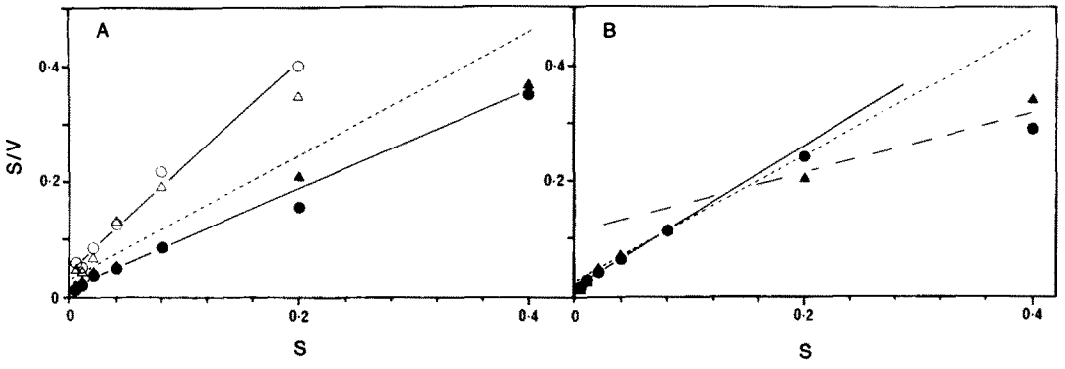

Fig. 4. (A) Plots of s/v against s for mouse liver microsomal amine formation at 33° (open symbols) and 41° (closed symbols) which exhibit Michaelis–Menten type kinetics. (B) A similar plot for amine formation at 44°, which shows deviation from Michaelis–Menten kinetics. The dotted lines represent comparable data for amine production at 37° (from Fig. 3). Two independent determinations were carried out at each temperature with 6–7 substrate concentrations in each. Units of v and s are nmol min^{-1} mg^{-1} protein and mM respectively. Lines were fitted to the data by eye.

[23], or from two different enzymes acting upon a single substrate [24]. The latter explanation is an attractive possibility in view of the reported involvement of both cytochrome P-450 and NADPH: cytochrome P-450 (cytochrome *c*) reductase in the initial 1-electron reduction step [18, 25]. The parameters K_m and V_{max} were not determined for this reaction as substrate values were chosen for computerised Lineweaver–Burk analysis of amine formation. The rate of microsomal amine formation was increased by an average of 45% at 37 compared to 33° and to a similar extent (22%) at 41 compared to 37°. These heat-enhanced bioactivation rates were associated with an increase in the corresponding apparent V_{max} though apparent K_m values were unaltered. At 44° microsomal amine production was greatly enhanced only at the highest substrate concentrations, with rates similar to those at 37° occurring at 0.2 mM BENZO and below.

Stoichiometric studies showed that roughly 2 moles of BENZO are utilised in the production of each mole of BENZO amine by liver microsomal enzymes at 37–44°, suggesting that intermediate steps were stimulated to the same extent as BENZO loss and amine formation. At 33°, however, amine generation was consistently less efficient.

As noted previously [18], KHT tumour enzymes were also able to reduce BENZO to its amine under anaerobic conditions *in vitro*, though the rates were some 20-fold lower than those occurring in microsomes under similar conditions. Because of the comparatively slow reaction rates full kinetic characterisation was not attempted. Tumour nitroreduction rates were enhanced to a similar extent to those for liver microsomal preparations over the range 33–41°. However, at 44° tumour amine generation was accelerated by 41% compared to 37°, an effect not seen with liver microsomes. This is likely to be a result of a differential effect of 44° hyperthermia on the disparate nitroreductases catalysing BENZO metabolism in liver and KHT tumours [18].

In conclusion, hyperthermia clearly enhanced BENZO nitroreduction by liver and tumour enzymes under anaerobic conditions *in vitro*. These results are consistent with the hypothesis that hyperthermia-enhanced nitroimidazole toxicity is a result of increased nitroreductive bioactivation to toxic metabolites [7], though heat-enhanced metabolite reactivity may also be involved. Tumour-selective enhancement of the cytotoxicity of bioreductively activated drugs might be achieved not only through localisation of the hyperthermia treatment, but also by exploiting potential differences in the degree of heat-stimulated bioactivation resulting from the presence of different activating enzymes in tumour and normal tissues. Studies are now in progress to determine the extent of nitroreductive bioactivation in KHT tumours subjected to localised hyperthermia *in vivo*.

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REFERENCES

1. G. E. Adams, in *Radiosensitizers of Hypoxic Cells* (Eds. A. Breccia, C. Rimondi and G. E. Adams), p.13. Elsevier/North-Holland, Amsterdam (1979).
2. K. C. George, D. G. Hirst and N. J. McNally, *Br. J. Cancer* **35**, 372 (1977).
3. G. E. Adams, I. J. Stratford and S. Rajaratnam, *Natnl. Cancer Inst. Monogr.* **61**, 27 (1982).
4. N. M. Bleehen, D. J. Honess and J. E. Morgan, *Br. J. Cancer* **35**, 299 (1977).
5. A. M. Rauth, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1293 (1984).
6. D. S. Hewick, in *Metabolic Basis of Detoxication, Metabolism of Functional Groups* (Eds. W. B. Jakoby, J. R. Bend, J. Caldwell), p. 151. Academic Press, New York (1982).
7. P. Olive, *Cancer Biochem. Biophys.* **2**, 155 (1978).
8. J. R. Coura, P. J. Brindeiro and I. Ferreira, in *Current Chemotherapy*, Vol. 1 (Eds. W. Siegenthaler and R. Luthy), p. 161. American Society for Microbiology, Washington, DC (1978).

9. N. M. Bleehen, J. T. Roberts and H. F. V. Newman, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1401 (1986).
10. M. Walton and P. Workman, *J. Chromatogr* **375**, 190 (1986).
11. J. Overgaard (Ed.), *Proceedings of the 4th International Symposium on Hyperthermic Oncology*. Taylor & Francis, London (1984).
12. C. J. Gomer and R. S. Johnson, *Radiat. Res.* **78**, 329 (1979).
13. M. D. O'Hara, F. W. Hetzel and S. Frinak, *Int. J. Radiat. Oncol. Biol. Phys.* **11**, 817 (1985).
14. M. C. Joiner, S. A. Hill, J. C. M. Bremner, K. A. Smith and J. Denekamp, *Int. J. Radiat. Biol.* **48**, 235 (1985).
15. M. K. Wolpert, J. R. Althaus and D. J. Johns, *J. Pharmac. exp. Ther.* **185**, 202 (1973).
16. P. Borton, R. Carson and D. J. Reed, *Biochem. Pharmac.* **23**, 2332 (1974).
17. P. R. Twentyman, R. F. Kallman and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1255 (1979).
18. M. Walton and P. Workman, *Biochem. Pharmac.* **36**, 887 (1987).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. P. J. F. Henderson, in *Techniques in Protein and Enzyme Biochemistry* (Eds. H. L. Kornberg, J. C. Metcalfe, D. H. Northcote, C. I. Pogson and K. F. Tipton), Vol B1/11, p. 1. Elsevier/North-Holland Biomedical Press, Amsterdam (1978).
21. J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 675 (1982).
22. M. Dixon and E. C. Webb, in *Enzymes* (3rd Edn). Longman, London (1979).
23. A. Levit and D. E. Koshland, *Proc. natn. Acad. Sci. U.S.A.* **61**, 1121 (1969).
24. E. G. Loten and J. G. T. Sneyd, *Biochem. J.* **120**, 187 (1970).
25. M. Masana, G. D. de Toranzo and J. A. Castro, *Biochem. Pharmac.* **31**, 1041 (1984).