hexachlorobenzene induced porphyria [4] and as to why there are inter-lobe differences in susceptibility to cell transformation and tumour production, as has been recently demonstrated for diethylnitrosamine-induced tumours [16].

In summary we have determined the concentration of total cytochrome P-450 and the major PB and 3 MC induced isoenzymes in microsomes prepared from the lobes of control and induced rat liver. We have found an asymmetry with respect to the distribution of total cytochrome P-450 in control animals. Treatment of animals with PB enhanced the asymmetry with respect to total cytochrome P-450 while MC treatment provided a reversed gradient. The contribution of the PB and MC induced isoenzymes remained a constant proportion of the total cytochrome P-450 within the lobes. Our data support the current concept of intra-lobular heterogeneity in the liver and may provide a rational for the differences in lobe susceptibility to xenobiotic induced damage.

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REFERENCES

1. A. Y. H. Lu and S. West, *Pharmac. Rev.* **31**, 277 (1980).
2. K. Jungerman and N. Katz, *Metabolic Compartmentation* (Ed. H. Siess), p. 21, Academic Press, New York (1982).
3. T. Matsubara, S. Mori, A. Touchi, Y. Masuda and Y. Takeuchi, *Jap. J. Pharmac.* **33**, 435 (1983).
4. A. G. Smith, J. E. Francis and F. De Matteis, *Biochem. Pharmac.* **29**, 3127 (1980).
5. T. Matsubara, A. Touchi and A. Ogawa, *Jap. J. Pharmac.* **32**, 999 (1982).
6. J. W. De Pierre and G. Dallner, *Biochemical Analysis of Membranes* (Ed. A. H. Maddy), p. 79. Chapman & Hall, London (1976).
7. R. Hebel and M. W. Stromberg, *Anatomy of the Laboratory Rat*, p. 50. The Williams & Wilkins Company, Baltimore, MD (1976).
8. F. P. Guengerich and M. V. Martin, *Archs Biochem. Biophys.* **205**, 365 (1980).
9. A. Johnstone and R. Thorpe, *Immunochemistry in Practice*. Blackwell Scientific Publications, Oxford (1982).
10. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. I. R. Phillips, E. A. Shepard, R. M. Bayney, S. F. Pike, B. R. Rabin, R. Heath and N. Carter, *Biochem. J.* **212**, 55 (1983).
13. T. A. Lawson and A. W. Pound, *Br. J. exp. Pathol.* **55**, 583 (1974).
14. R. W. Brauer, *Physiol. Rev.* **43**, 115 (1963).
15. T. T. Kawabata, F. P. Guengerich and J. Baron, *Molec. Pharmac.* **20**, 709 (1981).
16. F. C. Richardson, J. A. Boucheron, M. C. Dyroff, J. A. Popp and J. A. Swenberg, *Carcinogenesis* **7**, 247 (1986).

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The effect of electron-affinic radiosensitizers on ATP levels in V79 379A Chinese hamster cells

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The full potential of nitroaryl radiosensitizers to improve the efficiency of clinical radiotherapy in killing relatively radio-resistant hypoxic tumour cells [1] has not been realized because the toxicity of, for example, nitroimidazoles *in vivo* limits the maximum clinical concentration of misonidazole that can be achieved to ~0.1 mmol/dm^3 in a multifraction radiotherapy regime [2]. The 2-nitroimidazole, misonidazole, has many effects on cellular metabolism (especially at higher concentrations) including depletion of non-protein thiols (NPSH*) and protein thiols [3], stimulation of oxygen consumption [4–6], inhibition of glycolysis [7] and perturbation of purine metabolism [8].

The importance of ATP in repair processes [9–11] and other metabolic pathways means that large changes in cellular ATP levels may affect the ability of cells to survive stresses such as radiation or drug toxicity. Slight radioprotection has been observed with aerobic cells depleted of ATP by treatment with 2-deoxy-D-glucose and rotenone in growth medium with serum [11] or by post-irradiation treatment with 2,4-dinitrophenol in phosphate-buffered saline (PBS) [12, 13]; a more severe post-irradiation treatment with 2,4-dinitrophenol which greatly delayed recovery of ATP levels increased the cellular radiosensitivity [13]. In contrast, uncouplers including 2,4-dinitrophenol radiosensitize aerobic bacteria [14] and mammalian cells (Hodgkiss, unpublished data) when present during, but not after, irradiation.

Previous reports have indicated that misonidazole reduces the amount of ATP in mammalian cells during short (30 min) incubations [8]. In this paper we present data on the ATP content of mammalian cells incubated under a range of conditions commonly used in radiobiology and drug toxicity experiments, and the effect of electron-affinic radiosensitizers on the ATP and non-protein thiol content of mammalian cells.

Materials and methods

V79 379A Chinese hamster cells were maintained as exponentially growing suspension cultures in Eagle's Minimum Essential medium (MEM) modified for suspension culture, with 7.5% foetal calf serum (FCS). ATP monitoring kits were obtained from LKB Instruments Ltd. Misonidazole and Ro 05-9963 were obtained from Dr C. E. Smithen, Roche Products Ltd.; MOA-16 was obtained from Dr J. Parrick and Mr M. Moazzam, Brunel University, Uxbridge; metronidazole and nitrofurantoin were obtained from Sigma Chemical Co.; CMNI was obtained from Aldrich Chemical Co. and 2,4-dinitrophenol was obtained from BDH and purified by recrystallization from hot methanol. PBS was prepared from Dulbecco "A" tablets, Oxoid Ltd. Other reagents were BDH "AnalaR" grade.

* Abbreviations: ATP, adenosine 5'-triphosphate; MEM, Eagle's minimal essential medium; FCS, foetal calf serum; PBS, phosphate-buffered saline; EDTA, ethylene diamine tetra-acetic acid; NPSH, non-protein thiol.

For ATP measurements, cell suspensions stirred at 37° in 0.1 dm^3 Dreshel bottles, modified with a side arm for sampling, were gassed at 0.4 dm^3/min with air + 5% CO_2 or nitrogen + 5% CO_2 (<10 ppm O_2) obtained from the British Oxygen Co. Ltd. Cultures were pre-gassed for 1 hr before the addition of small volumes of drug solutions as appropriate. One-cm^3 samples of cell suspensions were withdrawn through the side arm at various times and immediately mixed with an equal volume of 0.1 mmol/dm^3 sodium hydroxide + 2 mmol/dm^3 ethylene diamine tetra-acetic acid (EDTA) to achieve rapid cell lysis. This was thought to be important in view of the rapid turnover of the ATP pool in mammalian cells [15]. Lysed cell samples were diluted with 8 cm^3 buffer (0.1 mol/dm^3 tris-acetate + 2 mmol/dm^3 EDTA pH 7.3). ATP concentrations were measured using LKB bioluminescence firefly reagent (0.1 cm^3 reagent + 0.4 cm^3 cell extract) in an LKB 1250 luminometer with internal and external standardization of each set of samples. ATP standard curves showed the luminometer reading to be linear with respect to ATP concentration from 10^{-9} mol/dm^3 to 2×10^{-6} mol/dm^3 ATP in the reaction mixture. All ATP measurements are the mean of two replicate determinations and include intra-cellular and extracellular ATP; the latter contributes <10% and usually 2–5% of the ATP in samples. ATP has a half-life of 19 min in growth medium at 37° and none is found in sterile medium. Thus any ATP in the medium has leaked out of the cells and may be considered a valid part of the sample, reflecting the ability of cells to make ATP. The origins and effects of extracellular ATP *in vitro* and *in vivo* have been reviewed [16].

The luciferase enzyme system in the LKB firefly reagent is inhibited by high concentrations of nitroaromatic compounds, e.g. 80% of control activity with 11 mmol/dm^3 misonidazole or 0.085 mmol/dm^3 2,4-dinitrophenol. Dilutions carried out during sample preparation reduce the drug concentrations to below that at which significant inhibition is observed, and drugs were included at appropriate concentrations in standards.

ADP and AMP in rapidly lysed cell samples were converted to ATP using methods described in ref. [17] and the resulting ATP measured as described above. Measurement of spiked samples showed that no significant loss of nucleotides occurred during the cell lysis procedure and that quantitative conversion to ATP occurred. For each cell sample, parallel assays were carried out on samples of growth medium from which cells had been removed by centrifugation; nucleotide concentrations in growth medium were subtracted from those for the corresponding cell samples before energy charge calculations were carried out. Adenylate energy charge was calculated as

$$([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]).$$

Cell samples taken before and at various times after addition of drugs were centrifuged to remove the drug solution and plated on 5-cm plastic petri dishes to assess viability in a 7-day colony forming assay. The method of estimating NPSH content of cells has been described [18].

Results and discussion

V79 379A Chinese hamster cells, grown as described, typically have 6–7 fmol/cell ATP with ~20% variation in the absolute level between experiments. No consistent differences were seen between cells grown in medium without drugs in aerobic and hypoxic conditions. In PBS, cellular ATP falls rapidly to 20% of controls in 4 hr under oxic conditions and 10% of controls in 1 hr hypoxia. Misonidazole (10 mmol/dm^3) has little detectable effect on the rate of loss of ATP in PBS but 0.1 mmol/dm^3 2,4-dinitrophenol accelerates it in both oxic and hypoxic conditions. Addition of 10 mmol/dm^3 glucose to hypoxic cells after 1 hr in PBS and in PBS with 2,4-dinitrophenol or misonidazole rapidly restores ATP levels demonstrating the importance of glucose as an energy source. By contrast, addition of 10 mmol/dm^3 pyruvate to PBS has little effect on the rate of loss of ATP in hypoxia as expected from the dependence of hypoxic cells on glycolysis as a source of ATP [15, 19, 20]; however, under aerobic conditions 10 mmol/dm^3 pyruvate allows some ATP production. In PBS with 10% FCS cellular ATP levels fall more slowly than in PBS alone, under control conditions and with misonidazole or 2,4-dinitrophenol. Addition of glucose to cells in PBS and 10% FCS after an incubation of 1 hr has a similar effect on ATP levels to that seen with PBS alone.

Radiobiological studies on electron-affinic radiosensitizers have been carried out both in full growth medium (e.g. [21, 22]) and in PBS (e.g. [23–25]). We have recently shown nitro-aromatic radiosensitizers to have a greater radiosensitizing efficiency in PBS compared with full growth medium [26]. Clearly care should be taken when comparing radiosensitizing efficiency data from different sources when the irradiation conditions are such that large differences in energy status may be anticipated.

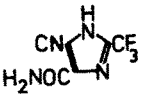
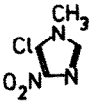
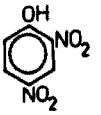
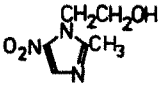
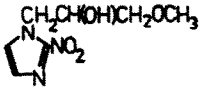
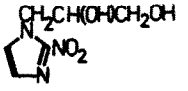
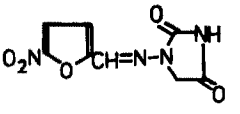
Structures of compounds used in this study together with their reduction potentials are summarized in Table 1. Figure 1 shows that in cells incubated in hypoxic growth medium with the electron-affinic compounds misonidazole, 2,4-dinitrophenol and MOA-16 there is a concentration-dependent increase in cellular ATP after a lag of 1–2 hr paralleling the time courses of toxicity and NPSH depletion, Fig. 2. Similar results are obtained with nitrofurantoin, CMNI and Ro 05-9963 (the desmethyl derivative of misonidazole). Nitrofurantoin (0.1 mmol/dm^3) has a similar effect on NPSH levels to 5 mmol/dm^3 misonidazole. The low electron-affinity compound metronidazole has no effect on ATP levels in hypoxic cells at 20 mmol/dm^3. Although some of these data appear to conflict with observations in ref. [18], this work used much shorter (30 min) incubations and obtained relatively small effects.

Ratios of adenylate nucleotides embodied in the parameter "energy charge" are sometimes used as a measure of the energy status of cells. However, hypoxia and misonidazole in hypoxic conditions has no effect on adenylate energy charge; values and standard errors of 0.87 ± 0.03 in aerobic medium, 0.88 ± 0.01 in hypoxic medium and 0.88 ± 0.02 in hypoxic medium with 5 mmol/dm^3 misonidazole were obtained after 5-hr incubations and similar values were obtained after shorter incubations. Bump *et al.* [31] reported that large changes in energy charge in CHO cells were accompanied by only small changes in radiation response; Calderwood *et al.* [32] found that energy charge was not correlated with cell survival during heat treatment although a correlation was found with viability in starved cells. In the present work, energy charge is not correlated with changes in viability, NPSH levels or ATP levels.

Anderson *et al.* [14] suggested that uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol overstimulate glycolysis, leading to elevated ATP levels in aerobic bacterial cells. This is consistent with our observation that no enhanced ATP levels are seen with pyruvate as a substrate when oxic or hypoxic mammalian cells are incubated with 2,4-dinitrophenol or misonidazole. However, Varnes and Biaglow [7] reported inhibition of anaerobic glycolysis by a rather toxic misonidazole treatment.

Rates of increase in cellular ATP levels in hypoxic growth medium were measured over the range of available concentrations for each compound (3–6 concentrations) and concentrations required to increase ATP levels by a fixed amount per hour are shown in Table 1 (an increase of 30% of the control level per hour allowed the inclusion of all compounds). The lack of effect of metronidazole on ATP levels compared with the effect of misonidazole and the stronger oxidant, nitrofurantoin, strongly suggest a redox relationship within the class of simple nitro-aromatic compounds as observed with several other biological endpoints

Table 1. Properties of compounds used in this paper

Compound	Structure	E_7^1*	C_{ATP}†	
MOA-16	1	<−1.0‡	0.48	
CMNI	2	−0.534§	0.1	
2,4-Dinitrophenol	3	−0.5¶	0.058	
Metronidazole	4	−0.468‖**	>20.0	
Misonidazole	5	−0.389‖**	4.0	
Ro-05-9963	6	−0.389‖	4.0	
Nitrofurantoin	7	−0.264‖	0.054	

* Reversible one-electron reduction potential in vitro *vs.* the normal hydrogen electrode.
† Drug concentration in mmol/dm³ required to increase ATP concentrations by 30%/hr.
‡ Ref. 21. § Ref. 25. ¶ Ref. 20. ‖ Ref. 22. ** Ref. 23.

[28] including toxicity. CMNI is anomalous in its reactivity towards thiols [33] whilst 2,4-dinitrophenol and MOA-16 share the properties of an acid function characteristic of many uncouplers of oxidative phosphorylation (the pK_a of MOA-16 is <2 [34]).

Misonidazole and other electron affinic compounds increase ATP levels in hypoxic cells incubated in full growth medium at as little as 1 mmol/dm³, a concentration that can be achieved *in vivo* and even in humans as a single dose [35]. We therefore believe that perturbation of ATP

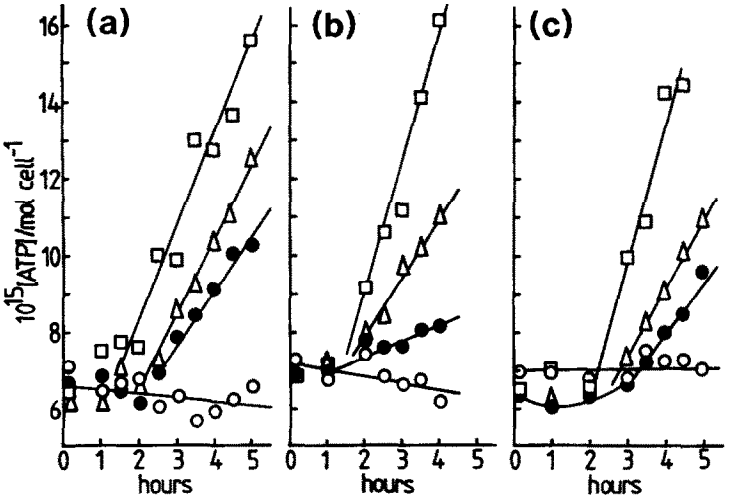


Fig. 1. ATP content of hypoxic V79 379A Chinese hamster cells incubated in MEM + 7.5% FCS. (a) misonidazole: (○) no drug; (●) 2.5 mmol/dm³; (△) 5 mmol/dm³; (□) 10 mmol/dm³. (b) 2,4-dinitrophenol: (○) no drug; (●) 0.025 mmol/dm³; (△) 0.05 mmol/dm³; (□) 0.1 mmol/dm³. (c) MOA-16: (○) 0 mmol/dm³; (●) 0.25 mmol/dm³; (△) 0.5 mmol/dm³; (□) 1 mmol/dm³.

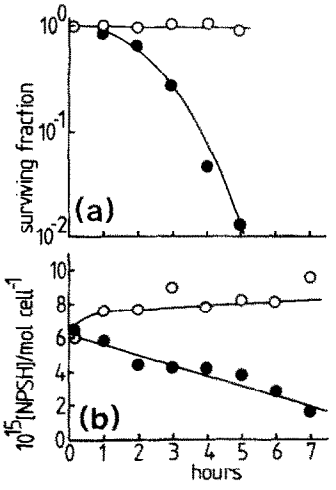


Fig. 2. Hypoxic cells incubated in MEM + 7.5% FCS. (a) Survival. (b) NPSH content: (○) untreated; (●) 5 mmol/dm^3 misonidazole. Points plotted represent the mean of two replicate experiments.

levels by electron-affinic compounds may be of significance *in vivo* and that its consequences should be considered in relation to *in vivo* toxicities such as the clinically observed peripheral neuropathy.

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REFERENCES

1. G. E. Adams, *Br. Med. Bull.* **29**, 48 (1977).
2. S. Diche, M. I. Saunders, I. R. Flockhart, M. E. Lee and P. Anderson, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 581 (1979).
3. J. E. Biaglow, in *Hyperthermia* (Eds. H. I. Bicher and D. F. Bruley). Plenum Press, New York, p. 347 (1982).
4. P. J. Ainsworth, M. Channon, R. Sridhar, B. Gushulak and E. R. Tustanoff, *Can. J. Biochem.* **56**, 457 (1978).
5. C. L. Greenstock, J. E. Biaglow and R. E. Durand, *Br. J. Cancer* **37**, 11 (1978).
6. J. E. Biaglow, M. E. Varnes, B. Jacobson and C. B. Schroy, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 27. Masson, U.S.A. (1980).
7. M. E. Varnes and J. E. Biaglow, *Biochem. Pharmac.* **31**, 2345 (1982).
8. J. F. Henderson and G. Zombor, *Biochem. Pharmac.* **29**, 2533 (1980).
9. V. K. Jain, I. Gupta and K. Lata, *Radiation Res.* **92**, 463 (1982).
10. A. J. Moss, J. R. Glenn, V. Dalrymple, J. L. Sanders, K. P. Wilkinson and J. C. Nash, *Biophys. J.* **11**, 158 (1971).
11. E. A. Bump, S. K. Calderwood, J. M. Sawyer and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1411 (1984).
12. G. V. Dalrymple, J. L. Sanders and M. L. Baker, *Nature, Lond.* **216**, 708 (1967).
13. K. Nishizawa, C. Sato and T. Morita, *Int. J. Radiat. Biol.* **35**, 15 (1979).
14. R. F. Anderson, K. B. Patel and M. D. Evans, *Int. J. Radiat. Biol.* **48**, 495 (1985).
15. S. Skog, B. Tribukait and G. Sundius, *Exp Cell Res.* **141**, 23 (1982).
16. J. L. Gordon, *Biochem. J.* **233**, 309 (1986).
17. J. F. Martin, P. Liras and A. L. Demain, *Biochem. biophys. Res. Commun.* **83**, 822 (1978).
18. R. J. Hodgkiss and R. W. Middleton, *Biochem. Pharmac.* **34**, 2175 (1985).
19. M. Donnelly and I. E. Scheffler, *J. Cell Physiol.* **89**, 39 (1976).
20. M. J. Morgan, K. M. Bowness and P. Faik, *Biosci. Rep.* **1**, 811 (1981).
21. G. E. Adams, E. D. Clarke, P. Gray, R. S. Jacobs, I. J. Stratford, P. Wardman and M. E. Watts, *Int. J. Radiat. Biol.* **35**, 151 (1979).
22. G. E. Adams, E. D. Clarke, I. R. Flockhart, R. S. Jacobs, D. S. Sehmi, I. J. Stratford, P. Wardman, M. E. Watts, J. Parrick, R. G. Wallace and C. E. Smithen, *Int. J. Radiat. Biol.* **35**, 133 (1979).
23. B. C. Millar, E. M. Fielden and J. J. Steele, *Int. J. Radiat. Biol.* **36**, 177 (1979).
24. B. C. Millar, E. M. Fielden and J. J. Steele, *Radiat. Res.* **82**, 478 (1980).
25. B. C. Millar, E. M. Fielden and J. J. Steele, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 450. Masson, U.S.A. (1980).
26. M. E. Watts, M. F. Dennis, R. J. Hodgkiss, N. R. Jones and M. Woodcock, *Proc. 7th Int. Congress Rad. Res.* (Eds J. J. Broerse, G. W. Barendsen, H. B. Kal, A. J. Van Der Kogel), B6-30 (1983).
27. P. Wardman, R. F. Anderson, R. J. Hodgkiss, J. Parrick, C. E. Smithen, R. G. Wallace and M. E. Watts, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 399 (1982).
28. M. E. Watts, R. F. Anderson, R. S. Jacobs, K. B. Patel, P. Wardman, M. Woodcock, C. E. Smithen, M. Moazzam, J. Parrick and R. G. Wallace, in *Radiation Sensitizers* (Eds by L. W. Brady), p. 175. Masson, U.S.A. (1980).
29. G. E. Adams, I. J. Stratford, R. G. Wallace, P. Wardman and M. E. Watts, *J. natn. Cancer Inst.* **64**, 555 (1980).
30. E. D. Clarke and P. Wardman, *Int. J. Radiat. Biol.* **37**, 463 (1980).
31. E. A. Bump, S. K. Calderwood, J. M. Sawyer and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1411 (1984).
32. S. K. Calderwood, E. A. Bump, M. A. Stevenson, I. V. Kersen and G. M. Hahn, *J. Cell. Physiol.* **124**, 261 (1985).
33. P. Wardman, *Int. J. Radiat. Biol.* **41**, 231 (1982).
34. P. Wardman, E. D. Clarke, R. S. Jacobs, A. Minchinton, M. R. L. Stratford, M. E. Watts, M. Woodcock, M. Moazzam, J. Parrick, R. G. Wallace and C. E. Smithen, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 83. Masson, U.S.A. (1980).
35. A. J. Gray, S. Dische, G. E. Adams, I. R. Flockhart and J. L. Foster, *Clin. Radiol.* **27**, 151 (1976).