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SELECTIVE MANIPULATION OF TUMOUR CELL ENERGY METABOLISM

by

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Submitted in partial fulfilment for the degree of
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University of Glasgow

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University of Glasgow

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SUMMARY

Rapidly growing, glycolytic tumours which are poorly differentiated were shown to contain functional mitochondria capable of oxidative phosphorylation. Mitochondria were isolated and assessed for use in screening potential antimitochondrial agents in vitro. As this model proved to be unsuitable, a second model, intact cells were employed for screening purposes. The effect of antimitochondrial agents on the metabolism of cultured tumour cells was compared to that in freshly isolated rat hepatocytes.

Rhodamine-6G (R6G) a cationic, fluorescent mitochondrial probe, altered oxygen consumption rates and increased lactate production in tumour cells in vitro. At the same concentrations R6G had little effect on the metabolism of isolated hepatocytes. Lamprene, an antileprosy agent also altered cellular oxygen consumption rates in tumour cells at comparable concentrations to R6G. Classical mitochondrial inhibitors were used to demonstrate that R6G has a biphasic action upon cellular oxygen uptake. Initially oxygen uptake is uncoupled from ADP phosphorylation, followed by subsequent inhibition of oxygen consumption suggesting that electron transport is also inhibited. Lamprene behaves like a classical uncoupler of tumour mitochondria, stimulating oxygen consumption in the presence of an inhibitor of phosphorylation.

Growth of tumour cells in vitro in the presence of 5 fluorouracil (5FU) did not appear to inhibit mitochondrial biogenesis as assessed by the activity of specific mitochondrial enzymes. It was concluded that either the drug concentration required to inhibit mitochondrial biogenesis was cytotoxic or the duration of exposure to drug was too short to adequately dilute mitochondrial protein.

Tumour cells grown in glutamine as the major sources of carbon depend upon their mitochondria for energy production. Although the phosphorylation inhibitor oligomycin did exhibit an enhanced toxicity toward cells grown in glutamine, R6G showed identical toxicity to

cells grown either in glucose and glutamine or glutamine alone. This suggests that the toxicity of R6G might not be due to an anti-mitochondrial action.

Combining lamprene with the antiglycolytic agent 2-deoxyglucose did appear to delay growth of a human tumour xenograft implanted in athymic mice, though this was not clearly observed, due to a reduced food intake by animals. However, R6G and 5FU did significantly delay tumour growth, although the mechanism of action remains to be established.

This study has shown that antimitochondrial agents may be of some value in anticancer chemotherapy and highlights methods by which potential agents might be identified.

CHAPTER 1

GENERAL INTRODUCTION

Limitations of Conventional Cancer Chemotherapy

Conventional anticancer agents tend to act non-selectively, altering the metabolism of both neoplastic cells and non-tumour cells within the cancer patient (Malpas, 1986). It is generally assumed that all tumours grow rapidly with cell turnover rates which are far greater than host cells within a patient. Those tumours successfully treated with conventional chemotherapy do have rapid growth rates such as for example, Hodgkin's disease, testicular teratoma or Burkitt's lymphoma (Pitot, 1986). There are however, normal tissues within the body, such as, GI tract and bone marrow which would be affected by the drugs used.

Conventional chemotherapy is directed at ^{tissues} with a large growth fraction. Such tissues have a substantial ^A percentage of cells undergoing division at any one time. Therefore, use of cytostatic or cytotoxic drugs is limited by the toxicity of such agents to bone marrow or GI tract, which ^{also} have a large growth fraction (Pitot, 1986; Tannock, 1987). Slow growing tumours such as pulmonary or gastric cancers and others, where the growth fraction is small are less affected by inhibitors of DNA synthesis which comprise a large fraction of the drugs used in chemotherapy (Pitot, 1986).

Energy Metabolism in Normal and Neoplastic Tissues

A major aim of this thesis is to explore a selective approach to chemotherapy. Mammalian cell survival is dependent upon the ability of the cell to metabolize suitable carbon sources to generate Adenosine triphosphate (ATP) for biosynthetic or maintenance purposes, a feature common to both neoplastic and normal cells. Turnover between ATP, Adenosine diphosphate (ADP) and inorganic phosphate (Pi) enable both tumour and normal cells to regulate their environment.

The oxygenated state of the cell is thought to determine by which metabolic pathways ATP is generated. Under anaerobic conditions

glycolysis yields 2 molecules of ATP from a hexose substrate such as glucose and 2 molecules of NADH. NAD may be regenerated following reduction of pyruvate to lactate.

Utilisation of ATP by membrane bound Na^+ K^+ ATPases will regenerate ADP and this together with NAD^+ will stimulate further glycolysis (Saier, 1987). Under aerobic conditions mitochondrial pathways will be activated, oxidation of the glucose derived carbon skeleton in the tricarboxylic acid (TCA) cycle will generate reducing equivalents NADH or FADH. According to the chemiosmotic theory, oxidation of reduced nucleotides, obtained via both glycolysis and the TCA cycle, to water, with oxygen as the final electron acceptor generates a proton gradient which is used to synthesize ATP (Mitchell, 1975; Mitchell, 1976). Complete oxidation of glucose yields 36 molecules of ATP. Thus whether pyruvate is oxidised through TCA cycle activity or reduced to lactate is dependent on oxygenation of the cell and thus is the key to respiratory control of metabolic flux.

In 1930, Warburg observed that malignant tumours exhibited high rates of glycolysis. Furthermore, whereas normal cells in the presence of oxygen underwent aerobic respiration with reduced or no lactate production (Pasteur effect), tumour cells exhibited high rates of lactate production even in the presence of oxygen (Warburg, 1930; Weinhouse, 1972; Weinhouse, 1982). Warburg postulated that cancer was essentially related to the reduced capacity of oxygen to reduce glycolysis. However, recent work ^{has shown} that although rapidly growing tumours were highly glycolytic, such tumours were poorly differentiated (Pedersen, 1978). Slower growing, well-differentiated tumours could utilise oxygen, coupling oxygen consumption to phosphorylation, thus indicating the intactness of the mitochondrial respiratory chain (Gregg, 1972; Racker, 1976; Pedersen, 1978). Explanations for how slow growing, well-differentiated hepatomas exhibited abnormal patterns of energy metabolism were based on altered expression of glycolytic enzymes. Such tumours were thought to have a mitochondrially bound hexokinase (ATP:hexose 6-phosphotransferase E.C. 2.7.1.1). Thus the tumour could use glycolytic pyruvate for mitochondrial metabolism without expressing

the high rates of glycolysis seen in poorly differentiated hepatomas (Pedersen and Bustamante, 1982; Bustamante et al, 1981). High rates of glycolysis in poorly differentiated tumours have been accounted for, according to some workers by abnormally increased activity of membrane-bound $\text{Na}^+ \text{K}^+$ ATPases generating large quantities of ADP. Thus glycolysis is stimulated by ADP (Racker and Spector, 1981). To some extent detailed evidence for this has been discredited (Racker, 1981). It is clear that while some tumours exhibit high rates of glycolysis, this is not a universal feature as confirmed by studies in vivo (Reichard et al, 1964; Weinhouse, 1982; Sauer and Dauchy, 1986). A study of fatty acid oxidation in both well-differentiated and poorly differentiated hepatomas indicated that lower substrate oxidation rates obtained, in comparison to normal liver, were explained more by defects in fatty acid shuttles rather than a defect in mitochondrial respiratory function (Cederbaum and Rubin, 1976).

Although the degree of differentiation of a particular tumour cell may indicate preferred metabolic paths for ATP generation, substrate availability plays a key role in determining whether tumour cells in vitro preferentially undergo glycolysis at the expense of mitochondrial metabolism. Glucose and other α -glucopyranoses such as galactose, mannose or fructose are primary glycolytic substrates (Lehninger, 1975). Ample evidence correlates glycolysis with glucose utilisation in tumour cells (Weinhouse, 1972; 1982). However recent evidence has shown that cultured HeLa cells and other tumour cells utilise glutamine, even when glucose is present with up to 35% of the amino acid used to derive ATP from oxidative metabolism (Kovacevic and Morris, 1972; Reitzer et al, 1979; Moreadith and Lehninger, 1984). In some cultured cell lines however, although glutamine might support ATP production, glucose was a preferred substrate. Cells are unable to maintain ATP if deprived of glucose (Demetrakopoulos et al, 1978; 1982; Nakashima et al, 1984). In order for mitochondrial substrates to be utilised, oxygen must be present. Access to oxygen in tumours in vivo is dependent upon how well supplied with blood the tissue is. Hypoxic regions, as shown by certain models in vitro cannot utilise glutamine and thus glucose is of more importance (Sutherland et al, 1986; Tannock et al, 1986; Hlatksy et al, 1988). However, the largest

tumour cell growth fraction is often in oxygenated regions, where glutamine utilisation could in theory occur. Evidence for utilisation of mitochondrial substrates has been obtained for rapidly growing, poorly differentiated tumours (Sauer et al, 1982; Sauer and Dauchy, 1983; Mares-Perlman and Shrago, 1988). In summary, the relative contributions of glycolysis or mitochondrial metabolism to ATP generation in the tumour cell is dependent not only on the differentiated state of the cell, but also on the availability of substrates and oxygen.

Mitochondria are found in both poorly differentiated and well differentiated tumour cells. Morphological differences observed in glycolytic, poorly differentiated tissues compared to normal cells of the same cell lineage have been attributed to a reduced capacity to oxidise carbon substrates. Certain substrates such as B-OH butyrate show reduced oxidation rates or cannot be metabolised when compared to preparations of isolated normal mitochondria (Kaschnitz et al, 1976; Cederbaum and Rubin, 1976; Nakashima et al, 1984). Yet in their capacity to oxidise other substrates tumour mitochondria obtained from either well differentiated or poorly differentiated tumours do not show any differences compared to normal tissues of the same lineage. Thus generalisations about tumour mitochondria, as compared to normal mitochondria, are difficult to make (Pedersen, 1978).

Inhibition of Tumour Cell Energy Metabolism

Mitochondrial Inhibitors

It was thought that the cytotoxicity of alkylating agents, used in conventional cancer chemotherapy, could be explained partly by an antimitochondrial action (Gosalvez et al, 1974; 1976; Bernal et al, 1982(a); Belousova, 1984). However until recently, few compounds had been shown to exert a selective toxicity toward tumour cells.

The rhodamine dyes rhodamine 123 (R123) and rhodamine 6G both cationic fluorescent dyes are thought to exert a selective toxicity toward tumour cells. R123 has been shown to accumulate in living cells only and its associated fluorescence was localised in mitochondria for the rodent cell lines screened (Johnson et al, 1980). Selective

accumulation in mitochondria of living cells was interpreted as dependence on the high transmembrane potential generated across the inner-mitochondrial membrane (Johnson et al, 1981). The retention time of R123 was increased in virus transformed mink fibroblast cells compared to untransformed cells, indicating that transformed cells have a defective transmembrane potential. Nigericin an ionophore exchanging H^+ ions for K^+ ions eliminates the mitochondrial membrane pH gradient, but increases the electrical gradient and increased R123 retention. Thus R123 can be used as probe for the abnormal metabolic state of the mitochondrion (Johnson et al, 1982). The compound appeared to halt cell cycle activity as inhibition of cell energy metabolism blocked replication causing reduction in accumulation of ribosomal RNA (Darzykiewicz et al, 1982). The compound was used as a probe to indicate whether mitochondrial function was inhibited by anticancer drugs (Bernal et al, 1982(a)). However evidence for possible anticancer activity was indicated by the unusually long retention of R123 in transformed epithelial cell lines or carcinoma cells compared to normal cells (Summerhayes et al, 1982). Exposure to R123 selectively reduced the clonal growth of carcinoma cells in vitro and selective toxicity was observed in carcinoma cells relative to untransformed cell lines in vitro (Bernal et al, 1982(b); Lampidis et al, 1983; Nadakavukaren et al, 1985).

Combining R123 with antiglycolytic agents for treatment against Ehrlich ascites tumours implanted in mice, showed some improvement in animal survival (Bernal et al, 1983). Thus the differences in retention times of R123 between normal and neoplastic cells is attributed to differences in transmembrane potential across the inner-mitochondrial membrane between cells. Depolarising agents decrease R123 fluorescence and retention, causing tumour cells to resemble normal cells, whereas hyperpolarising the membrane of non-neoplastic cells increases R123 fluorescence and retention (Davis et al, 1985). The antimitochondrial action of R123 is thought to be directed at the F₁F₀ ATPase complex with possible secondary effects on electron transfer (Modica-Napolitano et al, 1984; Emaus et al, 1986; Modica-Napolitano and Aprille, 1987).

Glioma's induced in rat brain with avian sarcoma virus have shown to retain R123 as compared to normal rat brain in vivo (Beckman Jr et al, 1987). It is clear that precise comparisons of tumour and non-tumour sensitivity to R123 are only valid between tissues from the same species, as well as comparisons of tumour with normal cells from the same lineage (Gupta and Dudani, 1987). However, to be of value in chemotherapy a general reduction in toxicity to all host tissues is important.

R6G was shown to inhibit coupling of phosphorylation with oxygen consumption in rat liver mitochondria, interpreted as due to an inhibition of ADP translocation (Gear, 1974). However further work indicated that R6G inhibited H⁺ion ejection during electron transport across the mitochondrial membrane (Higuti et al, 1980). There is some evidence to suggest that at low concentrations R6G and R123 inhibit mitochondrial protein synthesis in tumours as well as in normal cells (Kuzela et al, 1986; Abou-Khalil et al, 1986).

Certain agents in clinical use have been described as exhibiting antimitochondrial activity. The antileprosy agent clofazamine (lamprone) has been shown to exert an inhibition of cell respiration in bacteria and yeast (Barry et al, 1948; Rhodes and Wilkie, 1973). Studies on isolated rat liver mitochondria and yeast were thought to indicate that lamprone acted as an electron acceptor bypassing cytochrome oxidase. In support of this theory, lamprone was thought to have structural similarities to the well characterised electron acceptor phenazine methosulphate (Rhodes and Wilkie, 1973). Other agents thought to inhibit eukaryotic mitochondria were the tricyclic antidepressants chlorimipramine (Anafranil) and maprotiline (ludiomil). The compounds are also similar in structure to phenazine methosulphate. Fairly high concentrations of Anafranil used to inhibit yeast respiration ranged between 70uM and 280uM (Hughes and Wilkie, 1970). A selectively toxic effect between transformed fibroblasts and untransformed cells was demonstrated. Primary activity suggested that the drug restricted energy metabolism, reducing respiration. Inhibitory effects of the drug could be reversed by addition of ATP to cultured cells (Wilkie and Delhanty,

1970). Figure 1.1 illustrates the chemical structures of the drugs investigated.

Inhibitors of Glycolysis

Early studies, attempting to use glycolytic inhibitors in anticancer chemotherapy focussed on inhibitors which had been used to examine metabolic flux in glycolysis. As neoplastic transformations were viewed in the light of Warburg's observations, agents such as iodoacetate and iodoacetamide were investigated. However, not only were the compounds non-selective, affecting both tumour and host tissue, but were also very toxic (Aisenberg, 1961; Webb, 1966).

2-Deoxyglucose an antimetabolite of glucose was one of the earliest glycolytic inhibitors which appeared to show a specific effect against animal tumour models (Woodward and Hudson, 1954; Ball et al, 1957). More recent studies have indicated that when administered with R123, the combination potentiated the survival of mice implanted with the ehrlich ascites tumour compared to mice administered with R123 alone (Bernal et al, 1983).

Other compounds implicated include the indazole-carboxylic acid lonidamine. Lonidamine depressed aerobic glycolysis in tumour cells, but elevated glycolysis in non-tumour cells. This action was related to possible inhibition of the mitochondrial hexokinase, an enzyme thought to be absent from normal differentiated cells (Floridi, et al, 1981(a); Floridi, et al, 1981(b); Pedersen and Bustamante, 1982; Bustamante et al, 1981). Another agent Quercetin was used to inhibit glycolysis indirectly by inhibiting membrane bound Na^+K^+ ATPases, thus reducing ADP for stimulation of glycolysis (Suolinna et al, 1974). An antihelminthic drug levamisole has recently been shown to exert a selective effect upon ehrlich ascites tumour cells without affecting mouse liver and muscle. This activity is thought to be related to similar effects of the amino acid L-cysteine, exhibiting structural similarity to a part of the levamisole molecule (Kedryna et al, 1983; Guminska et al, 1986).

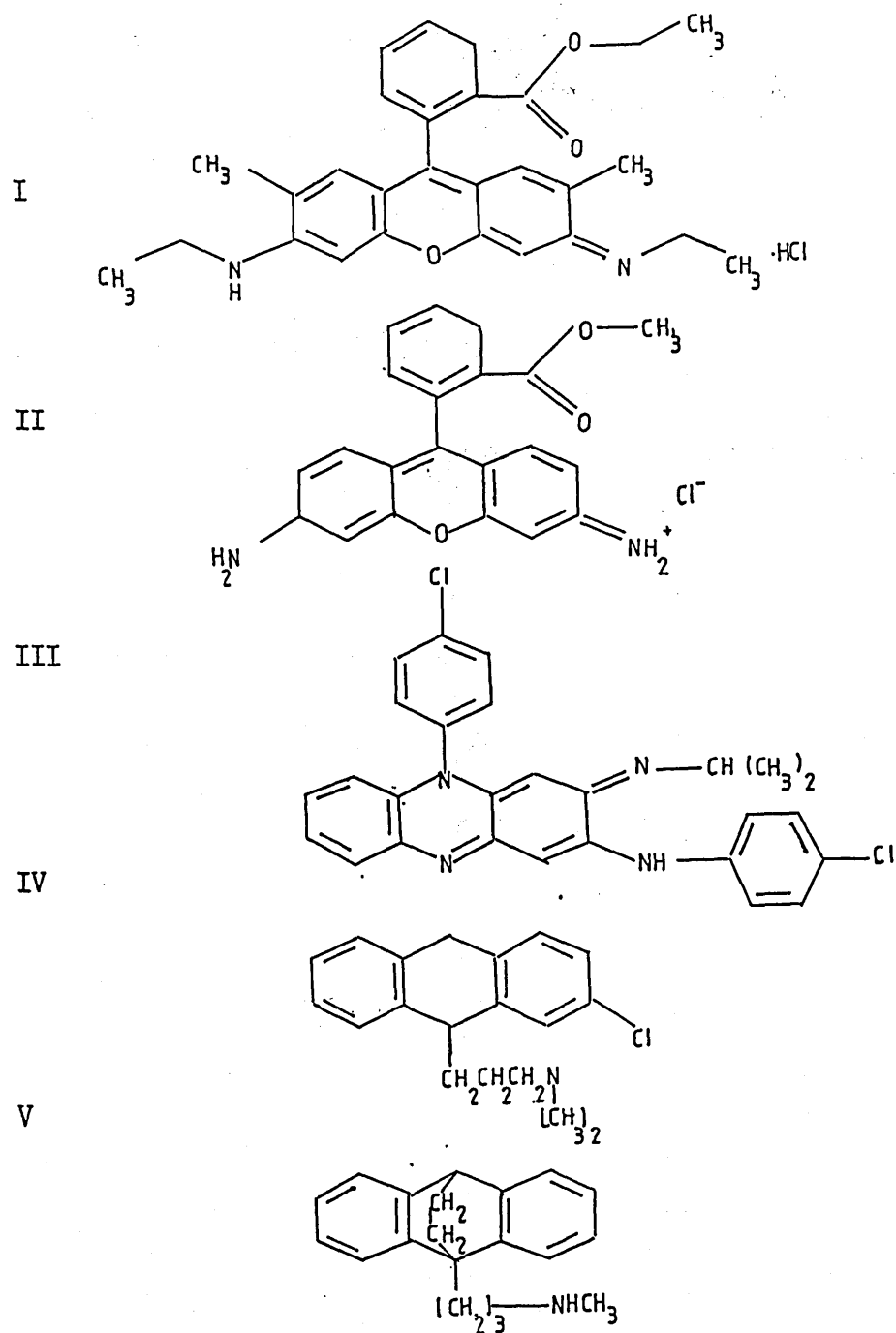


Figure 1.1: The Chemical Structures of Compounds investigated in these studies:-

- I Rhodamine 6G
- II Rhodamine 1,2,3
- III Clofazamine (Lamprene)
- IV Chlorimipramine (Anafranil)
- V Maprotiline (Ludiomil)

Combinations of antiglycolytic and antimitochondrial agents used to inhibit tumour energy metabolism in vivo

Some attempts have been made to use antiglycolytics with antimitochondrial agents to selectively inhibit tumour cell energy metabolism in vivo.

Methylglyoxal Bis (guanyl hydrazone) (MGBG) has been used to block mitochondrial replication in tumours in vivo, and this effect has been potentiated with hypoglycaemic agents such as phenethylbiguanide (Byczkowski et al, 1982). As mentioned some potentiation of the antimitochondrial agent R123 has been achieved with 2-deoxyglucose in prolonging survival times of mice bearing the ehrlich ascites tumour (Bernal et al, 1983). R6G combined with either the antiglycolytic, 2-deoxyglucose or the hypoglycaemic agents, 3-mercaptopycolinic acid or hydrazine sulphate reduce tumour growth in rats bearing the W256 tumour (Fearon et al, 1985(a); 1987).

Aims of Thesis

Evidence reviewed thus far, implies that there may be differences between the energy metabolism of normal and neoplastic cells. It might be possible to inhibit tumour cell energy metabolism with agents which show a specificity for tumour mitochondria. However, leaving the glycolytic component of the cell unaltered could allow the cell to maintain ATP through breakdown of substrate carbon to lactate. Thus it is hoped that combining tumour selective antimitochondrial agents with inhibitors of glycolysis, tumour cell energy metabolism might be blocked without deleterious effects upon host metabolism (Wilkie, 1979; Fearon, 1984; Wilkie and Fearon, 1985). Highly glycolytic tumours targeted by such therapy would have their ATP production limited by the nature of the antiglycolytic agents used. Normoxic regions of the tumour undergoing oxidative metabolism would receive antimitochondrial therapy. As the ratio of ATP production generated by anaerobic metabolism relative to aerobic respiration is 1:18, even reduced mitochondrial function in such tumours would generate substantial quantities of ATP.

Slow growing, well differentiated tumours tend to resemble normal host tissue in structure and metabolic function. Effectiveness of the therapy would be more dependent upon the selectivity of the antimitochondrial agents used. If mitochondrial metabolism is more critical for highly differentiated tumours it might be possible to use a combination of functional inhibitors of tumour mitochondria together with tumour specific inhibitors of mitochondrial replication or biogenetic inhibitors to block energy metabolism in tumours (Van den Bogert, 1983).

Thus the aims of the experimental work described in this thesis may be stated as follows:

A screening system is required, whereby the selectivity of antimitochondrial agents used in this study might be evaluated against tumour and non tumour system in vitro. A further series of studies is required to confirm that those agents selected inhibit mitochondrial metabolism in vitro. Finally the ability of such agents in combination with recognised tumour selective antiglycolytic agents to halt the growth of tumours in vivo needs to be confirmed.

Thesis Layout

Each Chapter is self-contained, containing introduction, methods, results and discussion. Chapter 2 describes the general methods applied to experiments carried out in more than one chapter. A background to parameters of mitochondrial function examined is also described.

Experiments described in Chapter 3 evaluated whether mitochondria isolated from a rat tumour and normal rat liver are suitable models for evaluating the effectiveness of possible antimitochondrial agents directed at tumour energy metabolism. Results indicated that although viable, coupled organelles could be obtained from a poorly differentiated, glycolytic tumour, certain disadvantages of this system, discussed in Chapter 3, made it unsuitable for an investigation of antimitochondrial agents.

A second model system was evaluated in Chapter 4, for assessing whether antimitochondrial agents described in the literature had potential as tumour selective inhibitors of energy metabolism. Whole tumour cells obtained from continuous cultured cell lines were compared with freshly isolated cells obtained from normal rat tissue in their responses to antimitochondrial agents. It was clear that both mitochondrial function and anaerobic metabolism could be assessed in such a model and was thus adopted for assessment of antimitochondrial agents.

From experiments in Chapter 4, the fluorescent cationic dye R6G and the antibacterial agent lamprene showed promise as possible metabolic inhibitors of tumours. However, evidence for antimitochondrial activity was indirect. A series of experiments described in Chapter 5 were designed to assess the potency of the antimitochondrial effect on tumour cells by comparing these agents with classical anti-mitochondrial agents.

Experimental evidence presented in Chapter 6 was an evaluation of the conventional anticancer agent ^{5-fluorouracil (5FU)} which at non-cytotoxic concentrations was thought to induce inhibition of mitochondrial biogenesis. Unfortunately, the response to continuous exposure of 5FU on expression of mitochondrial enzymes did not provide conclusive evidence of inhibition of mitochondrial biogenesis.

Any inhibitor of mitochondrial function might have other effects upon tumour cell metabolism. It was therefore necessary to examine whether the cytotoxic effect upon the tumour cell caused by the inhibitor was in fact due to specific inhibition of mitochondrial metabolism or due to some other effect. The approach in Chapter 7 attempted to determine this by making cells dependent upon carbon sources which required mitochondrial activity, and to see if such cells showed any enhanced sensitivity to inhibitors investigated. Although the approach was successful, a selective antimitochondrial effect with R6G was not demonstrated upon tumour cell toxicity.

Chapter 8 described experiments which evaluated how successful combining inhibitors of tumour mitochondrial function and, either inhibitors of tumour cell glycolysis, or inhibitors of tumour mitochondrial biogenesis, were in reducing the growth rate of a human tumour xenograft implanted in athymic mice. Of the combinations used, both lamprene in combination with the antiglycolytic agent 2-deoxyglucose and 5FU combined with R6G achieved some delay in tumour growth.

Finally, Chapter 9 is a general summary and discussion of the experimental evidence presented in this thesis, together with conclusions of the studies undertaken. Although this approach to manipulating tumour cell energy metabolism was shown to be partly successful, a truly conclusive study required a more effective selection of inhibitors, preferably from agents already in clinical use, rather than experimental compounds.

CHAPTER 2

GENERAL METHODS

INTRODUCTION

Most of the experimental methods described in this chapter are applicable to more than one chapter of this thesis. These techniques may be broadly classified into six sections. The initial sections will describe routine maintenance of animals used in these studies as well as the tumour types selected. General tissue culture methods for maintenance and growth of continuous tumour cell lines will then be described. Assays applicable to studies in more than one chapter will also be described. Finally, methods for investigating the respiratory activity of mitochondrial preparations or intact whole cells by oxygen polarography will be described.

METHODS

Animals

Female Wistar rats, weighing between 200 and 300 grams were purchased from Charles Rivers, Margate, Kent. Alternatively, animals were obtained from stocks bred in the Department of Medical Oncology. Animals were maintained in the animal house of the Department of Medical Oncology. Animals had free access to fresh water and food (Wm Macarthur & Co, Glasgow).

Tumours

Isolated mitochondria were obtained from the Walker 256 carcinosarcoma. This rat tumour is a poorly differentiated, rapidly growing tumour characteristic, of the highly glycolytic tumours originally described by Warburg (1930). Tumour tissue for subcutaneous implantation in Wistar rats were obtained from two sources. Cells were obtained from cells grown in suspension culture. Alternatively tumour fragments stored in liquid nitrogen (-160°C) were thawed and used to initiate tumour growth. Animals were anaesthetised with anaesthetic grade diethyl ether. Animals were then placed with the dorsal surface facing upward. Tumour sections measuring between 2 and 3mm^3 were surgically inserted onto the surface of hind limb muscle beds. The wound was secured with autoclips (Vicary Davidson, Glasgow). Animals were then allowed to recover. Where cell suspensions were used to initiate tumour growth, cells were injected into the region above the hind limb with a sterile syringe and needle (Becton Dickinson, Cowley, Oxfordshire). Tumour was passaged at 7 day intervals thereafter. Tumour was obtained from freshly killed animals and tumours were removed to sterile petri dishes. Fat, connective tissue and necrotic material were removed. Viable tissue obtained from the tumour surface was divided into sections measuring between 2 and 3mm^3 . Tumour passage was continued as described.

General Tissue Culture Methods

Tissue culture was carried out under sterile conditions in a vertical air flow hood.

Maintenance and Growth of Tumour Cell Lines

WIL, a human non small cell lung tumour line was used for the experiments described in this thesis. It is characteristic of tumours which resemble the host tissue, showing differentiation and yet exhibiting rapid growth rates. The cell line was maintained in monolayer culture in flasks (Nunc, 75cm² and 150cm², GIBCO, Paisley). Cells were routinely grown in Hams F10 and Dulbecco's Modified Eagles Medium (F10:DMEM, 50:50, GIBCO, Paisley) supplemented with glutamine, 2mM and foetal calf serum (FCS), 10% (Biocon, Eardiston, Worcestershire). Stocks of this cell line were originally obtained from the Ludwig Institute, Sutton. Cells were stored in liquid N₂ in medium supplemented with dimethyl sulphoxide, 10% as preservative. Cells were routinely passaged twice weekly following treatment with trypsin [0.25% in phosphate buffered saline (PBS) (V:V), containing 1mM ethylene diamine tetra-acetic acid (EDTA)]. Cells were harvested from at least two N75 flasks, pooled and resuspended in fresh medium in sterile 30ml universal containers (Sterilin, Middlesex). Cell number was determined with a Coulter Counter (Model ZB1, Coulter Electronics, Luton, Beds). Cell viability was assessed by dye exclusion (0.02% Trypan Blue in PBS) and viewed under phase contrast (Olympus C.K.2 Olympus Optical Co, London). Microscope, Viability routinely exceeded 95%. Fresh cultures were seeded into sterile flasks at a dilution of 1:10. Monolayers reached saturation density after 4 days. At 6 month intervals fresh stocks were obtained from the liquid nitrogen freezer and old stocks were discarded.

Walker 256 (W256), a rat carcinosarcoma tumour described previously as characteristic of poorly differentiated, highly glycolytic tumours was maintained in suspension culture. Cells were grown in flasks (Nunc, 75cm² and 150cm²) containing F10:DMEM (50:50) supplemented with glutamine, 2mM and FCS, 10%.

Cells were maintained by routine passage as described for WIL. Passage was routinely carried out at 3 day intervals during which time viable cell number reached a maximum in each flask. Cells were harvested by decanting cells into sterile 30ml universal containers. Cells were sedimented in a bench top centrifuge at 160g (MSE

Instruments). Cell number and viability was determined as described for WIL. Cell viability routinely exceeded 90%. Fresh cultures were seeded at a dilution of 1:10 into fresh medium. At 6 month intervals stocks were discarded and fresh cultures were seeded from stocks obtained from the liquid nitrogen freezer.

Photographic Studies

Cells (WIL) plated into tissue culture flasks (25cm²) were routinely photographed under phase contrast with a 35mm single lens reflex (SLR) camera attachment (Olympus C.K.2 Microscope; Olympus O.M.2 SLR Camera). Microscope settings gave magnification of X33 and X66. Plates were obtained with 35mm ASA 160 film. Exposure times were automatically adjusted for lighting conditions. Routinely exposure times were between 0.25 and 1 second at different aperture settings.

Cells were prepared for fluorescent photography as described in Chapter 7 (p 110). Fluorochrome uptake was examined with a polyvar microscope with a 35mm camera attachment (Reichert Jung, Cambridge Instruments, Bar Hill, Cambs). For R6G as the fluorochrome the G-1-filter setting was used (546nm). R6G, under aqueous conditions exhibits an absorption maximum in the range 460nm to 560nm and emits in the range 530nm to 605nm, (Loveland, 1970). Plates were taken with Ektachrome 35mm ASA 1600 film. Film exposure was automatically corrected for light conditions and exposure times varied between 0.125 and 1.00 seconds. From microscope settings, magnification was calculated at X225.

Estimation of Cell Growth Rates

Cells (WIL) were seeded onto 24-well plates at a concentration of 3×10^4 cells per well in tissue culture medium described previously. Cells were allowed to attach and grow for 72 hours. Medium was then removed from plates and cells were incubated either in fresh medium (control) or in fresh medium containing drug. After 24 hours drug was removed and all plates were fed with fresh medium. Thereafter plates were fed daily. Cell number was determined daily in triplicate wells from all plates. Following treatment with trypsin, cell number could be determined with the Coulter Counter. Experiments were carried out for 7 to 8 days (Chapter 5).

Experiments described in Chapter 7 were modified as follows:

Growth medium consisted of Minimal Essential Medium (MEM) supplemented with FCS, 10% containing either glucose, 5.5mM and glutamine, 2mM or glutamine, 2mM only. Cell number was determined at 48 hour intervals as described previously. Experiments were continued for 14 to 16 days.

Assay of Cytotoxicity

Chemosensitivity was determined by an assay recently established within the Department of Medical Oncology. The assay is based upon a method described by Mossman (1983) (see also Cole, 1986; Carmichael et al, 1987).

The assay is dependent upon the reduction of a tetrazolium dye, MTT (3-(4, 5-dimethylthiazolyl-2-yl)-2, 5 diphenyl-tetrazolium bromide) to a purple formazan product by live, but not dead cells. Cells are plated onto 96 wells and allowed to attach and grow. Cells are then exposed to cytotoxic drug for a fixed period of time. Drug is removed and cells are allowed to recover and divide. Surviving cells are then indicated following incubation with MTT. Formazan production is thus an endpoint to this assay.

Cells (WIL) were seeded onto the central 80-wells of 96-well plates at a density of 500 cells per well. Cells were incubated at 37°C in an atmosphere of 2% CO₂, in air and allowed to attach and grow for 48 hours. Rows 1 and 12 of each plate contained medium only. Medium was then removed and cells were fed with fresh medium, 0.2ml. Drugs were diluted in tissue culture medium. Drug, 0.05ml was added to cells in row 3 of the plate. The drug was then serially diluted along each row of the plate using 0.05ml aliquots. The procedure was continued upto row 10. Rows 2 and 11 were fed medium only and were used as controls. One of the drugs studied, lamprene, required solubilisation in DMSO prior to dilution in tissue culture medium. For this drug only 6 drug concentrations were used (rows 5 to 10). Cells in row 4 were fed with medium containing DMSO. Cells in row 3 were fed with medium and wells in row 2 were cell free, fed with medium containing lamprene (500uM). After 24 hours drug was removed from each plate. Plates were fed with

fresh medium on consecutive days thereafter. Three days after removal of drug 0.05ml MTT, 5mg per ml in PBS was added to each well. Plates were wrapped in foil and placed in a 2% CO₂ incubator at 37°C for 4 hours. Medium was removed and the formazan produced was solubilised by addition of DMSO, 0.2ml. To each well was also added Tris-HCl buffer (0.1M, pH 9.5), 0.025ml. All formazan present was as a single species with an absorption maxima at 570nm. Formazan crystals were dissolved as plates were subjected to gentle agitation on a plate shaker (Titertrek, Flow Laboratories, Irvine, Scotland). Absorbance was measured at a wavelength of 570nm with an ELISA plate reader (Biorad, ^{Bromley, Kent} ~~London~~). From each row of 8 wells, corresponding to a particular drug concentration a mean absorbance at 570nm was calculated and this was plotted against drug concentration. The amount of drug required to kill half the cells was determined as the concentration of drug required to reduce the absorbance of the wells to half that of the control, drug free, cells.

Experiments described in Chapter 7 were modified as follows. Cells were plated out at an initial cell density of 1000 cells per well. Exposure to drug was carried out for 24 hours (R6G) or 1 hour (oligomycin). Oligomycin was solubilised in ethanol, 100% and diluted in culture medium. The final concentration of ethanol was less than 1% and was not toxic.

This assay is dependent upon a linear relationship between cell number and absorbance, for each cell line examined. A plot of absorbance verses cell number to show linearity of the assay is shown in figure 2.1 (courtesy of Dr J A Plumb). From this plot a linear relationship exists between absorbance and cell numbers ranging between 1×10^3 and 20×10^3 cells.

A typical dose response curve for WIL exposed to R6G for 24 hours and recovery measured by the MTT assay after 3 days is shown in figure 2.2. At a drug concentration of 10^{-8} M or less, no toxicity was observed. At drug concentrations between 1×10^{-8} M and 5×10^{-6} M the final absorbance per well is reduced. From this curve an ID₅₀ value

is obtained at the point where 50% of the absorbance value intersects the curve.

Total Cell Protein

Total cell protein was determined by the method of Lowry et al (1951). Cell protein was solubilised in sodium deoxycholate, 5%, prior to protein determinations as described by Wang et al (1985). Samples were stored at -20°C for subsequent analysis.

Total Cell DNA

Total cell DNA content was determined by the method of Le Pecq and Paoletti (1966) with modifications according to Karsten and Wollenburger (1972) and Gardner and Plumb (1979).

The method is based on the enhancement of the fluorescence of ethidium bromide by DNA. Cell homogenates are treated with pronase to remove histones and other proteins associated with DNA. Endogenous nuclease activity is also inhibited following cell disruption. RNA is removed from the sample by digestion with ribonuclease (RNase) free of any deoxyribonuclease (DNase) activity. Ethidium bromide intercalates with released DNA in a stoichiometric manner and can be measured at 580nm following excitation of the sample at a wavelength of 360nm.

Fresh cell homogenates were prepared by suspending cells in sterile distilled water at a concentration of 1×10^6 cells ml^{-1} . Samples were homogenized in a Potter-Elvehjem type homogenizer (teflon on glass) with 20 to 30 strokes.

Reagents were as follows:-

Phosphate buffered saline (PBS):

	g/L	mM
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.196	0.90
KCl	0.200	2.70
KH_2PO_4	0.200	1.50
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.100	0.50
NaCl	8.00	137
$\text{Na}_2 \text{HPO}_4$	1.15	8.0

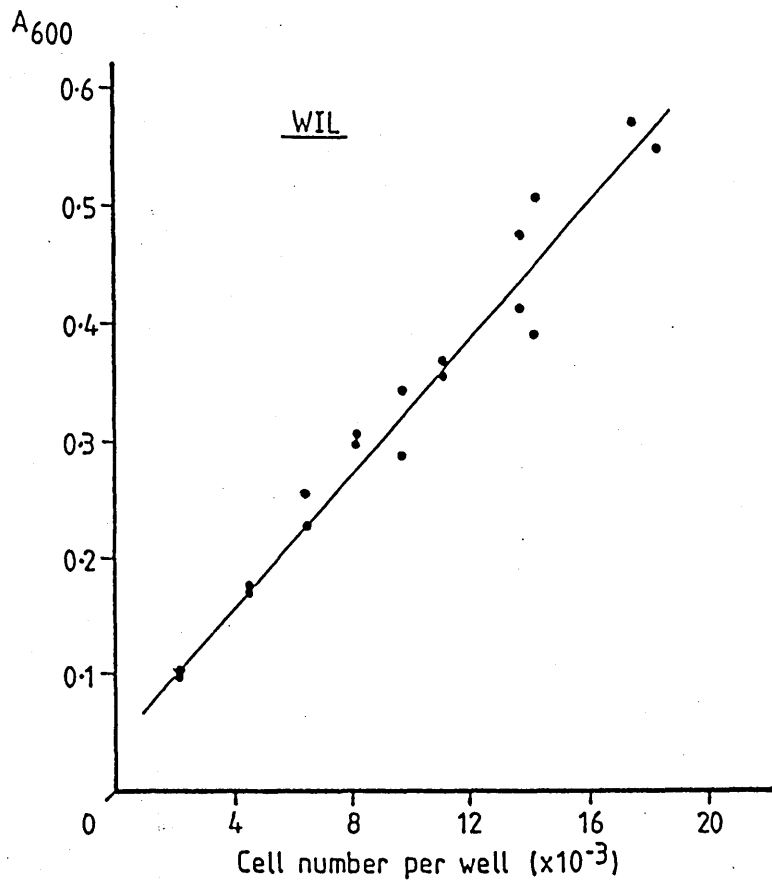


Figure 2.1: Absorbance at 600nm following Formazan production by WIL expressed against cell number per well. A linear relationship between absorbance and cell number is obtained for the MTT assay.

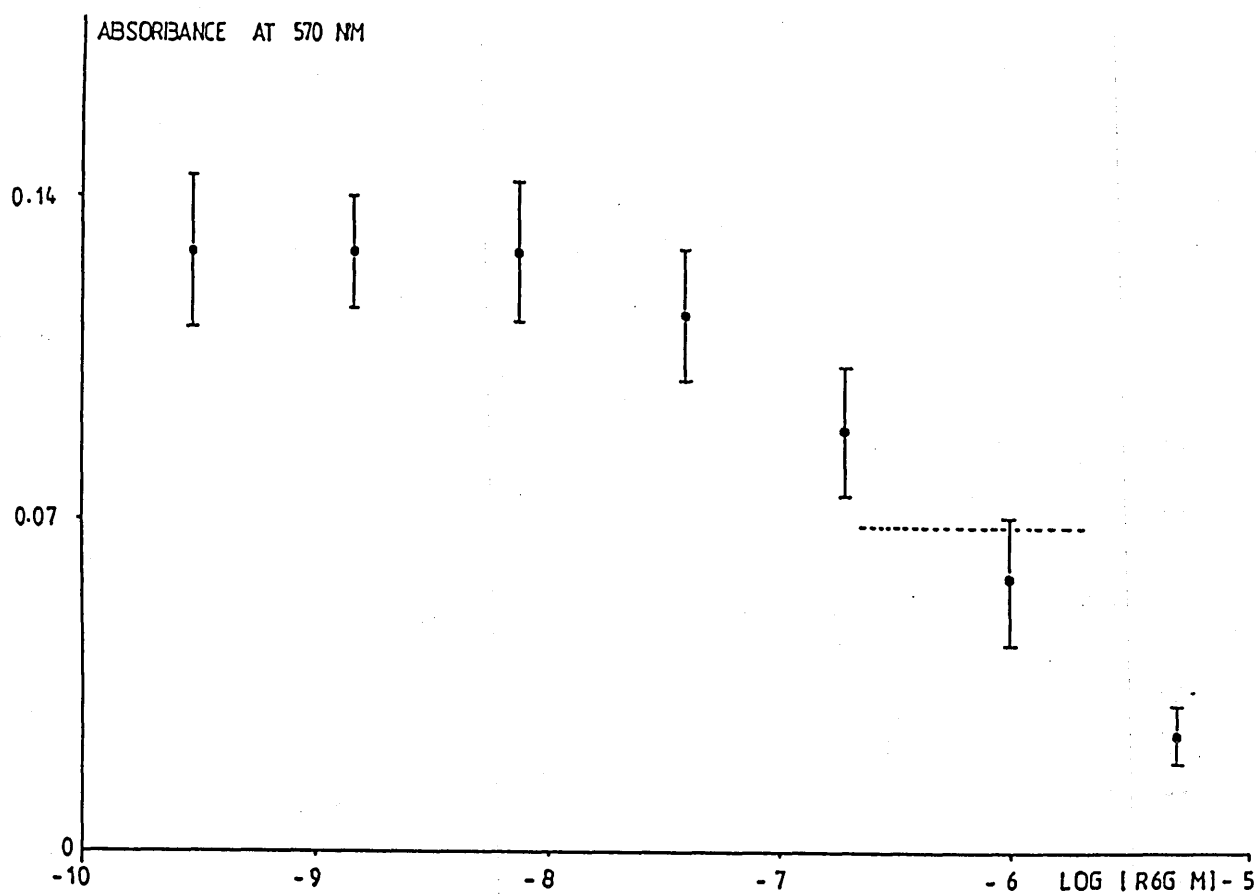


Figure 2.2: Absorbance at 570nm following Formazan production by WIL expressed against concentration of R6G. For each drug concentration $N = 8 \pm SEM$. A typical dose response curve for WIL exposed to R6G for 24 hours. Formazan production was recorded 3 days after cell exposure to drug by the MTT assay. An ID_{50} value was obtained where the dotted line intersected the curve.

Buffer was adjusted to pH 7.5 with NaOH, 1M prior to making upto volume.

The following solutions were prepared just before use:-

Pronase : Pronase, 6mg dissolved in PBS, 100ml

RNase : RNase, 20mg dissolved in distilled water, 1ml

DNA Standard : DNA, 5mg dissolved in saline, 0.9%, 100ml.

DNA solubilised at 4°C, 6 to 8 hours before use.

Ethidium Bromide : Ethidium bromide, 2.5mg dissolved in PBS, 200ml.

Samples (0.2ml) were placed in pyrex test tubes in duplicate. To each tube was added PBS, 1ml, pronase, 0.8ml and RNase, 0.04ml. Tube contents were mixed thoroughly and tubes were sealed with parafilm and incubated at 37°C for 60 minutes in a water bath. Ethidium bromide (0.025mg in 2ml) was then added to each tube. Samples were read in a fluorescence spectrophotometer (Perkins-Elmer, ^{Beaconsfield, Bucks} at an excitation wavelength of 360nm and an emission wavelength of 580nm. A standard curve was prepared with calf thymus DNA. DNA content was determined from the regression line fitted to the standard curve. The reaction was linear upto 10 micrograms of DNA.

Measurement of the Respiratory function of whole cells or isolated mitochondrial preparations

The respiratory activity of freshly isolated mitochondria or preparations of intact whole cells were measured by a polarographic method (Estabrook, 1967; Knapp, 1983).

The Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambs) is summarised in figure 2.3. The electrode consisted of an airtight chamber enclosing a platinum electrode which is in contact with a thin teflon membrane. Dissolved oxygen in the chamber diffuses through the membrane and is reduced at the platinum surface. The other half cell incorporates a silver-silver chloride electrode. A salt bridge containing saturated potassium chloride completes the circuit. In stirred, air saturated aqueous solution at 30°C a current flows through the circuit which is proportional to the partial pressure of

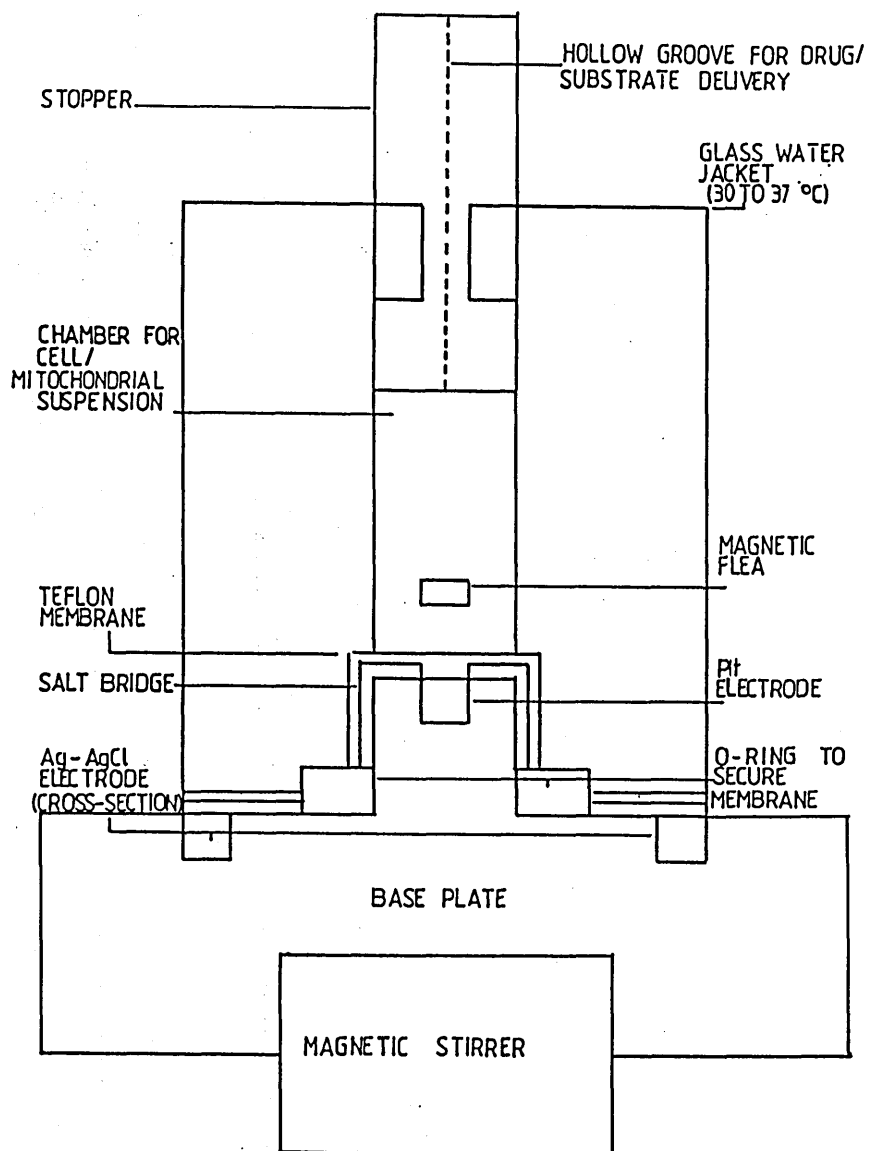


Figure 2.3: Schematic representation of the Clark-type oxygen electrode, seen in cross-section.

oxygen in the solution. When the solution becomes anaerobic, i.e. all the oxygen is utilised, the current falls to zero. The electrode was calibrated with air saturated buffer to 80 to 90% full scale deflection on a chart recorder (Rikadenki, ^{Mitsui Electronics, Chessington} Surrey). To obtain a value of zero deflection, a few crystals of sodium dithionite was added to the chamber, depleting the oxygen contents. The dissolved oxygen concentration in aqueous buffer was obtained from published tables (Forstner and Gnaiger, 1983). At 30°C, oxygen content was estimated at 236 nanomoles oxygen per ml air saturated buffer. At 37°C, oxygen content was estimated at 210 nanomoles oxygen per ml of air saturated buffer.

Parameters of Mitochondrial Function

The parameters of mitochondrial function used to assess rat tumour or rat liver mitochondrial preparations are defined as follows (Estabrook, 1967):

$$\text{Oxygen Consumption} = \frac{\text{(chart recorder) slope} \times 236 \times 2 \times \text{Total volume}}{\text{(Recorder) deflection in divisions}} \times \text{in electrode}$$

$$\text{Oxygen Consumption} = \frac{\text{oxygen consumption} \times \text{volume of mitochondria}}{\text{ng atoms min}^{-1} \text{ mg}^{-1} \text{ protein}} \times \frac{\text{introduced}}{\text{protein content of volume introduced}}$$

$$\text{Respiratory Control Ratio} = \frac{\text{Oxygen Consumption in State 3}}{\text{Oxygen Consumption in State 4}}$$

$$\text{ADP:O ratio} = \frac{\text{ATP produced}}{\text{Oxygen consumed}}$$

Since ADP consumed = ATP produced

and Oxygen consumed obtained from State 3 respiratory rate

$$\text{ADP:O ratio} = \frac{\text{ADP consumed}}{\text{Oxygen consumed}}$$

A typical trace of the respiratory activity of isolated rat liver mitochondria is shown in figure 2.4. Addition of isolated rat liver mitochondria to the oxygen electrode, show mitochondria respiring at a basal level utilising endogenous substrates and ADP to respire. Addition of oxidisable substrate such as succinate enables mitochondria to use endogenous phosphate to undergo phosphorylation, although exhibiting a respiratory rate limited by availability of ADP. State 4 respiration describes this phenomenon. Subsequent addition of a known amount of excess ADP, enables phosphorylation hence oxygen consumption to occur unrestricted. ATP synthesis is coupled to an increased oxygen consumption rate. This describes State 3 respiration. Once the ADP is used up, State 4 respiration is regained. Oxygen consumption is reduced due to ADP depletion limiting phosphorylation.

Parameters of Whole Cell Respiratory Activity

The parameters of cell respiratory activity with which to assess intact rat hepatocytes or intact tumour cell preparations are as follows:

$$\text{Oxygen consumption rate} = \frac{(\text{chart recorder}) \text{ slope}}{\text{divisions min}^{-1}} \times \frac{(210 \times 2)}{\text{Recorder deflection}} \times \frac{\text{Total volume in electrode}}{\text{volume in electrode}}$$

$$\text{Oxygen consumption rate} = \text{Oxygen consumption rate} \times \frac{\text{Volume of cells introduced}}{\text{protein/DNA content in volume introduced}}$$

or

$$\text{ng atoms min}^{-1} \text{ ug}^{-1} \text{ DNA}$$

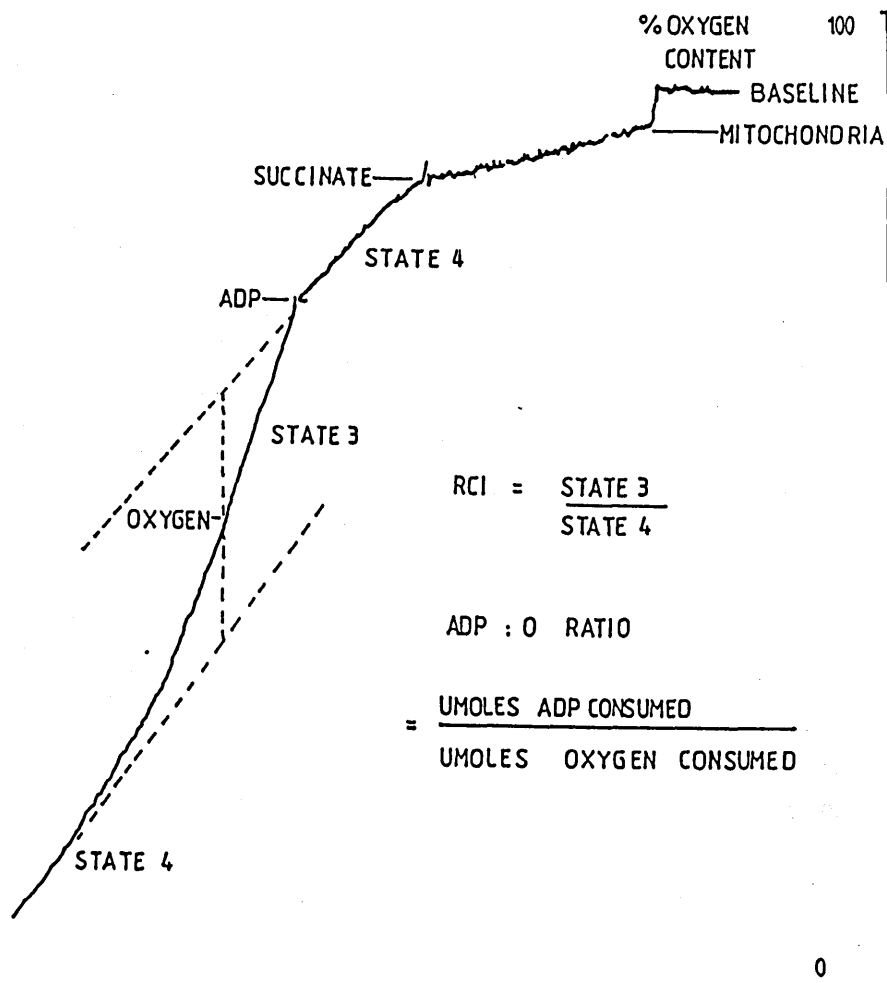


Figure 2.4: Rate of oxygen consumption by isolated rat liver mitochondria preparation incubated in respiration buffer at 30°C. The reaction was started by addition of mitochondria (1-2mg protein) to buffer, 3ml (pH 7.4). After a steady state rate of oxygen consumption was achieved, succinate (10mM) and ADP (600nmoles) were added sequentially at points indicated. Parameters of mitochondrial function were calculated as described in the text.

CHAPTER 3

A COMPARISON OF THE RESPIRATORY PROPERTIES OF MITOCHONDRIA FROM TUMOUR AND NON-TUMOUR CELLS

INTRODUCTION

In mammalian cells, the main site of cellular oxidative energy metabolism is the mitochondrion. A major aim of the studies described in this thesis is to identify specific antimitochondrial agents which might alter the energy metabolism of tumour cells. One way to achieve this aim was to study isolated mitochondria obtained from tumours and compare standard respiratory parameters with those of isolated mitochondria obtained from normal tissue. Thus an examination of mitochondrial metabolism would highlight differences between tumour and non-tumour organelles and could be exploited as a consequence. A critical assessment of the methods employed to obtain mitochondria from both tumour and normal tissues would determine whether such methods would be suitable for studying the behaviour of selective antimitochondrial agents.

An essential feature of such a study is a screening method whereby it would be possible to identify possible antimitochondrial agents for ultimate use in humans. Therefore it would be especially useful to identify compounds already in clinical use which may have tumour specific antimitochondrial activity. There is some evidence to suggest that antidepressants such as chlorimipramine (anafranil) and maprotiline (ludiomil) may have antimitochondrial activity (Wilkie and Delhanty, 1970; Wilkie, 1979). A second approach is to assess novel compounds which might have antimitochondrial activity, such as, for example, Rhodamine 6G. Attempts have been made in anticancer therapy to use novel inhibitors (Webb, 1966). The antimitochondrial agent 2,4 Dinitrophenol has been used unsuccessfully in attempts to alleviate obesity(!) (Racker, 1976).

Animal studies as a basis for selecting agents which would have potential as mitochondrial inhibitors of tumour cells are slow and give little indication as to the particular aspects of cell metabolism which are blocked. Techniques selected in this study enable the metabolism of intact tumour mitochondria to be examined, whereby a high degree of control may be exerted over organelle function. There can however be no critical assessment of the efficacy of a particular antimitochondrial at the level of the intact animal. The approach is admirably suited to obtaining data indicating the potential of a particular mitochondria inhibitor and whether it might then exert a selective action upon mitochondrial activity, hence energy metabolism of tumours. Ultimately, an examination of the effectiveness of the compounds that then show most promise must be examined in more complex systems, i.e. the intact cell or intact animal or human, where the degree of respiratory control that can be exerted is much less. It would then be possible to assess the possible therapeutic role such an agent might have in cancer chemotherapy.

Rat liver is the source of the most intensively studied mitochondria and has become the standard to which other mitochondrial preparations are compared. Mammalian liver has a high concentration of mitochondria (30 to 33% of liver protein) and the tissue can be easily homogenised to obtain isolated mitochondria with minimal damage (Schneider and Hogeboom, 1951; Johnson and Lardy, 1967; Gregg, 1972).

The experiments described in this chapter were designed to determine whether the ultrastructural and quantitative differences observed between tumour and non-tumour mitochondria are reflected in differences in respiratory function. Previous studies with mitochondria isolated from both rapidly growing, poorly differentiated hepatomas and slow growing, well differentiated hepatomas indicated that such mitochondria were capable of coupled respiration with normal phosphorylating capacity and respiratory control ratios ranging from 2 to considerably more than 10 (Kaschnitz et al, 1976). Similar results have been obtained from mitochondria isolated from other tumours (Sordahl et al, 1969; Pedersen, 1978).

The parameters of respiratory function which may be compared when isolated mitochondria from each source are examined, provide information about energy metabolism in each type of organelle. Mitochondria may be supplied with different oxidisable substrates when incubated in the Clark-type oxygen electrode to determine which substrates can be utilised. The coupling of ADP phosphorylation to oxygen consumption can also be examined when preparations of isolated tumour mitochondria or normal mitochondria are incubated in the oxygen electrode (Estabrook, 1967; Gregg, 1972; Nakashima et al, 1984).

Models required for this study are mitochondria from a well characterised normal tissue. As described previously rat liver mitochondria are the accepted standard as a source of 'normal' isolated organelles. The respiratory activity of isolated mitochondria obtained from rat liver were thus selected for this study.

The Walker 256 (W256) rat carcinosarcoma exhibits growth characteristics indicative of a highly glycolytic, poorly differentiated tumour. Previous studies have already shown that a significant delay in tumour growth can be achieved following administration of agents which are thought to inhibit tumour cell energy metabolism, hence mitochondrial metabolism in vivo (Fearon et al, 1985a). Mitochondria were isolated from W256 tumours implanted subcutaneously in Wistar rats.

Isolated mitochondrial preparations from both liver and W256 tumours were examined with a polarographic oxygen sensor. The response to a variety of exogenous oxidisable substrates was determined from respiratory control indices, ADP:O ratios and oxygen consumption rates.

Isolation of Mitochondria

All stages carried out at 4°C.

Solid Tumours

Liver

1. Tissue minced, suspend in isolation medium (225mM Mannitol, 150mM Sucrose, 400uM EGTA, 2mM MOPS, 0.5% BSA, PH7.2), 25% W/V.
2. Homogenize 5 strokes (Teflon on glass).
3. Centrifuge 10 mins, 1100g.

Supernatant

- Centrifuge 15 mins, 6780g.

Sediment

1. Resuspend in isolation medium to original volume.
2. Centrifuge 10 mins, 1100g.

Supernatant

- Centrifuge 10 mins, 6780g.

Sediment

1. Wash pellet 1-2x respiration medium (200mM Mannitol, 50mM Sucrose, 10mM NaH_2PO_4 , 20mM MOPS, PH 7.2).
2. Resuspend in 1ml respiration medium, keep on ice.

MITOCHONDRIA

Figure 3.1: A summary of the procedure used to obtain preparations of isolated mitochondria from both rat liver and walker 256 tumours.

METHODS

Animals

Rat liver mitochondria were prepared from female Wistar rats weighing between 200 and 300 grams. Prior to each experiment, animals were given food (SDS, Essex) and water ad libitum. Tumour mitochondria were isolated from W256 tumours grown subcutaneously in female Wistar rats. Animals were given food and water ad libitum prior to each experiment.

Preparation of Isolated Mitochondria

Isolated mitochondria were prepared essentially as described by Cummings *et al*, 1984 (see also Wehrle and Pedersen, 1982). The procedure for isolation of mitochondria is summarised in figure 3.1.

Animals were killed by cervical dislocation and tissues were rapidly removed. All subsequent steps were carried out at 4°C. Tissues were weighed and placed in isolation medium (25% weight : volume) consisting of mannitol (225mM), sucrose (150mM), EGTA (400uM), MOPS (2mM) and bovine serum albumin (BSA, 0.5%). The pH of the medium was adjusted to 7.2 before use. Fat and connective tissue were removed with a fine pair of scissors. In the case of tumour, only regions of viable tissue contained in the outer rim of the neoplasm were included. Necrotic material from the core of the tumour was discarded. Tissue was minced in cold isolation buffer and washed 2 to 3 times. The tissue was then suspended in fresh isolation medium and homogenised by means of a Potter-Elvehjem type homogeniser (teflon on glass) (Jencons Scientific, Bedfordshire). Five strokes were used. Differential centrifugation was carried out with a high speed refrigerated centrifuge (Sorvall RC-5B, Du Pont, Connecticut, USA) to obtain a mitochondrial pellet as follows. Homogenate was centrifuged for 10 minutes at 1100g and 4°C to obtain a cell supernatant containing cytoplasmic organelles. This was centrifuged for 15 minutes at 6780g and the crude mitochondrial pellet was collected. Purification of the pellet was carried out to remove both nuclear and cytoplasmic contaminants (particularly lysosomal). The pellet was resuspended in isolation medium and centrifuged for 10 minutes at 1100g. The supernatant containing the mitochondrial fraction was

centrifuged for 10 minutes at 6780g to obtain a relatively pure mitochondrial pellet. The pellet was then washed in respiration buffer consisting of mannitol (200mM), sucrose (50mM), NaH_2PO_4 (10mM) and MOPS (20mM). The pH was adjusted to 7.2 prior to use.

Assay of Mitochondrial Protein

Protein content of the mitochondrial suspension was measured by the biuret method, essentially as described by Layne (1957).

Assay

Biuret reagent was prepared as follows:

Solution A 1.5g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 6g $\text{K}_4 \text{Na C}_4 \text{H}_4 \text{O}_6 \cdot 4\text{H}_2\text{O}$
 Dissolved in 500ml distilled water

Solution B 10% (weight:volume) NaOH in distilled water
 300ml

Solution C 1g KI solubilised in 10ml distilled water.

Solution A was mixed with Solution B. Stirring was continued and Solution C was added. The reagent was diluted to 1 litre with distilled water and stored at room temperature. This solution was stable for several months. Reagent was discarded when a red precipitate of oxidised copper formed in the solution. A standard solution of BSA (Cohn Fraction V), 10mg per ml was prepared. 0.1ml of the mitochondrial suspension was diluted to 1ml with distilled water. The suspension contained 1 to 10mg of protein. 4mls of biuret reagent was then added. The solution was mixed thoroughly and allowed to stand in glass test tubes at room temperature for 30 minutes. A standard curve containing BSA, 1 to 10mg was prepared. The assay blank contained 1ml distilled water and 4ml biuret reagent. The absorbance of samples and standards were read against distilled water at a wavelength of 550nm in a spectrophotometer. The reaction was linear up to 10mg of BSA. Unknowns were determined from the standard curve.

Measurement of Respiratory Activity

The respiratory activity of freshly prepared mitochondria were measured by a polarographic method as described in chapter 2. 1 to 2mg of mitochondrial protein (0.075 to 0.2ml of suspension) in a final volume of 3ml of respiration buffer, air saturated at 30°C was incubated in the electrode.

The final concentration of substrates included in the incubation were pyruvate (8mM) with malate (2mM), succinate (10mM) or B-OH butyrate (10mM). ADP (800 nanomoles) was added to stimulate respiration. Respiratory activity was measured following sequential addition of oxidisable substrates to isolated mitochondrial preparations in the oxygen electrode.

RESULTS

Respiratory Activities of Isolated Rat Liver Mitochondria

Table 3.1 shows the respiratory control index, ADP:O ratio and oxygen consumption rates for preparations of isolated rat liver mitochondria supplied with different oxidisable substrates following incubation in the oxygen electrode. Liver mitochondria were able to oxidise each of the three substrates supplied (malate/pyruvate, succinate and B-OH butyrate). ADP:O ratios for pyruvate and B-OH butyrate approached 3, while the ratio for succinate was close to 2. With all substrates utilised, oxygen consumption rates increased following stimulation with ADP (state 3 respiration). For both B-OH butyrate and malate/pyruvate the state 4 respiratory rate was similar prior to and after stimulation with ADP. However, state 4 respiratory activity with succinate, following ADP utilisation was much greater than the initial state 4 respiration rate.

Respiratory Activity of Isolated Walker 256 Mitochondria

Table 3.2 shows the respiratory control index, ADP:O ratio and oxygen consumption rates for preparations of isolated Walker 256 mitochondria incubated in the oxygen electrode following addition of different oxidizable substrates. Respiratory control indices for malate/pyruvate and succinate as fuels were similar to values obtained with rat liver mitochondrial preparations. The ADP:O ratio when mitochondria were supplied with malate/pyruvate was close to 3. However the ADP:O ratio when succinate was supplied was greater than the expected value of 2. Oxygen consumption rates for both state 3 and state 4 respiration were much lower than rates obtained with liver mitochondrial preparations when either succinate or malate/pyruvate were supplied as respiratory fuels. In both cases the state 4 respiration rates prior to and after ADP stimulation were comparable. A striking feature of these results is the inability of W256 mitochondria to metabolise B-OH butyrate.

Substrate	RCI	ADP:O Ratio	Oxygen Consumption ng atoms/mg/min			N
Malate / Pyruvate	2.92 (0.15)	3.14 (0.10)	State 4	18.03	(2.06)	23
			State 3	57.28	(5.08)	23
			State 4	22.13	(2.03)	23
Succinate	3.62 (0.11)	2.16 (0.07)	State 4	37.42	(2.79)	28
			State 3	144.00	(8.05)	28
			State 4	60.24	(17.20)	28
B-OH Butyrate	3.05 (0.16)	2.94 (0.08)	State 4	15.10	(1.37)	11
			State 3	47.71	(4.72)	11
			State 4	18.44	(1.89)	9

Table 3.1: Respiratory Control Indices (RCI), ADP:O ratios and oxygen consumption rates for isolated rat liver mitochondria incubated in the oxygen electrode at 30°C. Isolated organelles were supplied with Malate/pyruvate, succinate or B-OH butyrate. Oxygen consumption rates are expressed per milligram protein. Figures in parenthesis are standard errors of mean values recorded.

Substrate	RCI	ADP:O Ratio	Oxygen Consumption ng atoms/mg/min			N
Malate / Pyruvate	3.54 (0.64)	3.38 (0.31)	State 4	4.04	(0.65)	4
			State 3	14.21	(1.20)	5
			State 4	5.52	(1.67)	4
Succinate	3.17 (0.37)	2.88 (0.35)	State 4	10.27	(1.26)	4
			State 3	32.10	(6.95)	4
			State 4	13.58	(1.21)	4
B-OH Butyrate	Not Metabolized					

Table 3.2: Respiratory Control Indices (RCI), ADP:O ratios and oxygen consumption rates for isolated W256 mitochondria incubated in the oxygen electrode at 30°C. Isolated organelles were supplied with Malate/pyruvate, succinate or B-OH butyrate. Oxygen consumption rates are expressed per milligram protein. Figures in parenthesis are standard errors of mean values recorded.

DISCUSSION

These results indicate that isolated rat liver mitochondria can be prepared which exhibit tight coupling between ADP phosphorylation and oxygen consumption. When compared with published work these results are consistent in terms of ADP:O ratios and oxygen consumption rates (Johnson and Lardy, 1967; Kaschnitz et al, 1976). Mean RCI values were lower than published values, however individual determinations did approach the values of 4 and 5 quoted in the literature (Pedersen et al, 1978; Pedersen, 1978). It should be noted that published figures often indicate an individual result which authors claimed was representative of the data obtained from the study (Sordahl et al, 1969; Pedersen et al, 1970). Mean values of the RCI obtained in this study for substrates succinate and B-OH butyrate were 3.62 and 3.05 respectively. These values were well within the range of RCI values published by other groups (Kaschnitz et al, 1976).

Results obtained with W256 mitochondria also indicated that tightly coupled preparations could be obtained with a characteristic oxidative function. Although oxygen consumption rates expressed per mg protein were much lower than liver, ADP:O ratios and RCI values compared favourably.

Comparison of the respiratory parameters with rapidly growing poorly differentiated hepatoma mitochondria for the substrate succinate indicated that RCI values were in agreement, but not ADP:O ratio which was greater than values published (Kaschnitz et al, 1976; Pedersen et al, 1970; Sordahl et al, 1969). State 3 respiratory rates were lower than reported for hepatoma. Similar ADP:O ratios for malate and RCI values of succinate and malate were reported with lymphoma mitochondria and Ehrlich ascites tumour mitochondria (Gregg, 1972; Wu and Sauer, 1967). State 3 respiratory rates were however, lower than reported for lymphoma. From sources quoted in the literature, the mean RCI values obtained for substrates malate/pyruvate and succinate of 3.54 and 3.17 respectively were within the range of published figures for tumour mitochondria and individual results compared favourably with data from the literature (Gregg, 1972; Wu and Sauer,

1967; Kaschnitz et al, 1976; Pedersen et al, 1970; Sordahl et al, 1969).

Liver mitochondrial suspensions supplemented with succinate registered a state 4 respiratory rate which was not regained following complete utilisation of exogenous ADP. This implies that a contaminating ATPase was present in the preparation which was able to regenerate ADP for continued phosphorylation. A possible source of ATPase could have been mitochondria damaged during preparation in such a manner that the ATP synthase responsible for ATP synthesis was now operating in the reverse direction, proton flow being generated to stimulate ATPase activity. Yet coupling ADP phosphorylation to consumption of oxygen was still observed with substrates malate/pyruvate and B-OH butyrate. It is more likely that the higher state 4 respiratory rate resulted from an over estimate of the state 4 respiratory before a steady state had been reached. Thus complete ADP utilisation had not in fact occurred. Subsequent estimations of the state 4 respiratory with other substrates following ADP utilisation were more accurate because the state 3 respiratory rates were less and therefore, complete utilisation could be more precisely assessed.

W256 tumour mitochondrial preparations showed a higher than expected ADP:O ratio with succinate. The value of 3 obtained was reproducible. In classical terms B-OH butyrate or malate/pyruvate are respiratory fuels which utilise the NAD-linked pathway of electron transport. The ratio of moles of ADP consumed to moles of oxygen consumed is 3:1 which results, according to the chemiosmotic theory, from the 3 sites which protons can pass through the mitochondrial membrane and be utilised by the ATP synthase (Mitchell, 1976). Succinate however is an FAD linked substrate and electrons released following substrate utilisation feed into the electron transport pathway entering at a point further along the mitochondrial electron transport chain. The ratio of moles of ADP utilised to moles of oxygen consumed is only 2:1. Results obtained with malate/pyruvate fit this model well. Results with succinate give a much higher ADP:O ratio unlike other tumour mitochondria which have ADP:O ratios of between 1 and 2 (Gregg, 1972; Wu and Sauer, 1967; Kaschnitz et al, 1976; Pedersen, 1970;

Sordahl et al, 1969). The ADP:O ratio obtained when succinate was utilised as the respiratory fuel implies that the preparation may have been uncoupled somewhat. Low oxygen consumption rates with tumour mitochondria probably reflect the difference in protein content of the preparation rather than defects in oxygen utilisation. Rates obtained with hepatoma correlate more closely with liver as expected if protein content of both tissues were similar (Kaschnitz et al, 1976; Sordahl et al, 1969).

Other explanations for low oxygen uptake rates obtained with W256 mitochondria as compared to liver mitochondria could also be possible. The preparations may have contained a low yield of viable organelles. It was not possible to assess the viability of the preparation incubated in the oxygen electrode. However a reduction in the size of the mitochondrial pellet was noted implying fewer mitochondria were present. To obtain a measurable response a 2 to 3 fold increase in the volume of suspension relative to the liver mitochondrial suspension was required. Alternatively tumour mitochondria may have a lower functional efficiency in terms of their ability to metabolize exogenous oxidisable substrates.

Tumour mitochondria are thought to be more fragile than organelles isolated from normal cells. This results from differences in mitochondrial membrane composition between normal cell and neoplastic cell organelles (Pedersen, 1978; Wehrle and Pedersen, 1982). Hence viable organelles are more likely to be destroyed during preparation of tumour mitochondrial suspensions. Furthermore uncoupling agents and membrane damaging agents present in the homogenate may also be responsible for reducing the viability of the preparation (Pedersen, 1978; Wehrle and Pedersen, 1982).

W256 mitochondria were unable to oxidise B-OH butyrate. Other groups have documented both decreased and non-utilisation of B-OH butyrate as a respiratory fuel by isolated hepatoma mitochondria (Pedersen et al, 1970; Kaschnitz et al, 1976; Nakashima et al, 1984). This is not thought to be a result of any defect in the electron transport pathways of tumour mitochondria. Instead it has been postulated that

defects in fatty acid oxidation and associated transmembrane shuttles prevent entry of fatty acid substrates into the organelle (Cederbaum and Rubin, 1976). B-OH butyrate is a ketone body which under extreme conditions (e.g. starvation) is produced by the liver from fatty acids. It is as an energy source for utilisation in the brain and other tissues following depletion of carbohydrate sources (Tisdale, 1982). In order to utilise B-OH butyrate several enzymes are required in the cell which enable the substrate to be converted to Acetyl CoA, a TCA cycle intermediate. The pathway proceeds as follows in mitochondria:

B-OH Butyrate -----	Acetoacetate -----	Acetoacetyl CoA -----	Acetyl CoA
B-OH butyrate	3-Oxo Acid	Acetoacetyl CoA	
Dehydrogenase	CoA transferase	Thiolase	
[3-Hydroxybutyrate]	[Succinyl-CoA:]	[Acetyl-CoA]	
[Dehydrogenase]	[3-Oxo-acid]	[transferase]	
	[CoA transferase]		
E.C. 1.1.1.30	E.C. 2.8.3.5	E.C. 2.3.1.9	

(Williamson et al, 1971; Robinson and Williamson, 1980).

Recent evidence indicates that activities of both 3-Oxo acid CoA transferase and acetoacetyl CoA thiolase are reduced in W256 cells. B-OH butyrate dehydrogenase activity does compare favourably with normal tissue (Tisdale and Brennan, 1983; Fearon et al, 1985(b)). Not all tumour tissues are depleted of these enzymes and expression of the 3-Oxo acid CoA transferase is found in other tumour cells including hepatoma (Tisdale and Brennan, 1983; Fenselau et al, 1976).

It is possible that cleaner preparations, obtained by alternative methods of purification might have resulted in mitochondria both from liver and tumour with greater viability which exhibited higher RCI values for example. In retrospect, with tumour organelle preparations, intactness of the individual components of the electron transport chain could have been assessed by addition of selective inhibitors of mitochondrial function with exogenous oxidisable

substrates. Examples include inhibitors such as rotenone which inhibits NAD^+ -linked electron transport. Thus when administered with succinate the integrity of the FAD^+ -linked path could have been determined in tumour mitochondrial preparations (Chance and Williams, 1956; Slater 1967).

The W256 carcinosarcoma is a poorly differentiated, highly glycolytic tumour. There are tumours which show varying degrees of differentiation and such tumours have normal or near normal mitochondria showing closer correlation in metabolic function to normal cell organelles (Pedersen, 1978).

The studies described in this chapter may be summarised as follows. A comparison of the respiratory properties of freshly isolated rat liver mitochondria and W256 tumour mitochondria reveal tightly coupled preparations which appear to be functionally intact. Certain metabolic differences are apparent, W256 mitochondria appear to be unable to metabolise B-OH butyrate lacking enzymes which enable utilisation of this substrate. However, mitochondria isolated from W256, a highly glycolytic poorly differentiated tumour do appear to be functional with respect to RCI and ADP:O ratios. Lower rates of oxygen consumption may be attributed to low protein content of the suspensions rather than a defect in electron transport itself.

This type of study does not reveal much information about the integration of the mitochondrial pathways of energy metabolism into whole cell energy metabolism in tumours. Therefore the effects of antimitochondrial agents upon metabolism within this system could not assess any anaerobic compensation, essentially a cytoplasmic process. Whole cell energy metabolism, that is both glycolysis and mitochondrial energetic function can only be studied in the intact cell. Limited information is provided about mitochondrial metabolism, and little could be determined about the state of mitochondria in tumour tissue. In conclusion, for the amount of information obtained, this study would be unsuitable for screening antimitochondrial agents against tumour cell energy metabolism. A better approach might be to study energy metabolism in the intact cell. Although the degree of

control that can be exerted over the system in vitro, is less than over isolated mitochondria, the effect of antimitochondrial agents upon bioenergetic function in the intact tumour cell can be assessed. This approach is described in chapter 4.

CHAPTER 4

THE EFFECT OF POTENTIAL ANTIMITOCHONDRIAL AGENTS ON CELLULAR ENERGY METABOLISM

INTRODUCTION

The main aim of the studies described in this chapter is to identify compounds which may be used to selectively inhibit tumour cell energy metabolism leaving normal cell energy function relatively intact. By exploiting differences between the cellular energy metabolism of normal and neoplastic cells, it is hoped that antimitochondrial agents can be selected which will alter the bioenergetic function of tumour cell mitochondria at doses which will have little or no effect upon mitochondria within the intact normal cell.

Clearly the ultimate aim of the work described in this thesis is to inhibit tumour cell energy metabolism in the whole animal. However, the study of energy metabolism in man or even in animal tumour models is complex and hence the mode of action of a potential antimitochondrial would be very difficult to establish. In chapter 3 an attempt was made to study the metabolism of isolated mitochondria from both normal and tumour cells. The study highlighted several problems with this approach and it was concluded that the model was unsuitable for the study of potential antimitochondrial agents. Although the whole cell is a more complex system it still has a number of advantages over the major complexity of the whole animal. Thus, the studies described in this chapter examine the effects of selective antimitochondrial agents on whole cell energy metabolism. The effect of potential antimitochondrial agents upon whole cell oxygen consumption in vitro as a measure of mitochondrial activity was investigated. Although substrate level depletions are harder to achieve with this model, any compensation of the anaerobic component of cell energy metabolism following exposure of cells to antimitochondrial agents may be investigated.

Whole cells may be obtained from two sources. Tumour cells may be obtained from either solid tumours grown up as xenografts in athymic mice or malignancies implanted in rats, following dispersion of tumours with collagenase. However, cells obtained by such methods not only contain quantities of necrotic cells, but also much stromal tissue, raising doubt as to the cell type responding to the drugs under investigation. Furthermore, contamination of the cell preparation with necrotic material is likely to occur. Pure tumour cell populations could however be obtained from continuous cultured cell lines. Such cells would be homogeneous and representative of the original tumour albeit a specific subpopulation of cells obtained from the original tumour whose growth was favoured by culture conditions. The experiments described in this chapter examine the effects of antimetabolic agents for any selective action these compounds might show on aerobic or anaerobic metabolism of tumour cells obtained from a tissue culture source.

Pathways which exist in cells for generating energy in the form of ATP are dependent to some extent on the availability of oxygen. Where oxygen availability is limited, cell ATP production occurs primarily by anaerobic glycolysis. A major byproduct of anaerobic metabolism is lactic acid which in the whole animal in vivo is rapidly removed from cells. Under aerobic conditions, cell ATP production is generated largely through mitochondrial activity. Glycolysis generates the mitochondrial substrate pyruvate from hexoses and this in turn is used to generate mitochondrial ATP via TCA cycle activity and associated oxidative phosphorylation. A third pathway, the pentose phosphate pathway, associated both with glycolysis and mitochondrial pathways, exists for degradation of hexose carbon. The pentose phosphate pathway however is mainly used to generate reducing power in the extra mitochondrial cytoplasm as NADPH, to convert hexoses into pentoses for biosynthesis of nucleic acids and to complete oxidative degradation of pentoses converting them into hexoses which can subsequently enter the glycolytic sequence. It is not the main pathway for obtaining energy from oxidation of glucose in animal cells (Lehninger, 1975).

Both glycolytic and mitochondrial pathways do appear to be intact in tumour cells, whether tumour cells are characterised as poorly differentiated (chapter 1) or well differentiated (Pedersen, 1978).

Methods for examining glycolysis and mitochondrial bioenergetic function were selected from those which could be studied in the intact cell.

Glycolytic flux may be studied by monitoring activities of enzymes such as phosphofructokinase [E.C. 2.7.1.1] a key regulatory enzyme in the pathway. However, such an approach would require disruption of the cell and destruction of cell integrity (Guminska et al, 1986).

An alternative method applicable to whole, intact cells is to study the effect of drugs upon the metabolic endpoint of the pathway, i.e. lactate production following incubation of cells with drug (Racker and Spector, 1981; Nakashima et al, 1984).

Oxidative function in cells may be studied in isolated mitochondrial preparations or even submitochondrial particles consisting of some of the mitochondrial enzyme complexes. As described however, such methods would yield little information about whole cell oxygen metabolism. Early methods for studying mitochondrial function indirectly in either whole cell preparations or tissue slices relied upon manometric methods, which though accurate in determining respiratory function were not sensitive to slight fluctuations in respiratory activity (Umbreit et al, 1972). A more accurate method, oxygen polarography which was sensitive to respiratory fluctuations became available for studying whole cell oxygen uptake (Estabrook, 1967; Gregg et al, 1968). The effect of agents which altered cellular bioenergetic function could be studied following incubation of such agents with whole cell preparations in a Clark-type oxygen electrode (Knapp, 1983).

Cell types selected for this study were both neoplastic and non-neoplastic in origin.

WIL, a non small cell lung tumour line is representative of a relatively slow growing malignancy with some degree of differentiation. The WIL tumour both as a cell line and in xenograft form from studies in vitro and in vivo has been shown to be very resistant to conventional chemotherapy (Merry et al, 1986; Merry et al, 1987). WIL grew as an anchorage-dependent monolayer under culture conditions as a continuous cell line in vitro. Preliminary studies with W256 grown as a solid tumour in Wistar rats indicate that this tumour does respond to novel forms of chemotherapy directed at tumour energy metabolism (Fearon et al, 1985(b)). The W256 carcinoma is representative of a rapidly growing poorly differentiated tumour which is very dependent upon aerobic glycolysis for continued growth. Under tissue culture conditions W256 cells grew as a suspension culture in vitro.

All continuous cell lines to some extent are transformed cells. In order to propagate over generations normal cells from animal sources will lose their normal phenotype and transform in culture (Ruddle 1961; Yerganien and Leonard, 1961; Hayflick and Moorhead, 1961; Todaro and Green, 1963). Therefore a normal cell type for the purposes of this study had to be obtained from freshly isolated cells. One of the most well characterised cell types, whose metabolic, properties have been well documented are rat hepatocytes (Seglen, 1976; Lebreque and Howard, 1976; Pogson et al, 1984). Freshly prepared rat hepatocytes were used as a source of non-neoplastic cells for the purpose of drug screening.

Studies described in this chapter were designed to evaluate the potential of proposed tumour specific antimitchondrial agents. Agents selected for this study included Rhodamine-6G (R6G). The rhodamine dyes, more specifically Rhodamine 1, 2, 3 (R123) have been shown to exert a selective cytotoxic effect against tumour cells with little effect upon normal cells (Bernal et al, 1982(b); Lampidis et al, 1983; Nadakavukaren et al, 1985). Other compounds included in the study were clofazamine (lamprene), chlorimipramine (anafranil), and maprotiline (ludiomil). All these compounds are in clinical use and are thought to show antimitchondrial activity in both higher and

lower eukaryote systems (Hughes and Wilkie, 1970; Wilkie and Delhanty, 1970; Rhodes and Wilkie, 1973).

Compounds were screened for their effects upon whole cell oxygen consumption in tumour cell preparations, WIL and W256, as well as freshly prepared rat hepatocytes. Oxygen consumption rates were determined with a Clark-type oxygen electrode. A measure of the mitochondrial response to these agents could thus be obtained from such a study. The glycolytic component of cellular energy metabolism was assessed from the effect these agents had on whole cell lactate production, determined by an enzymatic method.

METHODS

Preparation of Isolated Rat Hepatocytes

Rat hepatocytes were prepared essentially as described by Seglen (1976) (see also Berry and Friend, 1969).

Female Wistar rats weighing between 200 and 300 grams were anaesthetised with ether and positioned on their back. An incision was made to expose the abdominal wall. Epidermal layers were separated from the abdomen wall with scissors and forceps. With fine tipped scissors, the abdomen was opened. The intestine was removed from the abdominal cavity and positioned to the left of the animal. The hepatic portal vein was identified and 3 loose ligatures were placed around the vessel. A 20 gauge Venflon Cannula (Viggo, Swindon, Wilts) was inserted into the vessel. Successful cannulation ensured that a flow of blood was observed along the length of the cannula as the needle was withdrawn, leaving the cannula in place. The cannula was secured with ligatures. The organ was cleared of blood by passing 50ml of calcium-free modified Berry medium (KCl 5.4mM, NaCl 140mM, MgSO₄.7H₂O 0.8mM, NaH₂PO₄ 0.8mM, Na HCO₃ 25mM, glucose 15mM adjusted to pH 7.4 (Berry, 1974). Buffer was supplemented with 25 international units of heparin per ml buffer, to keep blood vessels dilated enabling continued perfusion of the organ. This heparinized medium was passed through the organ by two discharges of a 20ml syringe. An incision in the posterior vena cava enabled fluid to drain into the abdominal cavity. Modified Berry medium was equilibrated for 30 minutes prior to use and during perfusion with O₂:CO₂ (95:5) at 37°C. A flow rate of 20ml per minute was maintained by a peristaltic pump (Shuco Scientific Ltd, London). As blood drained from the organ, the tissue changed from red to brown in colour. A bubble trap ensured that microbubbles in the buffer did not accentuate perfusion damage. The liver was excised out of the abdominal cavity, taking care not to disturb the cannula. It was suspended on a 100 micron nylon mesh. The mesh was attached across a plastic funnel suspended above a reservoir of collagenase 0.5% (collagenase Type IV, Sigma) in modified Berry medium supplemented

with CaCl_2 (5mM) equilibrated with $\text{O}_2:\text{CO}_2$ (95:5) and maintained at 37°C .

Recirculation of collagenase through the organ was achieved by means of a 3 way tap in the perfusion circuit.

After 20 to 30 minutes, the organ was removed to a petri dish and fresh buffer perfused through it. The liver was gently disaggregated with two pairs of fine forceps. The resulting suspension was filtered twice through 100 micron nylon mesh into 30ml universal containers. The vessels were centrifuged in a bench top centrifuge (MSE, FSA, Loughborough, Leics) at between 3 to 5g for 5 minutes in order to sediment cells. The supernatant was discarded and cells were resuspended in F10:DMEM (50:50) containing 2mM glutamine, 10% foetal calf serum (FCS) and 0.5ug per ml deoxyribonuclease (DNase). Addition of DNase prevented cells from adhering to one another by means of DNA released from damaged cells. Cells were counted in a haemocytometer under a phase contrast microscope. Cell viability was determined by dye exclusion (Howard et al, 1973).

0.2% trypan blue was made up in phosphate buffered saline (PBS). 0.1ml of dye was added to 0.5ml of cell suspension. To this was added 0.5ml of PBS. Only preparations where cell viability was at least 60% were used for further studies.

Tumour Cell Lines

Tumour cell lines WIL and W256 were maintained in tissue culture in vitro as described in Chapter 2. Harvesting of WIL, grown as a monolayer in vitro was facilitated with trypsin as described in chapter 2. Trypsinised cells from each flask were pooled and resuspended in fresh F10:DMEM and placed in 30ml universal containers. Cell number was determined with a Coulter Counter. Cell viability was assessed by dye exclusion (0.02% trypan blue) and viewed under phase contrast. Viability exceeded 95%.

W256 cells were maintained in vitro as described in chapter 2. Harvesting of W256 cells was carried out by decanting cells into

Tumour Cell Preparation

1. Walker 256 Rat carcinosarcoma grown in suspension culture
2. WIL Human lung tumour line grown as monolayers in culture

Both lines maintained in F10:DMEM+ Glutamine+ 10% FCS.



1. W256 cells sedimented at 1000g and resuspended in medium
2. WIL cells trypsinised and resuspended in medium

Viability determined by Trypan blue exclusion and cells counted in Coulter Counter.



Cells washed and resuspended in aerated Chance-Hess medium (154mM NaCl, 6.16mM KCl, 9.35mM Na₂HPO₄, 1.65mM NaH₂PO₄ PH 7.4) at a concentration of 1×10^7 cells per ml.



Measure Oxygen consumption of 1×10^7 cell aliquots in 3 ml of respiration buffer (above) total volume supplemented with 6mM Glucose in a Clark-type Oxygen electrode.

Figure 4.1 A summary of the procedure for preparing tumour cells for studies of whole cell oxygen uptake.

sterile 30ml universal containers. Cells were sedimented in a bench top centrifuge at 130g. Cells were resuspended in fresh medium. Cell number was determined with a Coulter Counter. Cell viability was assessed by dye exclusion (0.02%) trypan blue and viewed under phase contrast. Viability exceeded 90%. Cell preparation is summarised in Figure 4.1.

Total Cell Protein

Total cell protein was determined by the method of Lowry et al (1951) essentially as described in chapter 2.

Total Cell DNA

Total cell DNA content was assessed by the method of LePecq and Paoletti (1964) with modifications according to Karsten and Wollenburger (1972) and Gardner and Plumb (1979) as described in chapter 2.

Measurement of Cellular Respiratory Activity

Cellular respiratory activity was monitored by means of a Clark-type oxygen electrode (see chapter 2).

Cells were washed at least once in Chance-Hess respiration medium. This buffer consisted of NaCl 154mM, KCl 6.16mM, Na₂HPO₄ 9.35mM, NaH₂PO₄ 1.65mM, equilibrated to pH 7.4. Cells were resuspended in fresh buffer at a concentration of 1×10^7 cells per ml.

Cells were incubated in a total volume of 3ml of air saturated medium at 37°C in the oxygen electrode at a concentration of between 5×10^6 and 1×10^7 cells per 3ml. The medium contained glucose (6mM). Drugs were introduced by means of a hamilton syringe once the rate of oxygen consumption was established. Cellular oxygen consumption rates were determined prior to drug addition and throughout the experiment in order to assess the viability of the preparation. Incubations were carried out for between 15 and 20 minutes. The oxygen uptake rate varied between preparations. In order to compare results between preparations the oxygen consumption rate in the presence of drug was expressed as a percentage of the rate prior to drug addition.

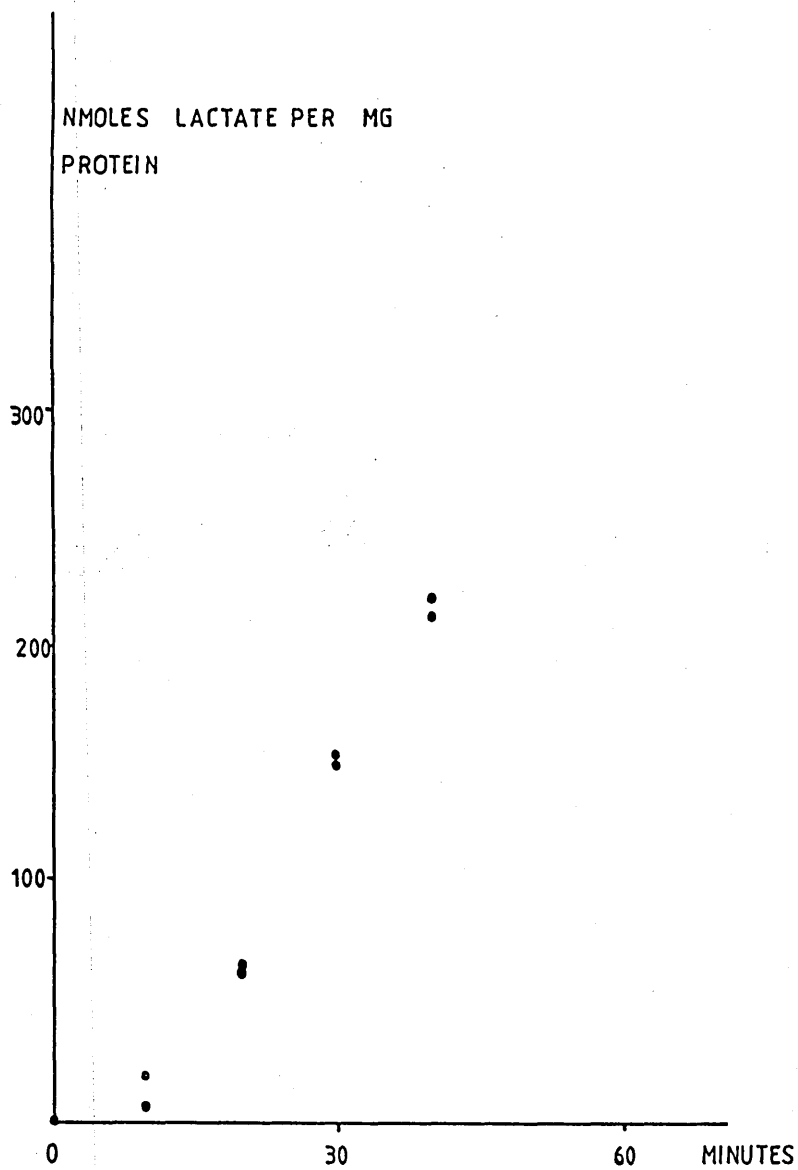


Figure 4.2: Lactate production as nanomoles of lactate per mg protein for WIL expressed against time in minutes. The production of lactate by WIL is linear with time over a 40 minute period. For each time point duplicate results were recorded.

Lactate Production Rates

Cells were prepared as described previously (chapter 2 p 29). Cells were resuspended in 3ml of fresh Chance-Hess medium at a concentration of 2.5×10^6 cells per ml.

Cells were preincubated at 37°C with drugs in sealed 25ml pyrex flasks in a shaking waterbath for 10 to 15 minutes. Addition of glucose (6mM) stimulated glycolysis and the reaction was thus initiated. Incubations were continued for 30 minutes, during which time the rate of lactate production was linear (see figure 4.2). At 10 minute intervals 0.2ml samples were removed, placed in eppendorf tubes on ice. Cells were sedimented immediately at 160g by means of a bench top refrigerated centrifuge (Jouan, Saint Herblain, France). The supernatant was collected and stored at -20°C. 0.1ml samples were assayed for lactate.

Lactate was measured by the method of Noll (1974) (see also Nakashima *et al*, 1984).

Assay Principle

The assay is based upon an enzymatic reaction which is linked to the reduction of NAD^+ to NADH. The reaction is followed on a spectrophotometer at a wavelength of 340nm. In order to drive the reduction of NAD^+ to NADH, when lactate is oxidized to pyruvate, the equilibrium conditions of the reaction must be shifted to the right. Pyruvate is used to transaminate glutamate to form L-alanine and α -keto glutarate (2-oxoglutarate), a reaction catalysed by the enzyme glutamate pyruvate transaminase [L-alanine:2oxo-glutarate amino-transferase E.C. 2.6.1.2]. Figure 4.3 summarises the procedure used and the basis of the reaction.

Estimation of Lactate

Samples (0.1ml) were placed in glass test tubes. Distilled water, 2.1ml followed by glutamic acid, 0.67ml (525mM in NaOH, 1M) and NAD^+ , 0.1ml (28mM) was then added. 0.015ml of glutamate pyruvate transaminase (GPT) stock suspension (L-alanine:2oxo-glutarate amino-transferase E.C. 2.6.1.2, from pigheart, 80 international units per mg

Lactate Production

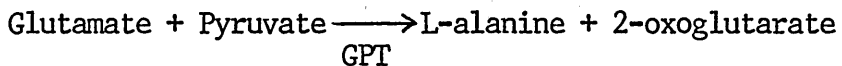
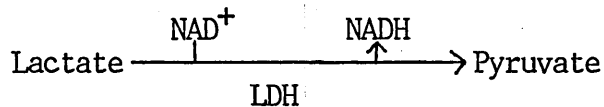
1. 2.5×10^6 cells per ml suspended in 3ml aerated Chance-Hess medium in sealed flasks and a shaking water bath at 37°C .
2. Preincubate appropriate drug concentration with cells for 10-15 minutes.
3. Add 6 mM glucose to start reaction.

Incubate for 30 minutes

1. At 10 minute intervals collect 200ul samples in eppendorf tubes at 4°C .
2. Centrifuge immediately at 160g to sediment cells.
3. Collect supernatant, use 100ul samples for Lactate Assay.

Enzyme Reaction:

Lactate Dehydrogenase (LDH) / Glutamate Pyruvate Transaminase (GPT) system.



GPT pulls the equilibrium of the LDH reaction to the right.

Absorbance read in a Gilford spectrophotometer at $\lambda = 340\text{nm}$ detecting $\text{NAD}^+ + \text{H} \rightarrow \text{NADH}$.

Figure 4.3: A summary of the procedure for measuring whole cell lactate production in tumour cell and rat hepatocyte preparations.

protein) was added to each tube. Tube contents were vortex mixed and the absorbance measured at a wavelength of 340nm in a spectrophotometer. The spectrophotometer was set to zero with water. Final concentration of reagents in the cuvette were glutamate 122mM, NAD^+ 0.97mM and GPT 12 international units.

An assay blank was set up containing distilled water 2.2ml, glutamate 0.67ml (525mM in NaOH, 1M), NAD^+ , 0.1ml (28mM) and ammonium sulphate, 0.15ml (3.2M).

Ammonium sulphate was used in place of GPT such that the reaction could not proceed in the assay blank, equilibrium conditions for the net reaction being unfavourable.

0.010ml of lactate dehydrogenase (LDH) enzyme suspension (L-lactate-NAD oxidoreductase E.C. 1.1.1.27, 550 international units per mg protein from rabbit muscle) was added to each tube. Each tube thus contained 28 international units final concentration of LDH. Samples were mixed with vortex mixer and allowed to stand at room temperature for 30 to 60 minutes by which time all the lactate had been converted to alanine and 2-oxoglutarate. The absorbance of the samples was read at 340nm. The change in absorbance was used to determine the amount of lactate in each sample following correction by the blank. The blank contained all reagents except GPT. Ammonium sulphate to correct for turbidity of this enzyme preparation was added to this reaction mixture instead. Any reduction of NAD^+ which took place without GPT could be corrected for. A standard curve was prepared with known concentrations of lactate in the range 0 to 160 nanomoles per tube. Lactate concentrations were determined from the regression line fitted to the standard curve (Figure 4.4). Total lactate production was expressed as nmoles lactate per mg of total cell protein or per microgram total cell DNA. The lactate production rate was determined from the slope of the regression line from a plot of the total amount of lactate produced against incubation time. Production rates are expressed as nmoles lactate per minute per mg protein or per microgram DNA.

To ensure that drugs under investigation did not interfere in the enzymatic reaction in the samples of supernatant assayed, a series of control experiments were run. The minute quantities of drug in each sample did not interfere in the absorbance change registered at a wavelength of 340nm.

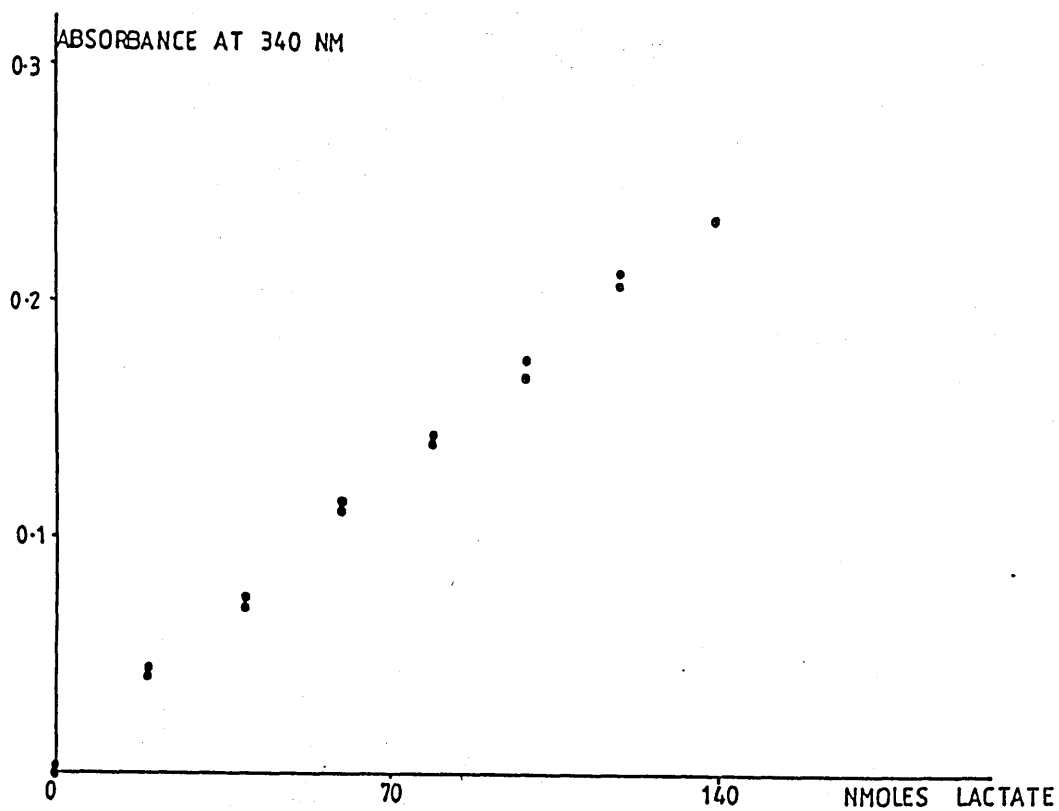


Figure 4.4: Absorbance at 340nm expressed against nanomole amount of lactate standard used in the enzymatic determination of lactate. A linear relationship exists between absorbance and nanomole amounts of lactate up to 140 nanomoles. Duplicate determinations were recorded for each lactate concentration.

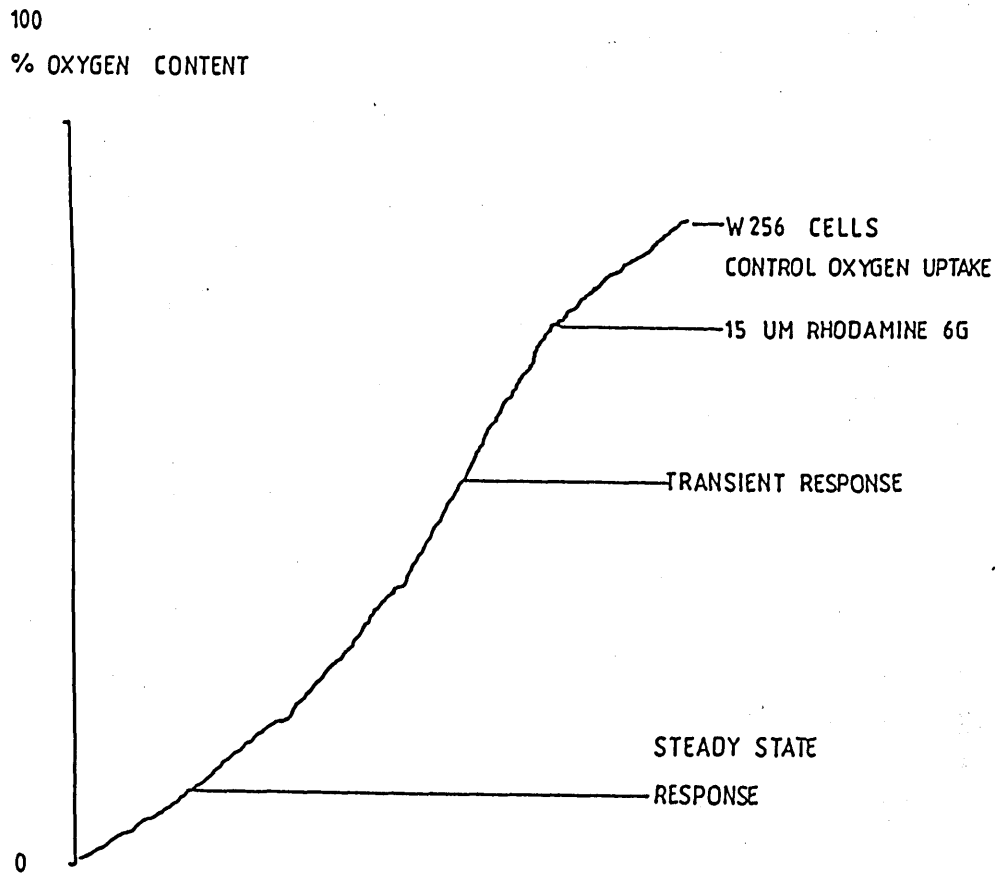


Figure 4.5: The rate of oxygen consumption in intact W256 cells incubated in Chance-Hess Medium at 37°C. The reaction was started by addition of W256 cells (1×10^7) to Chance-Hess medium, 2 ml (pH 7.4) in the presence of glucose, 6mM. After a steady state rate of oxygen consumption was attained, R6G, 15uM was added as indicated. Following a transient stimulation of the respiratory rate, a final steady state response was observed.

RESULTS

Effects of Rhodamine 6G on Oxygen Consumption Rates

Figure 4.5 is a representative trace obtained with W256 cells incubated in the oxygen electrode. At T=0, W256 cells (1×10^7 cells) are added to give a total volume of 3ml in the electrode. Cells respire exhibiting a control rate of oxygen consumption. Addition of 15uM R6G causes a stimulation of respiration, with an increase in the rate of oxygen consumption. This is transient however, and a steady state inhibition of oxygen consumption occurs as shown by a rate of oxygen uptake which is less than the control rate.

Figures 4.6A and 4.6B show the rate of oxygen uptake by WIL and W256 respectively following addition of R6G. Addition of R6G (2-20uM) to both WIL and W256 resulted in a transient stimulation of oxygen uptake which lasted for about 5 to 10 minutes. This stimulation of the respiratory rate increased with increasing concentrations of R6G. Following this initial stimulation oxygen consumption decreased for both cell lines (Figure 4.7). For WIL, the inhibition of oxygen uptake is dose dependent for concentrations of R6G between 1 and 10uM. No further inhibition is observed at a higher concentration of R6G. The inhibitory response of W256 to R6G is maximal at a concentration of 10uM. At higher concentrations R6G appears to be less toxic. Isolated rat hepatocytes exhibited a significant inhibition of oxygen consumption at R6G concentrations of greater than 20uM. At concentrations of R6G between 1 and 15uM a slight, but not significant, inhibition of oxygen consumption was observed.

Effect of Anafranil on Oxygen Consumption Rates

Cellular oxygen consumption in the presence of anafranil for the cell lines WIL and W256 are shown in Figures 4.8A and 4.8B. Anafranil (25uM) inhibited cellular oxygen consumption in both WIL and W256. Lower concentrations of anafranil showed only a very small inhibition of oxygen consumption. Concentrations of between 25uM and 200uM anafranil resulted in further inhibition of oxygen uptake.

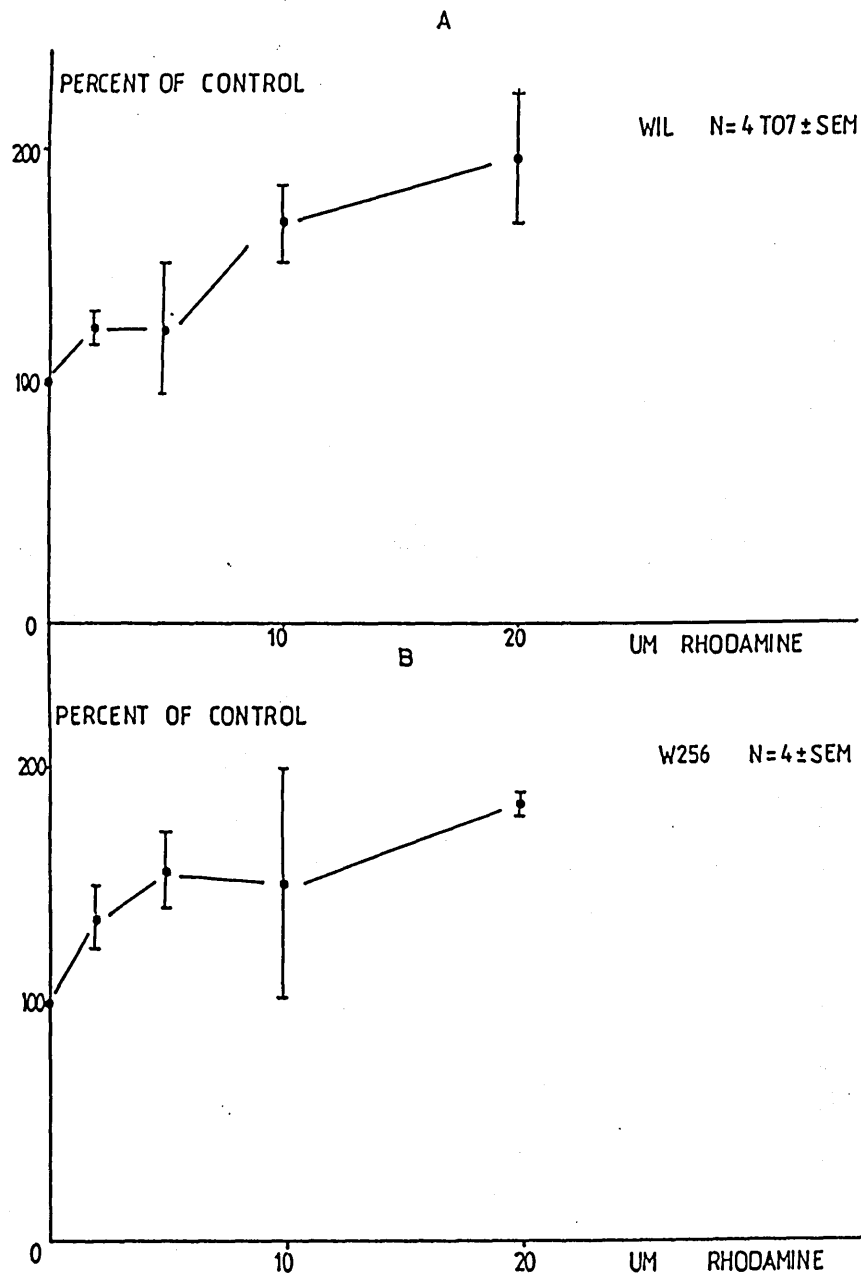


Figure 4.6A and 4.6B:

The rate of oxygen uptake by WIL (Figure 4.6A) and W256 (Figure 4.6B) respectively, measured in a Clark-type oxygen electrode following addition of R6G. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C. Transient response, measured as percentage change of oxygen consumption following addition of R6G, 1 to 20uM, over a 5 to 10 minute period.

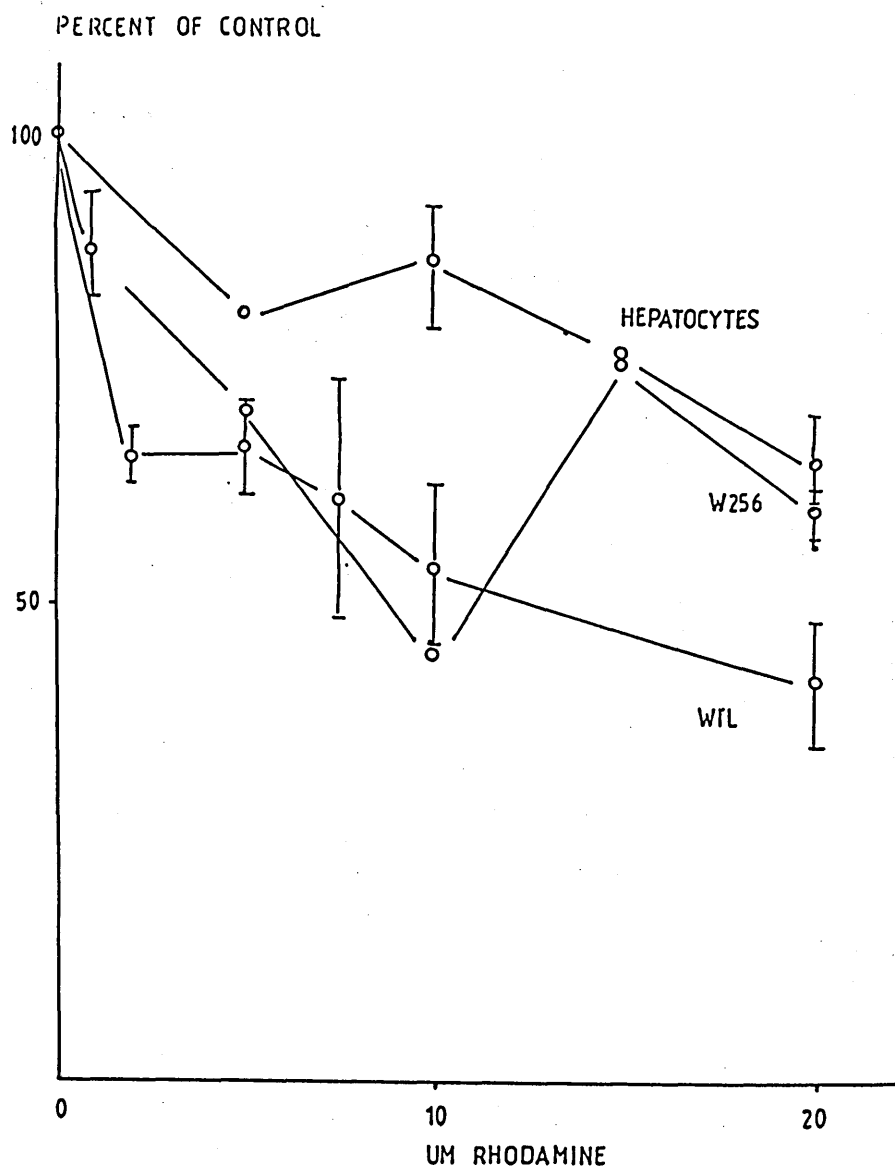


Figure 4.7: Steady state rate of oxygen uptake by WIL, W256 and rat hepatocytes, measured in a Clark-type oxygen electrode following addition of R6G. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C . Steady state response measured as percentage change of oxygen consumption following addition of R6G, 2 to 20 μM .

A

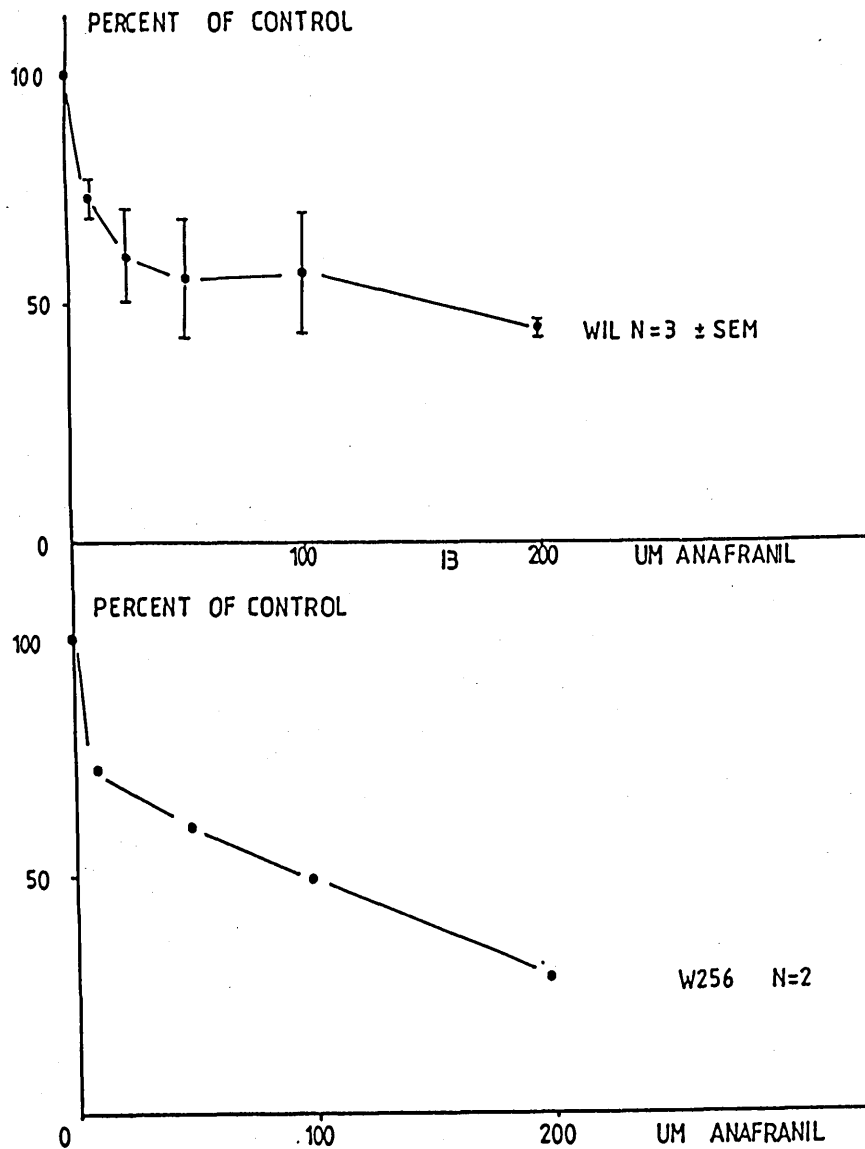


Figure 4.8A and 4.8B:

Rate of oxygen uptake by WIL (Figure 4.8A) and W256 (Figure 4.8B) respectively, measured in the oxygen electrode, following addition of anafranil. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C . Steady state response measured as percentage change of oxygen consumption following addition of anafranil, 10 to 200 μM .

Effect of Lamprene on Oxygen Uptake Rates

Figures 4.9A and 4.9B show the rate of oxygen uptake by WIL and W256 respectively following addition of lamprene. Incubation of lamprene (1 - 50 μ M) with both WIL and W256 resulted in a transient stimulation of oxygen uptake which lasted for 4 to 5 minutes. This stimulation of the respiratory rate increased with increasing concentrations of lamprene when incubated with WIL. The dose dependent stimulation of oxygen consumption by lamprene is not observed for W256. Following this initial stimulation, oxygen uptake exhibited a steady state stimulation of the respiratory rate. The steady state response (figure 4.10) is slightly lower than the initial respiratory burst for both cell lines WIL and W256. The stimulation of oxygen consumption by lamprene is dose dependent for the cell line WIL, but not for W256. Although lamprene was solubilized in dimethyl sulphoxide (DMSO), the presence of DMSO had no significant effect on oxygen uptake rates by either cell line.

Effect of Ludiomil upon Rates of Oxygen Consumption

Table 4.1 shows the rate of oxygen consumption following addition of ludiomil (1 - 100 μ M) for both cell lines WIL and W256. The rate of oxygen consumption by cells was decreased in the presence of ludiomil and this decrease was dose dependent for both cell lines WIL and W256.

Lactate Production Rates

Effect of R6G upon the rate of lactate production

Table 4.2 shows the effect of R6G on the rate of lactate production by WIL, W256 and freshly isolated rat hepatocytes. Production rates are expressed with reference to both cellular protein and DNA content. In the presence of R6G, lactate production rates for both WIL and W256 increased. When expressed either in terms of mg of cellular protein or micrograms of cellular DNA the production rate increased with increasing concentrations of R6G. However when rat hepatocytes were incubated in the presence of R6G (5 - 20 μ M) there was no significant increase in lactate production rates.

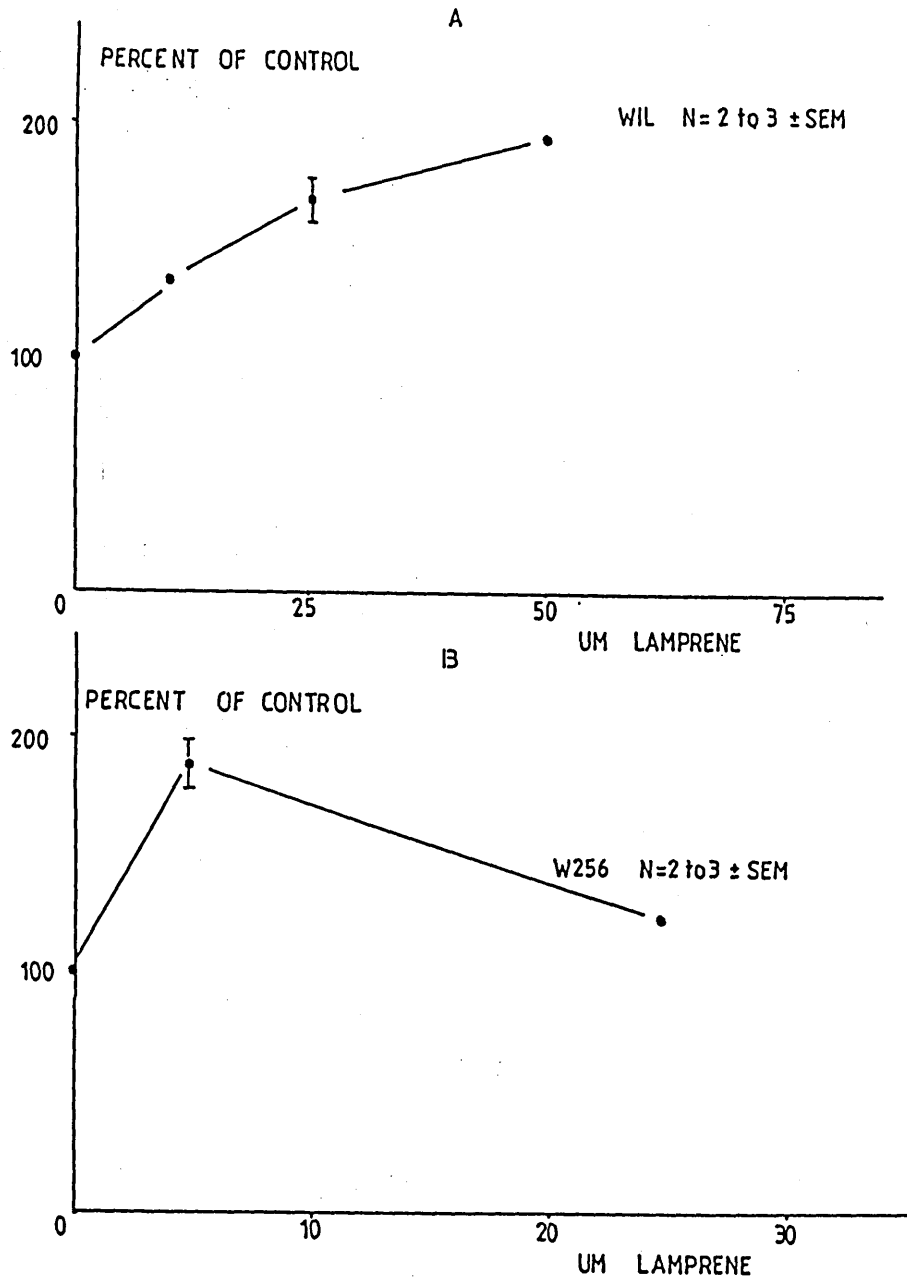


Figure 4.9A and 4.9B:

Rate of oxygen uptake by WIL (Figure 4.9A) and W256 (Figure 4.9B) respectively, measured in the oxygen electrode following addition of lamprene. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C. Transient response measured as percentage of oxygen consumption following addition of lamprene, 5 to 50uM (WIL) or 5 to 25uM (W256).

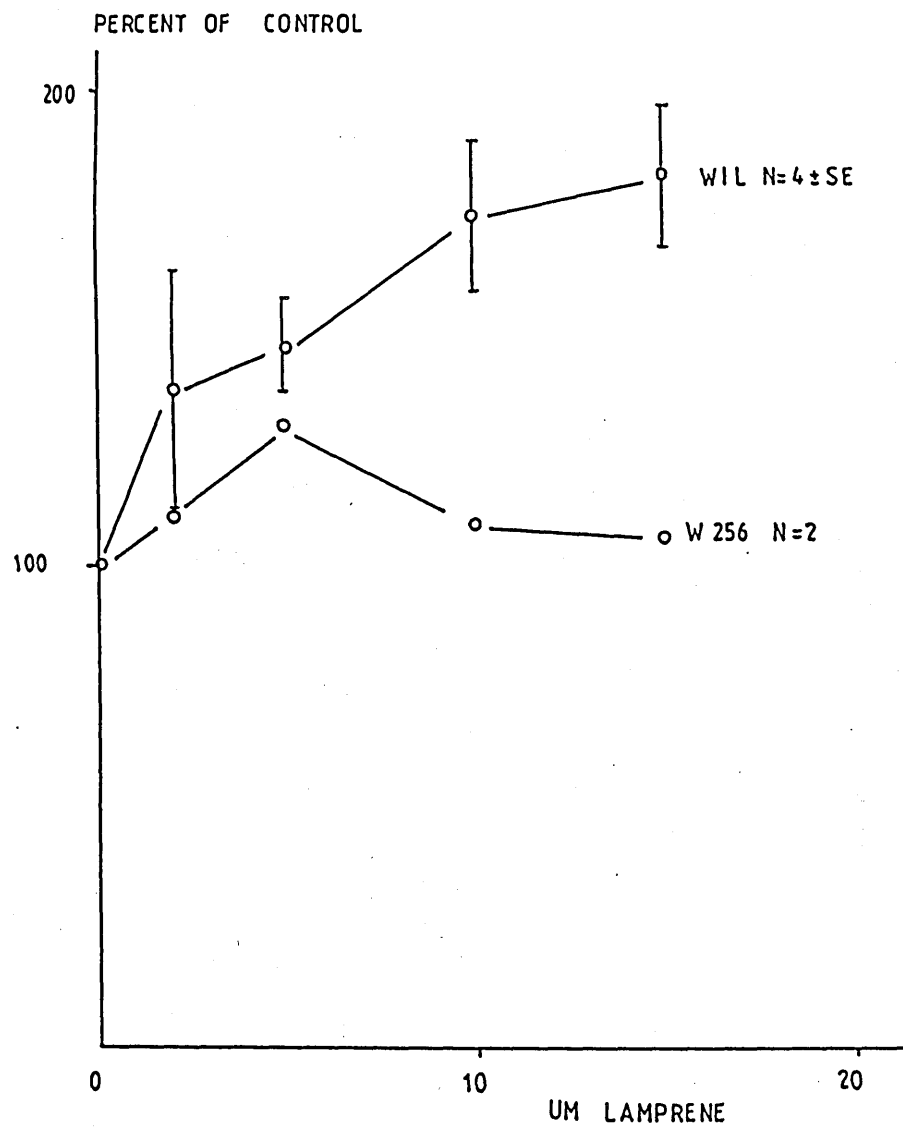


Figure 4.10: Rate of oxygen uptake by WIL and W256 measured in the oxygen electrode following addition of lamprene. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C. Steady state response measured as percentage change of oxygen consumption following addition of lamprene, 2 to 20uM.

W256	OXYGEN UPTAKE	OXYGEN UPTAKE	PERCENT OF CONTROL
CONDITIONS	ng atoms/mg/min	ng atoms/ug/min	
control	9.61	0.145	100
1uM Ludiomil	9.61	0.145	100
control	11.08	0.167	100
5uM Ludiomil	9.35	0.141	84
control	10.01	0.151	100
10uM Ludiomil	10.01	0.151	100
control	12.14	0.183	100
20uM Ludiomil	9.71	0.146	80
control	11.26	0.169	100
50uM Ludiomil	7.09	0.107	63
control	8.66	0.130	100
100uM Ludiomil	6.78	0.102	78
WIL	OXYGEN UPTAKE	OXYGEN UPTAKE	PERCENT OF CONTROL
CONDITIONS	ng atoms/mg/min	ng atoms/ug/min	
control	8.29	0.349	100
1uM Ludiomil	7.25	0.305	87
control	8.82	0.371	100
5uM Ludiomil	7.21	0.303	82
control	10.25	0.431	100
10uM Ludiomil	8.20	0.345	80
control	12.58	0.529	100
20uM Ludiomil	8.05	0.338	64
control	6.36	0.267	100
50uM Ludiomil	3.90	0.164	61

Table 4.1: Rate of oxygen uptake by WIL and W256 measured in the oxygen electrode following incubation with Ludiomil. Cells were incubated at a concentration of 1×10^7 cells in Chance-Hess medium, 3ml at 37°C. Steady state response measured as percentage change of oxygen consumption following addition of Ludiomil, 1 to 100uM. Oxygen uptake rates expressed as ng atoms oxygen per minute per mg protein or per ug DNA.

LACTATE PRODUCTION

nmoles lactate per min per ug DNA

R6G	0	5	10	20	uM
WIL	0.26	0.73	0.84	0.84	
W256	0.58	0.85	1.0	1.04	
Hepatocytes	0.1	0.1	0.1	0.14	

nmoles lactate per min per mg protein

R6G	0	5	10	20	uM
WIL	5.65	15.71	18.28	18.06	
W256	14.13	20.51	24.11	25.28	
Hepatocytes	3.62	4.08	3.61	5.04	

Table 4.2: Rate of lactate production by WIL and W256 measured by an enzymatic method following incubation with R6G. Cells were incubated at a concentration of 2.5×10^6 cells per ml in Chance-Hess medium, 3ml at 37°C, for 30 minutes in sealed flasks. Incubation medium sampled at 10 minute intervals. Samples assayed for lactate by an enzymatic method. Production rates expressed as nmoles lactate per min per mg protein or per ug DNA. Results are from individual experiments.

Effect of Lamprane on the rate of lactate production

Lactate production rates by WIL and W256 incubated in the presence of lamprane are shown in Table 4.3. There was an increase in the rate of lactate production when cells were incubated with lamprane (5-30uM). The rate of lactate production increased with increasing drug concentration for both cell lines studied.

Effect of Anafranil on the rate of lactate production

Table 4.4 shows the rate of lactate production in both cell lines WIL and W256 following incubation with anafranil (25-100uM). The lactate production rate was not affected by anafranil up to a concentration of 25uM. At greater concentrations there was a slight stimulation of lactate production in both cell lines.

Effect of Ludiomil on the rate of lactate production

Table 4.5 shows the rate of lactate production in WIL and W256 following addition of Ludiomil (5-50uM). Ludiomil had no consistent effect upon the rate of lactate production (when incubated) with WIL. Incubation of W256 cells with ludiomil resulted in a reduction in the rate of lactate production. This effect was dose dependent and increasing the concentration of ludiomil decreased the rate of lactate production further.

LACTATE PRODUCTION

nmoles lactate per min per ug DNA

Lamprene	0	5	10	20	30	uM
WIL	0.09	0.36	0.21	0.25	0.37	
W256	0.237	0.27	0.331	0.556	0.436	

nmoles lactate per min per mg protein

Lamprene	0	5	10	20	30	uM
WIL	3.7	14.45	8.33	10.0	18.5	
W256	13.68	15.63	19.16	32.16	25.20	

Table 4.3: Rate of lactate production by WIL and W256 following incubation with lamprene. Cells were incubated at a concentration of 2.5×10^6 cells per ml in Chance-Hess medium, 3ml in sealed flasks at 37°C for 30 minutes. Incubation medium was samples at 10 minute intervals. Samples were assayed for lactate by an enzymatic method. Production rates expressed as nmoles lactate per minute per mg protein or per ug DNA. Results are from individual experiments.

LACTATE PRODUCTION

nmoles lactate per min per ug DNA

Anafranil	0	25	50	100	uM
WIL	0.30	0.33	0.22	0.99	
W256	0.62	0.63	0.67	0.71	

nmoles lactate per min per mg protein

Anafranil	0	25	50	100	uM
WIL	3.20	3.53	2.33	10.62	
W256	20.02	20.38	21.89	23.07	

Table 4.4: Rate of lactate production by WIL and W256 following incubation with anafranil. Cells were incubated at a concentration of 2.5×10^6 cells per ml in Chance-Hess medium, 3ml in sealed flasks at 37°C for 30 minutes. Incubation medium was sampled at 10 minute intervals. Samples were assayed for lactate by an enzymatic method. Production rates expressed as nmoles lactate per minute per mg protein or per ug DNA. Results are from individual experiments.

LACTATE PRODUCTION

nmoles Lactate per min per ug DNA

Ludiomil	0	5	10	20	50	uM
WIL	0.85	0.69	0.58	0.89	0.71	
W256	0.72	0.70	0.58	0.56	0.46	

nmoles Lactate per min per mg protein

Ludiomil	0	5	10	20	50	uM
WIL	29	24	20	31	25	
W256	50	49	41	38	32	

Table 4.5: Rate of lactate production by WIL and W256 following incubation with ludiomil. Cells were incubated at a concentration of 2.5×10^6 cells per ml in Chance-Hess medium, 3ml in sealed flasks at 37°C for 30 minutes. Incubation medium was sampled at 10 minute intervals. Samples were assayed for lactate by an enzymatic method. Production rates expressed as nmoles lactate per minute per mg protein or per ug DNA. Results are from individual experiments.

DISCUSSION

These results confirm that rates of whole cell oxygen consumption rates obtained for cultured cells compare well with previously published results (Gregg *et al*, 1968; Nakashima *et al*, 1984). The results show that the compounds studied are able to alter cellular energy metabolism. Incubation of tumour cells with R6G resulted in a marked inhibition of oxygen uptake. In contrast the rate of lactate production by the cells was significantly increased. The results suggest that R6G is inhibiting oxidative phosphorylation in both tumour cell types under study. The selective inhibition of aerobic metabolism, in tumour cells by R6G *in vitro* may be compensated for, in part, by an increase in the rate of lactate production hence the rate of anaerobic metabolism. Other workers have shown that the exposure of tumour cells to specific inhibitors of mitochondrial function results in compensation by the anaerobic component of metabolism (Bickis and Quastel, 1965; Gregg *et al*, 1968; Racker and Spector, 1981). The effect of R6G upon oxygen uptake in freshly isolated rat hepatocytes was also inhibitory, but at concentrations that were greater than were required for tumour cell inhibition. Furthermore lactate production was not affected by the R6G concentrations selected. This suggests that although R6G may exert a toxic effect upon hepatocyte energy metabolism, this may be the result of a generalised cytotoxic effect rather than a specific effect directed primarily at energy metabolism.

The effect of lamprone on cellular oxygen uptake in the neoplastic cell lines examined was stimulatory. It is possible that ATP production and thus tumour cell mitochondrial activity was stimulated as a result of incubation with lamprone. However, results which show an increase in the rate of lactate production in WIL and W256 suggest that the anaerobic component of energy metabolism may be compensating for the inhibitory effect of lamprone on tumour cell mitochondrial activity.

Anafranil affected cellular oxygen consumption in WIL and W256 at concentrations 2 to 4 fold greater than R6G or lamprone. All effects

of the drug were inhibitory. Similar concentrations of anafranil have been shown to inhibit cellular respiration in both yeast and transformed fibroblasts (Hughes and Wilkie, 1970; Wilkie and Delhanty, 1971). However, a clear increase in the rate of lactate production with both cell lines was not apparent. This implies, particularly with the high concentrations required to reduce cell oxygen consumption, that any reduction in mitochondrial activity was more indicative of a cytotoxic effect on tumour cell metabolism rather than a more specific effect upon tumour cell energy production.

Exposure of cells to ludiomil resulted in an inhibition of cellular oxygen consumption in both cell lines. However, there was no consistent effect upon the rate of lactate production.

It is probable that any antimitochondrial effect observed with ludiomil, like the response to anafranil, were part of a generalised cytotoxic effect upon cellular metabolism. If this were so, an expected reduction in anaerobic metabolism may not have been observed within the time limits of the experiment, although for the W256 cell line a reduction in lactate production was observed.

It has been suggested, from recent evidence, that the differential effects of rhodamine dyes on different cell types might be explained by specific species related uptake and binding characteristics of these dyes. The uptake and binding of rhodamine is characteristic of cell types from a particular species. Therefore comparisons of the effects of rhodamine on tumour cells from one species with normal cells from another species might be invalid (Summerhayes et al, 1982; Bernal et al, 1982(b); Nadakavukaren et al, 1985; Gupta and Dudani, 1987).

In this study, comparison of results obtained with rat hepatocytes and either W256 (rat) tumour cells or WIL (human) cells indicate that in both comparisons R6G has an effect upon the energy metabolism of the tumour cells and not on that of the non malignant cell type. Furthermore evidence from other groups indicate that toxicity to rhodamine of human fibroblast cell or breast epithelial cell lines

obtained either from foetal or normal subjects is limited (Lampidis et al, 1983; Gupta and Dudani, 1987).

Clearly, the criticism that early studies did compare the effects of rhodamine on tumour and non tumour cells from different species is valid. However, results obtained from experiments described in this chapter and evidence cited in the literature indicate that rhodamine toxicity is selective to both rat and human tumour cells, and it is likely that this toxicity occurs at concentrations which appear to have little effect on normal cells.

The studies described in this chapter may be summarised as follows:-

The effects of potential tumour selective antimitochondrial agents upon the energy metabolism of intact tumour and non tumour cells was examined. Two tumour cell lines WIL and W256 were compared with freshly isolated rat hepatocytes, a source of non malignant cells. Of the compounds investigated R6G, lamprene, anafranil and ludiomil all had inhibitory effects upon tumour cell energy metabolism. Lamprene and R6G exerted their action at concentrations which were lower than anafranil and ludiomil. Furthermore the effect of anafranil and ludiomil upon cellular energy metabolism in tumour cells was thought to be part of a general cytotoxic action upon these cells, rather than a specific inhibition of tumour cell energy metabolism. The effects of R6G were found to be selective at the concentrations used. Furthermore there was ^{less} inhibition of the cellular energy metabolism of the non malignant cell type used in the study, relative to the tumour cells.

In conclusion, both R6G and lamprene are compounds which appear to have potential as possible inhibitors of tumour cell mitochondrial function. However, the evidence presented in this chapter which might indicate this is indirect. The studies have established a screening system by which potential agents might be selected for further study. A more direct measure of whether these agents are inhibitors of mitochondrial function in tumour cells can be obtained only by direct comparison of the effects of R6G and lamprene on tumour cell bioenergetic function with known classical inhibitors of mitochondria. The experiments in chapter 5 describe this approach.

CHAPTER 5

POSSIBLE MECHANISMS OF ACTION BY WHICH RHODAMINE 6G AND LAMPRENE INHIBIT TUMOUR CELL OXIDATIVE METABOLISM

INTRODUCTION

From studies described in Chapter 4, two compounds, R6G and lamprene were identified which altered oxygen uptake in isolated cell preparations. It was concluded that this was probably due to interference with mitochondrial function and at the concentrations used, this alteration of respiratory activity was shown to be selective for tumour cells. If the mechanism by which respiratory activity was altered were known, specific defects in tumour mitochondrial metabolism might be identified. Such defects could then be exploited and might lead to the development of compounds designed specifically to inhibit tumour mitochondrial function.

One approach to the study of mitochondrial oxidative function involves the use of specific inhibitors which block electron transport in mitochondria at certain points along the respiratory chain. In order to determine whether R6G and lamprene exert an action similar to classical inhibitors of mitochondrial function, it is necessary to understand the pathways by which cellular energy, in the form of ATP, is generated and how, following titration with specific inhibitors, these pathways might be defined.

Generation of ATP by normal mammalian mitochondria is dependent upon the integrity of the electron transport system which allows oxidation of nutrient carbon sources to be coupled to the synthesis of ATP. Following breakdown of pyruvate or other sources of carbon (e.g. amino-acids such as glutamine) via the TCA cycle, reducing potential is generated in the form of NADH_2 and FADH_2 . Oxidation of these intermediates is achieved along the mitochondrial electron transport chain, summarised in figure 5.1. Components of the chain are

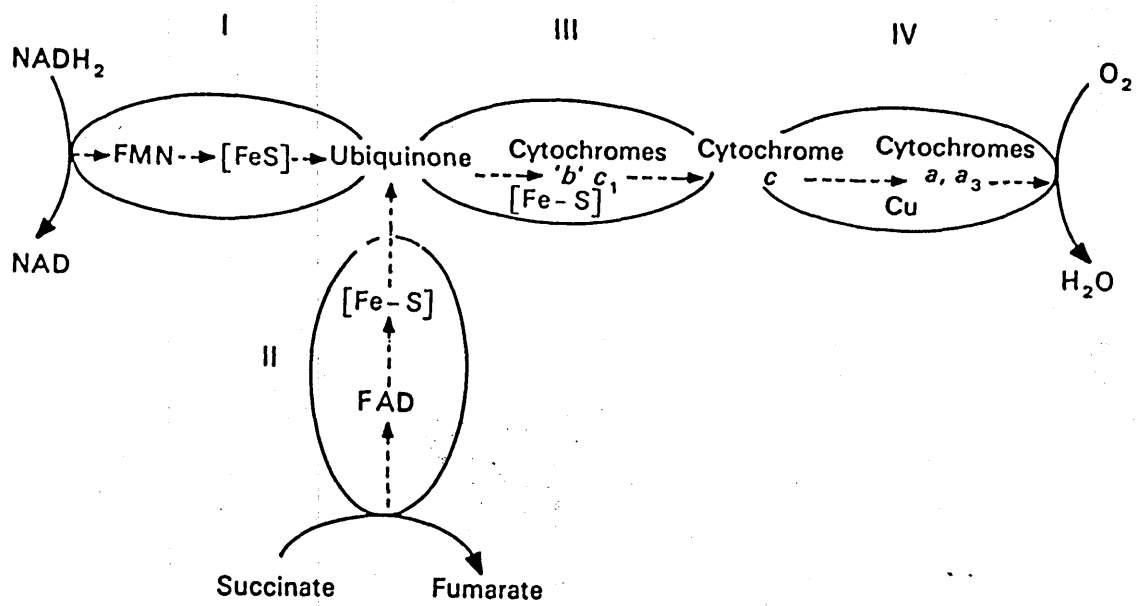


Figure 5.1: The complexes of the respiratory chain shown schematically together with the direction of electron flow along the chain (Prebble 1981).

intimately associated with the inner mitochondrial membrane. Tumour cells both from slow growing, well differentiated tumours and from rapidly growing, poorly differentiated tumours are thought to contain all the appropriate carriers required to carry out electron transport (Pedersen, 1978).

The electron transport chain can be dissected into regions. This is achieved by the use of specific inhibitors of mitochondrial metabolism. The combined use of different inhibitors has led to the elucidation of steps in the respiratory chain leading to the reduction of molecular oxygen (Slater, 1967; Lehninger, 1975; Prebble, 1981; Saier, 1987). With these well established inhibitors of mitochondrial function, it might be possible to identify how the potential antimitochondrial agents, R6G and lamprene inhibit respiratory function and subsequently identify points along the mitochondrial pathway at which these agents act.

The studies described in this chapter were designed to examine how inhibitors of tumour cell oxidative function exerted their action. In Chapter 4 it was shown that R6G and lamprene altered cellular oxygen uptake presumably by their action on mitochondrial function in tumour cell preparations. Furthermore there was some indication that this action is selective to tumour cells and is not a result of a general cytotoxic action upon tumour cell metabolism. If the mechanism by which R6G and lamprene inhibit tumour cell metabolism is understood, then it may be possible to identify potential sites and agents which would increase the selectivity of this approach.

There are two mechanisms by which an antimitochondrial agent may act. Either it may interfere with mitochondrial function impairing the organelle's ability to perform its' metabolic function. Alternatively it may inhibit mitochondrial biogenesis thus altering the ability of the organelle to reproduce itself. The present study is concerned with inhibition of mitochondrial function with classical mitochondrial inhibitors. Such inhibitors may be classified into three separate types; uncoupling agents, inhibitors of ADP phosphorylation and inhibitors of electron transport.

Uncoupling agents cause dissociation of electron transport with the associated consumption of oxygen from phosphorylation of ADP to ATP. Oxygen consumption occurs at an unrestricted rate since the electron transport chain is no longer limited by the availability of ADP. 2, 4 Dinitrophenol (DNP) is a classic uncoupler. This compound dissipates the proton gradient required for ATP formation and the inner mitochondrial membrane is made permeable to protons (Slater, 1967; Libermann et al, 1969; Heytler, 1979; Prebble; 1981).

Inhibitors of phosphorylation inhibit the conversion of ADP to ATP. In well coupled preparations, where oxygen consumption is indicative of phosphorylation, any block on ATP synthesis would be reflected in the reduced rate of cellular oxygen consumption. Oligomycin an inhibitor of phosphorylation blocks the mitochondrial ATP synthase responsible for harnessing proton generated energy to synthesize ATP (Slater, 1967; Shaw, 1967; Harold, 1972; Senior, 1979; Prebble, 1981).

Electron transport chain inhibitors interfere with the transfer of electrons between carriers of the mitochondrial electron transport chain. Examples of such inhibitors include rotenone and antimycin. Rotenone is thought to interfere with the ability of NADH_2 to transfer electrons to ubiquinone. Inhibition is at the site of the NADH dehydrogenase enzyme complex I [E.C. 1.6.99.3]. Antimycin blocks electron movement between cytochromes b and cI. Inhibition is thought to be at the site of the ubiquinone-cytochrome C reductase complex III (ubiquinol:ferricytochrome-C oxidoreductase, E.C. 1.10.2.2). The compound is thought to bind to cytochrome b preventing further electron transfer (Slater, 1967; Rieske, 1967; Harold, 1972; Singer, 1979; Prebble, 1981). Figure 5.2 summarizes inhibitors of mitochondrial oxidative function.

The effects of classical inhibitors of mitochondrial function upon the metabolism of the intact cell has been well established (Bickis and Quastel, 1965; Gregg et al, 1968; Gregg, 1972; Olivotto and Paoletti, 1981; Backer et al, 1982; Nakashima et al, 1984).

The cell types selected for this study have been described in some detail in previous Chapters (3 and 4). WIL, a non small cell human lung tumour line, shown to be resistant to conventional forms of chemotherapy was selected from a tissue culture model in vitro. W256, a rodent carcinoma cell line was obtained from a tissue culture source. This tumour, from studies conducted upon tumour implanted in animals does appear to respond to novel chemotherapies directed at tumour cell energy metabolism (Fearon et al, 1985(a)). Isolated rat hepatocytes were obtained and used in these experiments to determine whether tumour cell selectivity applied to the antimitochondrial agents under study. Hepatocytes are a well defined experimental model in vitro for studying the action of drugs upon the metabolism of normal cells.

From studies in chapter 4, it was observed that both R6G and lamprone stimulated tumour cell oxygen consumption. It was unclear, however, whether this action represented a stimulation of ATP synthesis by mitochondria or an uncoupling of respiration from ATP synthesis. The experiments described in this chapter were aimed at resolving this and confirming the mechanism of action of both of these compounds. In order to do this, it was necessary to determine whether preparations of tumour cells responded to classical inhibitors of mitochondrial function in the oxygen electrode, i.e. that respiratory activity was coupled to phosphorylation of ADP and could be uncoupled or inhibited with agents such as DNP, oligomycin, antimycin and rotenone.

With whole cells, examination of mitochondrial function is more difficult in that substrate level depletions are far harder to achieve. Therefore, parameters such as P : O ratios cannot be accurately assessed, nor can acceptor control ratios, involving control of respiration by ADP. However, control of respiration induced by oligomycin can be investigated and this type of respiratory control is referred to as oligomycin-induced respiratory control. Addition of oligomycin to a fresh cell preparation reduces the respiratory rate to a level comparable to the State IV rate of intact mitochondria. Addition of DNP reopens the mitochondrial membrane, rendering it leaky to protons and results in stimulation of

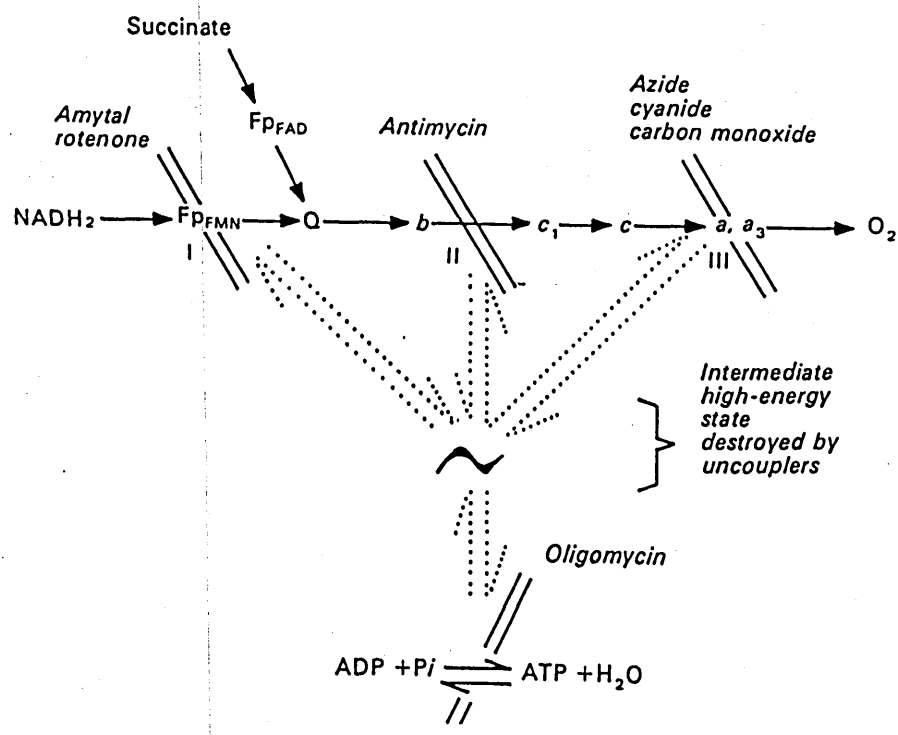


Figure 5.2 : Inhibitors of oxidative phosphorylation. Schematic representation of points on the electron transport chain at which inhibition occurs (Prebble, 1981).

respiration back to State III (Pedersen et al, 1978; Nakashima et al, 1984). Respiratory control indices obtained in this manner give some indication as to the respiratory capacity of a particular cell preparation and together with cellular oxygen consumption rates indicate to what extent antimitochondrial agents would influence respiration. Considerable evidence exists, which indicates that R6G might inhibit cellular bioenergetic function either by inhibiting translocation of adenine nucleotides across the mitochondrial membrane, altering the proton flux across the membrane and/or uncoupling phosphorylation from electron transport (Gear, 1974, Higuti et al, 1980; Conover and Schneider, 1981). Lamprene which shows some structural similarities to R6G has also been reported to have antimitochondrial activity (Rhodes and Wilkie, 1973). Other agents included in this study were anafranil (Cloimipramine hydrochloride) and ludiomil (maprotiline hydrochloride).

METHODS

Isolated rat hepatocytes were prepared as described previously (Chapter 4). Suspensions of hepatocytes, WIL tumour cells and W256 tumour cells were obtained as described in Chapters 2 and 4.

Measurement of Cell Respiratory Activity

Cell oxygen consumption was determined following incubation of cells in a Clark-type oxygen electrode as described in Chapter 2. Total cell protein was determined by the method of Lowry et al (1951). Total cell DNA was also determined as described in Chapter 2.

Characterisation of each cell preparation with classical inhibitors of mitochondrial function were carried out as follows. Cells were incubated in the oxygen electrode. A basal, control oxygen consumption was recorded over a 5 minute period. DNP was then added and the steady state response measured for 5 minutes. Oligomycin, was added for 5 minutes followed by antimycin or rotenone, for 2 to 3 minutes. A second characterisation procedure was carried out upon a fresh preparation of cells. A basal oxygen consumption rate was recorded for a fresh preparation of cells for 5 minutes. Oligomycin was added and the oxygen uptake rate recorded for a further 5 minutes. DNP was then added for 5 minutes followed by antimycin or rotenone for 2 to 3 minutes.

The antimitochondrial agents under study R6G, lamprene, anafranil and ludiomil were introduced into this system to determine if they exhibited respiratory inhibition or uncoupled respiration from phosphorylation. Drug concentrations were limited to 10uM as determined by previous screening experiments (Chapter 4). A basal oxygen uptake rate was recorded for fresh cell preparations incubated in the oxygen electrode for 5 minutes. Experimental drug was added and the oxygen consumption rate recorded for 5 minutes. Oligomycin was then incubated with cells for 5 minutes followed by antimycin or rotenone for 2 to 3 minutes. A second preparation of cells was incubated in the oxygen electrode. The basal rate of oxygen consumption was recorded for 5 minutes. Oligomycin was added and the

oxygen uptake rate was measured for 5 minutes. Experimental drug was added and the oxygen consumption rate was recorded for 5 minutes followed by addition of antimycin or rotenone to cells for 2 to 3 minutes.

Although experiments were repeated 2 to 4 times, in order to relate each individual response of drug to control, results from single experiments are shown. Final concentrations obtained in the oxygen electrode were DNP 10 μ M, oligomycin 4.2 μ M or 5 μ M, antimycin 5 μ M and rotenone 3 μ M.

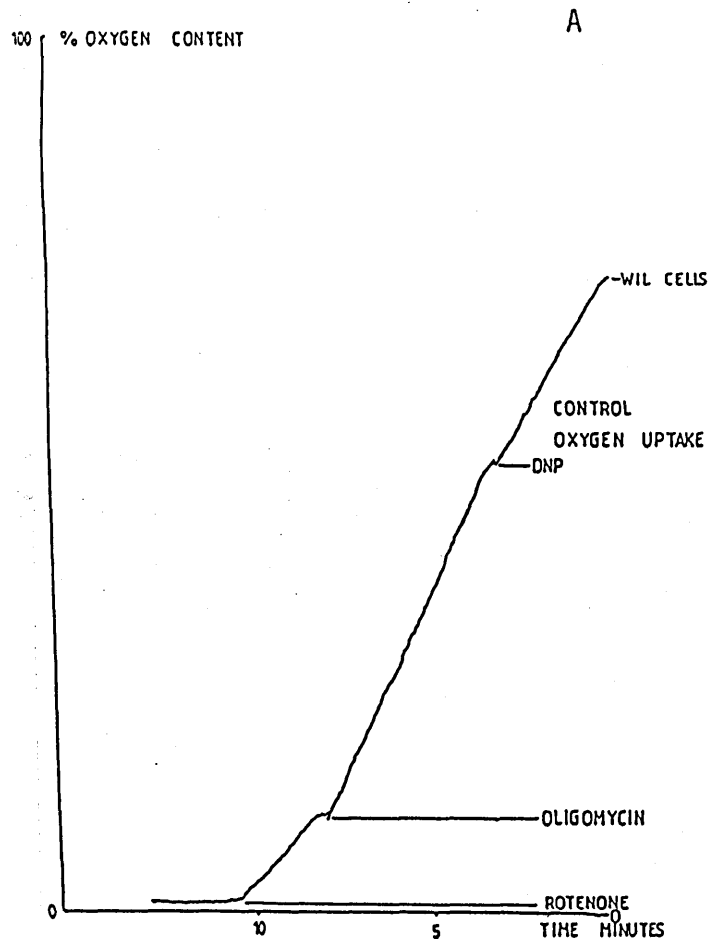
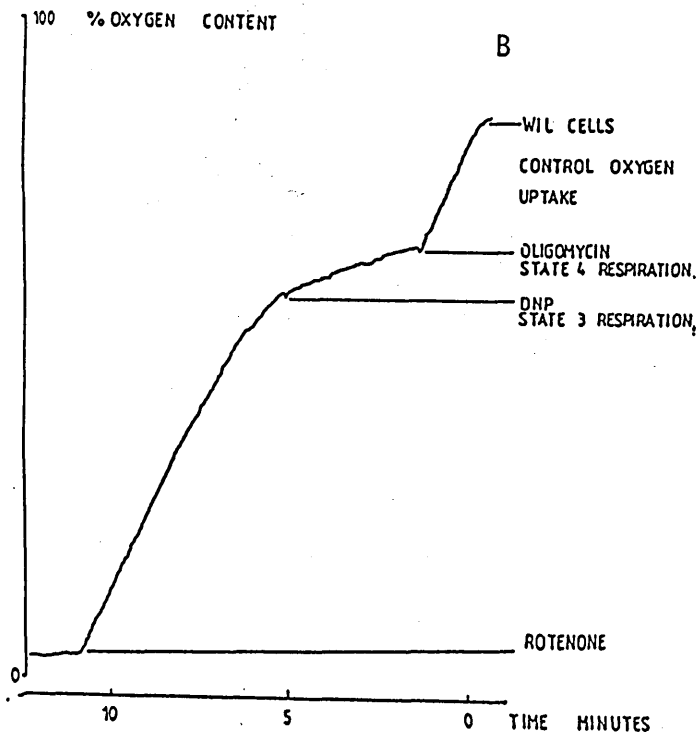


Figure 5.3: The rate of oxygen consumption by intact preparations of WIL. Cells were incubated in Chance-Hess medium at 37°C in the oxygen electrode. The reaction was initiated by addition of cells (1×10^7) to buffer (3ml) supplemented with glucose 6mM.

After a steady state rate of oxygen consumption was obtained (A), DNP (10 μ M), oligomycin (4.2 μ M) and rotenone (3 μ M) were added sequentially at the points indicated.

After a steady state rate of oxygen consumption was obtained (B), oligomycin (4.2 μ M), DNP (10 μ M) and rotenone (3 μ M) were added sequentially at points indicated. Respiratory control ratios were calculated from the DNP stimulated oxygen uptake rate (State III) and oligomycin inhibited oxygen uptake rate (State IV).



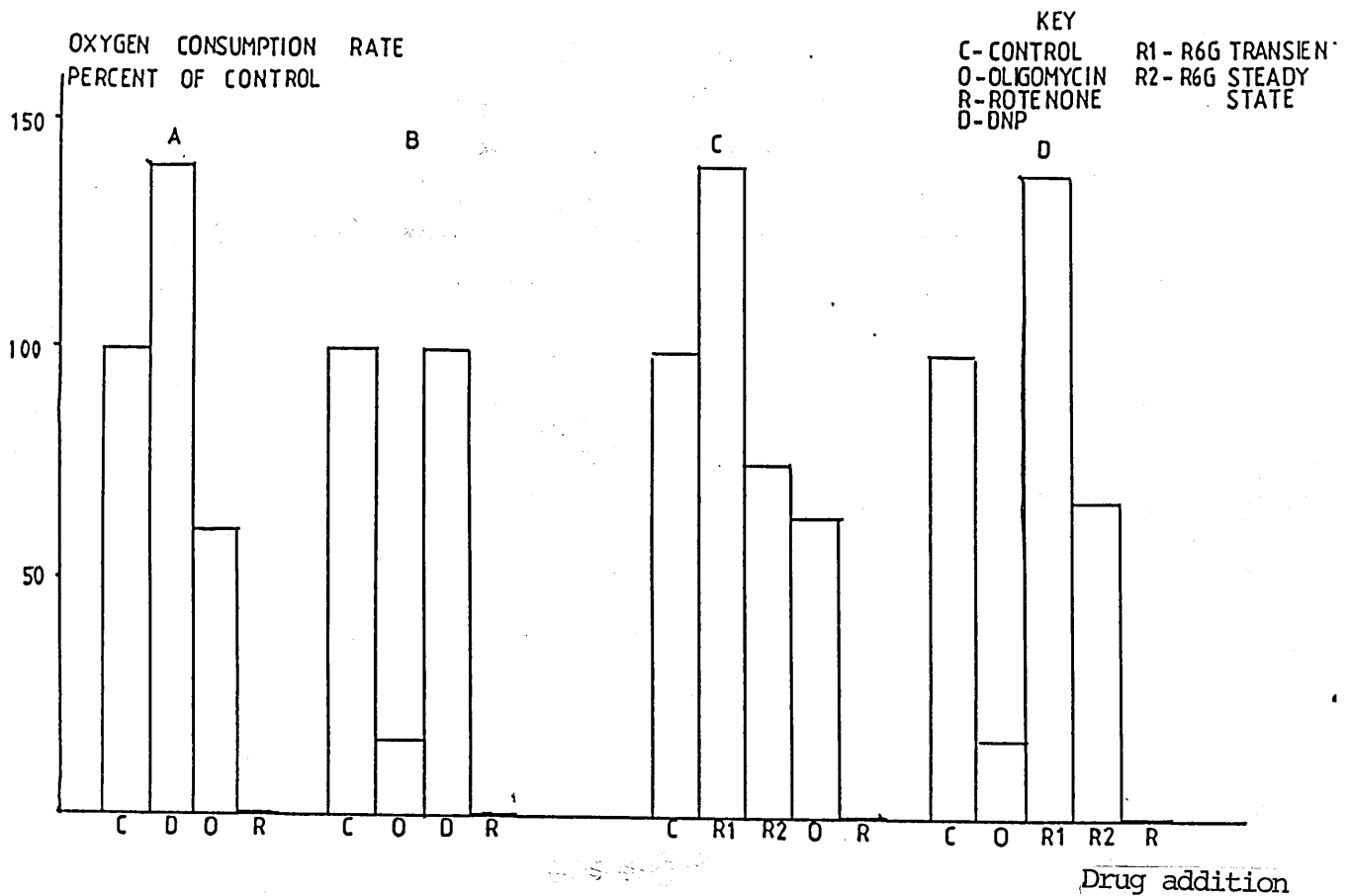


Figure 5.4: The effect of sequential addition of antimitochondrial agents upon the basal rate of oxygen uptake expressed as per cent of control in intact preparations of WIL. Following characterisation of fresh cell preparations with classical antimitochondrial agents DNP, oligomycin and rotenone (A and B), the effect of R6G upon the basal rate of oxygen uptake (C) and the oligomycin inhibited rate of oxygen uptake (D) in WIL was assessed. The dual response of cells to R6G is denoted by R1 and R2. An RCR value of 6.2 was obtained with this preparation.

RESULTS

Effect of Rhodamine 6G on the oxygen uptake of cells following manipulation with classic antimitochochondrial agents

Figure 5.3(A) shows the effect of sequential addition of DNP, oligomycin and rotenone to a fresh preparation of tumour cells (WIL) incubated in the oxygen electrode. Addition of DNP (10 μ M), increased the basal rate of oxygen consumption by 20% to 30%. Subsequent addition of oligomycin (4.2 μ M), decreased the oxygen uptake rate by 30 to 40% of that in the presence of DNP. Rotenone (3 μ M); inhibited oxygen uptake completely. Figure 5.3(B) shows the effect of sequential addition of oligomycin, DNP and rotenone to a fresh preparation of tumour cells (WIL). Addition of oligomycin (4.2 μ M) decreases the basal rate of oxygen uptake by about 80%. Addition of DNP (10 μ M), stimulated oxygen consumption to a value within 10% of the basal rate. Oxygen consumption is completely inhibited following sequential addition of rotenone (3 μ M).

Addition of DNP (Figure 5.4A) to a fresh preparation of WIL stimulated oxygen consumption to a rate 40% greater than the basal rate. However, addition of oligomycin (4.2 μ M) to this preparation reduced oxygen consumption to about 50% of the basal rate. Oxygen uptake was completely inhibited following addition of rotenone (3 μ M). When oligomycin was added to a fresh preparation of WIL, oxygen uptake was reduced by 80% (Figure 5.4B). Subsequent addition of DNP increased oxygen consumption to that of basal rates. Addition of rotenone (3 μ M) completely abolished oxygen uptake by WIL. Addition of R6G (10 μ M) to a fresh preparation of WIL cells resulted in an initial increase in the rate of oxygen consumption to 40% above that of the basal rate (Figure 5.4C). Subsequently the oxygen consumption rate was reduced to a steady state value of about 75% of the basal rate. Oligomycin addition resulted in a further decrease in the oxygen uptake rate by 10%. Oxygen consumption is abolished following addition of rotenone (3 μ M). Figure 5.4D shows the effect of addition of R6G to an oligomycin inhibited preparation of WIL. Initially R6G increased oxygen consumption to about 140% of basal values. This effect was transient and after between 5 to 10 minutes oxygen consumption was

reduced to a final steady state value of approximately 70% of the control oxygen uptake. Oxygen consumption was fully inhibited with rotenone (3 μ M).

Addition of DNP (10 μ M) to a fresh preparation of W256 tumour cells (Figure 5.5A) results in a stimulation of oxygen uptake to 35% above basal rates. The addition of oligomycin (4.2 μ M) to this preparation reduced oxygen consumption to only about 30% of the basal rate. Oxygen uptake was fully inhibited following addition of rotenone (3 μ M). When oligomycin (4.2 μ M), was added to a fresh preparation of W256 cells, oxygen uptake was reduced by 75% (Figure 5.5B). Subsequent addition of DNP (10 μ M) increased the oxygen consumption rate to within 7% of the basal rate. Rotenone (3 μ M) however, completely inhibited cellular oxygen uptake.

Addition of R6G (10 μ M), to a fresh preparation of W256 cells resulted in an initial increase in the rate of oxygen consumption by 3% (Figure 5.5C). However, the oxygen consumption rate subsequently decreased to 70% of the basal rate. Addition of oligomycin (4.2 μ M), reduced the rate of oxygen uptake only by a further 23%. Oxygen consumption is completely inhibited following addition of rotenone (3 μ M). Figure 5.5D shows the effect of adding R6G (10 μ M), to an oligomycin inhibited preparation of W256 cells. Oxygen consumption was stimulated by 25% reaching 40% of the basal rate. However, this response was transient, lasting for between 5 to 10 minutes. Cellular oxygen uptake rates decreased to the same value as the oligomycin inhibited rate, 35% of the basal uptake rate. Rotenone (3 μ M) abolished the response.

The effect of addition of DNP, oligomycin, rotenone and R6G to fresh preparations of rat hepatocytes is shown in Figure 5.6. DNP (10 μ M) stimulated oxygen consumption rates of fresh cell preparations by 15% (Figure 5.6A). Oligomycin (5 μ M) reduced this response by only 30%. Antimycin (5 μ M) inhibited cellular oxygen uptake completely. Antimycin in place of rotenone with freshly prepared hepatocytes was more effective at reducing cell oxygen consumption. Oligomycin (4.2 μ M) when added to fresh preparations of rat hepatocytes reduced

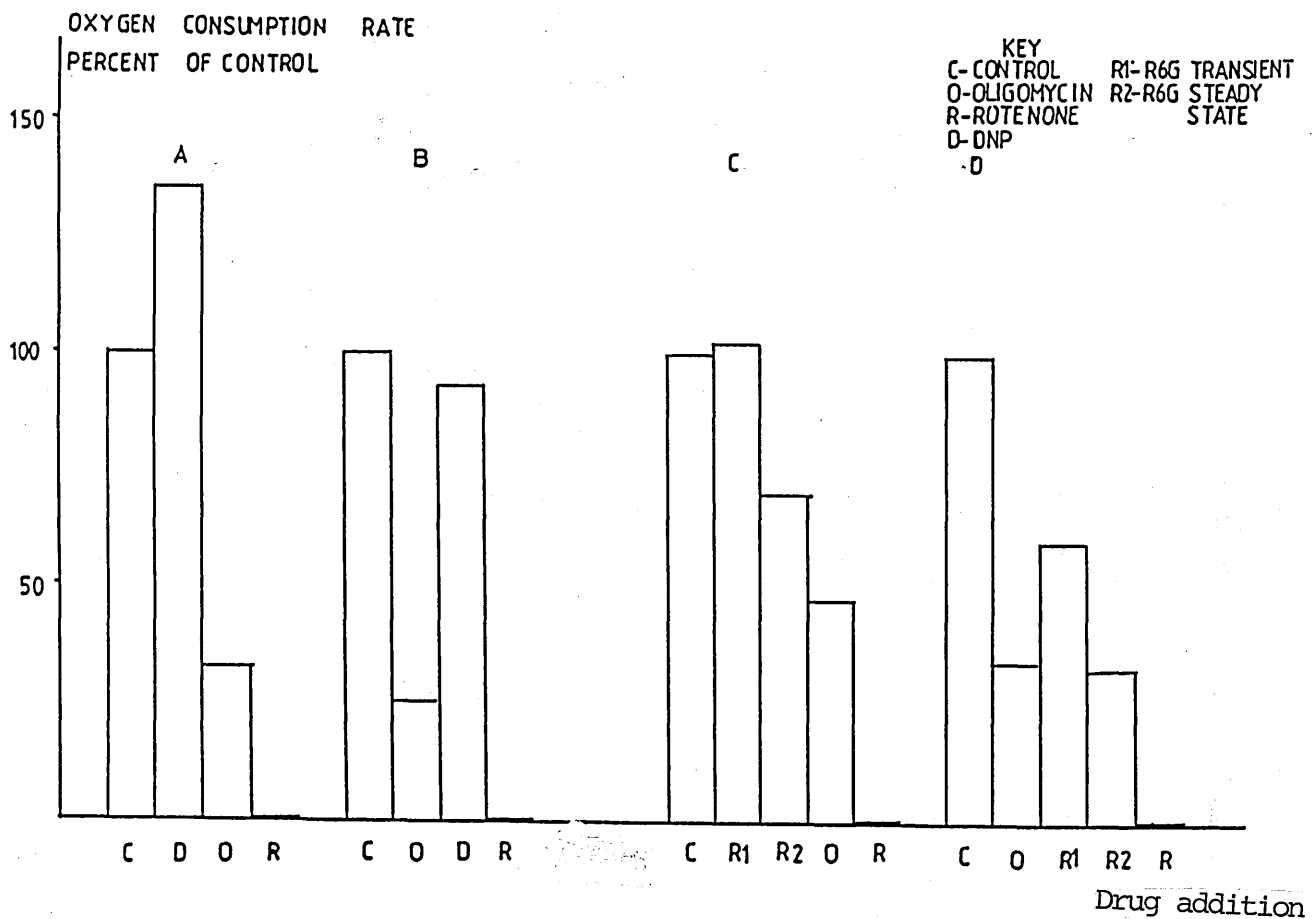


Figure 5.5: The effect of sequential addition of antimitochondrial agents upon the basal rate of oxygen uptake expressed as percent of control in intact preparations of W256. Following characterisation of fresh cell preparations with classical antimitochondrial agents DNP, oligomycin and rotenone (A and B), the effect of R6G upon the basal rate of oxygen uptake (C) and the oligomycin inhibited rate of oxygen uptake (D) in W256 was assessed. The dual response of cells to R6G is denoted by R1 and R2. An RCR value of 3.6 was obtained with this preparation.

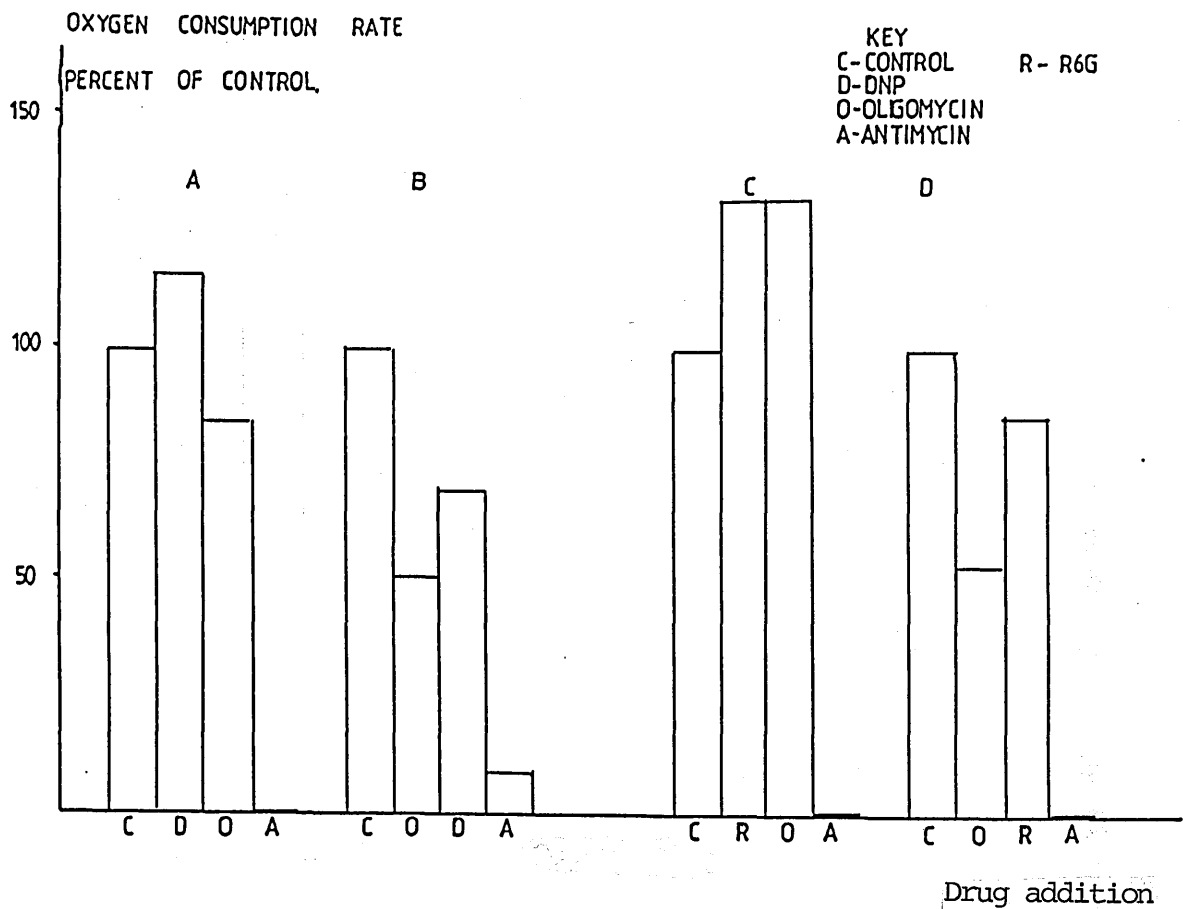


Figure 5.6: The effect of sequential addition of antimitochochondrial agents upon the basal rate of oxygen uptake expressed as percent of control in freshly isolated preparations of rat hepatocytes.

Following characterisation of fresh cell preparations with classical antimitochochondrial agents DNP, oligomycin and rotenone (A and B), the effect of R6G upon the basal rate of oxygen uptake (C) and the oligomycin inhibited rate of oxygen uptake (D) in rat hepatocytes was assessed. An RCR value of 1.4 was obtained with this preparation.

cell oxygen consumption by 50% (Figure 5.6B). DNP (10 μ M) stimulated cell oxygen consumption to within 30% of basal rates. Antimycin (5 μ M) almost completely inhibited cellular oxygen uptake, reducing oxygen consumption to 10% of basal rates. Addition of R6G (10 μ M) to fresh preparations of rat hepatocytes stimulated respiration by 30% above basal rates (Figure 5.6C). Oligomycin (5 μ M) had no effect upon this response, although antimycin (5 μ M) inhibited all respiratory activity.

Adding R6G (10 μ M) to an oligomycin inhibited preparation elicited a stimulation of respiratory activity to within 10% of the basal rate (Figure 5.6D). Antimycin sensitivity was indicated by antimycin mediated inhibition of respiratory activity.

Effect of Lamprene on the oxygen uptake of cells following manipulation with classic antimitochondrial agents

The effect of sequential addition of DNP, oligomycin and rotenone to fresh preparations of WIL in the oxygen electrode are shown in Figure 5.7A. Addition of DNP (10 μ M) results in stimulation of the oxygen uptake rate by 10%. Oligomycin (4.2 μ M) reduces the oxygen consumption rate to only 65% of the basal rate. Rotenone (3 μ M) fully inhibited oxygen consumption in this preparation. Oligomycin (4.2 μ M) when added to fresh preparations of WIL reduced oxygen consumption by 80% (Figure 5.7B). DNP (10 μ M) stimulated oxygen consumption to within 30% of the basal rate. Rotenone (3 μ M) fully inhibits the oxygen uptake rate. Lamprene (10 μ M) when added to a fresh preparation of WIL elicits a stimulation of the oxygen uptake rate to above 25% of the basal rate (Figure 5.7C). Oligomycin (4.2 μ M) reduces this by only 15% above the basal rate and the oxygen uptake rate is completely inhibited with rotenone (3 μ M). Addition of lamprene (10 μ M) to an oligomycin inhibited preparation (Figure 5.7D) caused an increase in the rate of oxygen consumption uptake to within 10% of the basal rate. Rotenone (3 μ M) fully inhibited oxygen consumption in this preparation.

The sequential addition of DNP, oligomycin and rotenone to fresh preparations of W256 cells is shown in Figure 5.8A. DNP (10 μ M) stimulated oxygen consumption to 50% above basal rates. Addition of

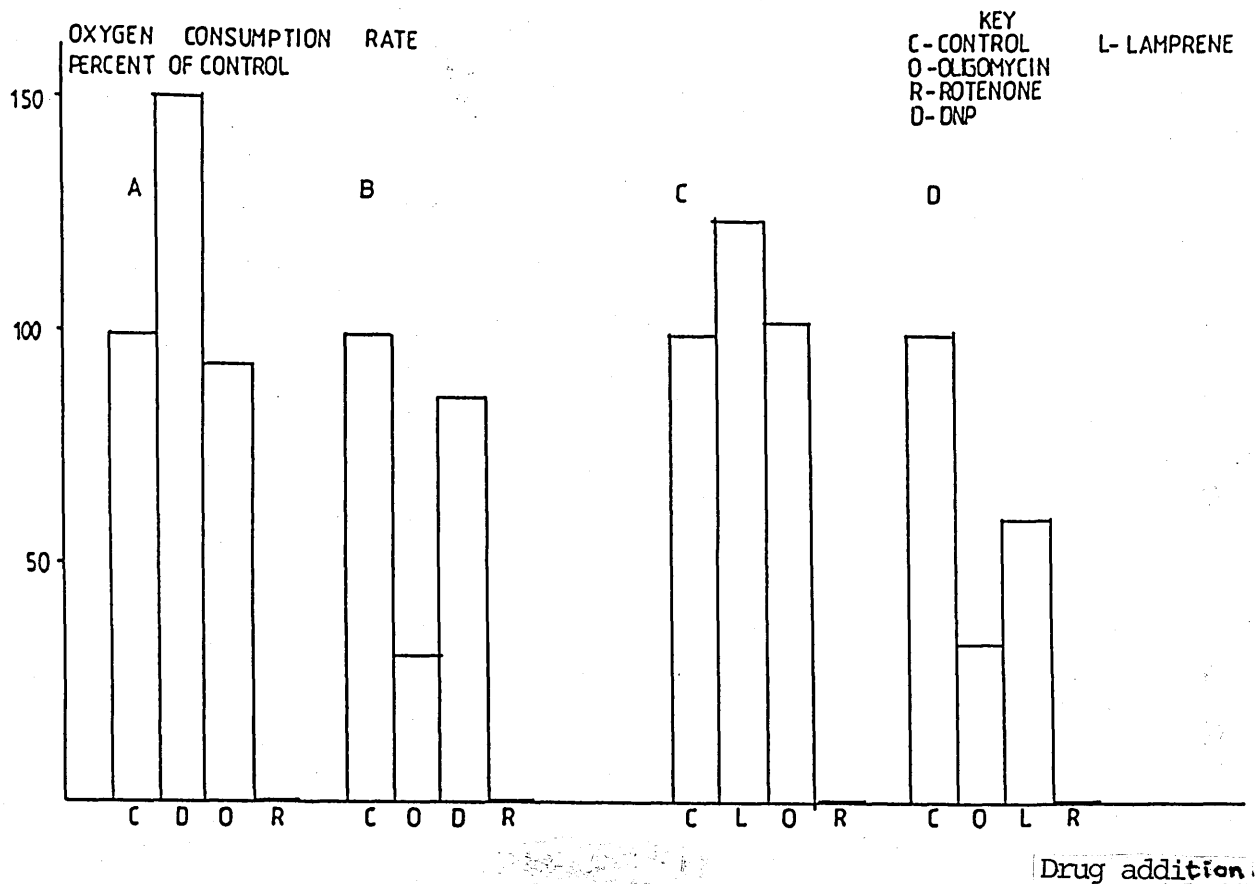


Figure 5.8: The effect of sequential addition of antimitochondrial agents upon the basal rate of oxygen uptake expressed as percent of control in intact preparations of W256. Following characterisation of fresh cell preparations with classical antimitochondrial agents DNP, oligomycin and rotenone (A and B), the effect of lamprene upon the basal rate of oxygen consumption (C) and the oligomycin inhibited rate of oxygen uptake (D) in W256 was assessed. An RCR value of 2.6 was recorded for this preparation.

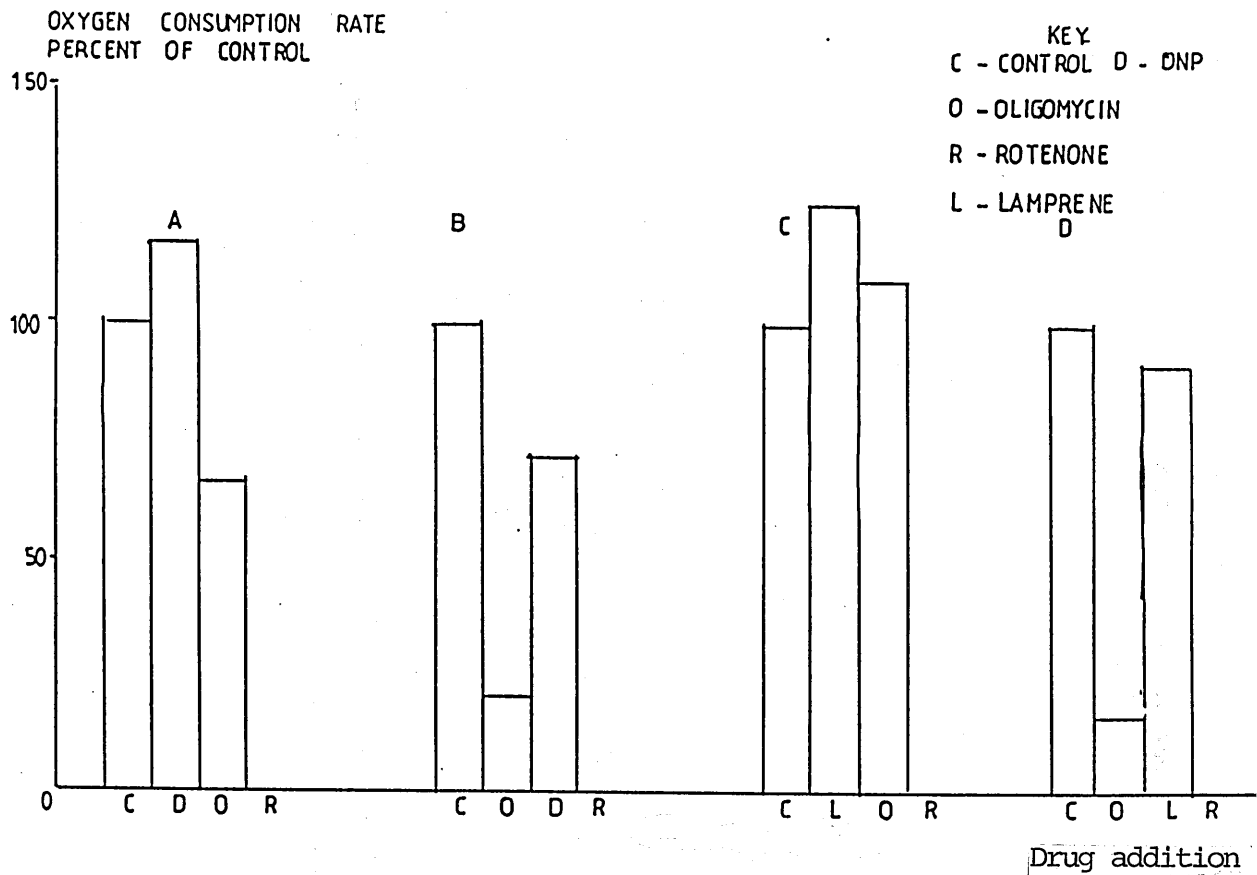


Figure 5.7: The effect of sequential addition of antimitochondrial agents upon the basal rate of oxygen uptake expressed as percent of control in intact preparations of WIL. Following characterisation of fresh cell preparations with classical antimitochondrial agents DNP, oligomycin and rotenone (A and B), the effect of lamprene upon the basal rate of oxygen consumption (C) and the oligomycin inhibited rate of oxygen uptake (D) in WIL was assessed. An RCR value of 3.6 was recorded for this preparation.

oligomycin (4.2 μ M) reduced this response to only within 5% of the basal rate. Cellular oxygen consumption was inhibited by rotenone (3 μ M). Oligomycin (4.2 μ M) reduced the oxygen uptake rate by 70% of the basal rate (Figure 5.8B). ^{DNP stimulates oxygen uptake by 40%} Rotenone (3 μ M) fully inhibited the oxygen uptake of W256 cells. Lamprene (10 μ M) when added to fresh preparations of W256 cells elicits an increase in the oxygen uptake rate by 25% above the basal rate (Figure 5.8C). Oligomycin (4.2 μ M) reduces this response to only 3% above the basal oxygen uptake rate, though rotenone (3 μ M) inhibits the consumption of oxygen by this cell preparation. Lamprene (10 μ M) when added to an oligomycin inhibited preparation of cells stimulated cellular oxygen consumption by 25% of the basal rate (Figure 5.8D). Rotenone 3 μ M abolished the oxygen uptake of this preparation. DMSO which was required to solubilise lamprene for these experiments had no effect upon respiratory activity in coupled or uncoupled preparations of WIL or W256.

Effect of Anafranil and Ludiomil on the oxygen uptake of cells following manipulation with classic antimitochondrial agents

Anafranil and Ludiomil at concentrations of 10 μ M had no effect upon coupled or uncoupled respiratory activity (Tables 5.1 and 5.2).

CONDITIONS	OXYGEN UPTAKE ng at/mg protein/min	OXYGEN UPTAKE ng at/ug DNA/min	PERCENT OF CONTROL	RCR
control	11.62	0.23	100	5.06
10 uM Anafranil	11.62	0.23	100	
4.2uM Oligomycin	1.80	0.04	16	
3 uM Rotenone	0	0	0	
control	10.30	0.20	100	5.06
4.2uM Oligomycin	2.18	0.04	21	
10 uM Anafranil	2.18	0.04	21	
3 uM Rotenone	0	0	0	
control	12.30	0.30	100	4.89
10 uM Ludiomil	12.30	0.30	100	
4.2uM Oligomycin	1.80	0.04	14	
3 uM Rotenone	0	0	0	
control	12.75	0.32	100	4.89
4.2uM Oligomycin	2.68	0.07	21	
10 uM Ludiomil	2.68	0.07	21	
3 uM Rotenone	0	0	0	

Table 5.1: The effect of sequential addition of antimitochochrial agents upon the basal rate of oxygen uptake as expressed as percent of control in intact preparations of WIL.

Cell preparations were characterised with classical antimitochochrial agents DNP, oligomycin and rotenone as described.

The effect of anafranil and ludiomil upon the oxygen consumption rate in WIL was then determined. RCR values following characterisation of each preparation were recorded as shown.

CONDITIONS	OXYGEN UPTAKE ng at/mg protein/min	OXYGEN UPTAKE ng at/ug DNA/min	PERCENT OF CONTROL	RCR
control	11.34	0.21	100	3.06
10 uM Anafranil	11.34	0.21	100	
4.2uM Oligomycin	3.26	0.06	29	
3 uM Rotenone	0	0	0	
control	10.06	0.18	100	3.06
4.2uM Oligomycin	4.02	0.07	40	
10 uM Anafranil	4.02	0.07	40	
3 uM Rotenone	0	0	0	
control	8.80	0.15	100	2.40
10 uM Ludiomil	8.80	0.15	100	
4.2uM Oligomycin	3.52	0.06	40	
3 uM Rotenone	0	0	0	
control	8.09	0.13	100	2.40
4.2uM Oligomycin	3.41	0.06	43	
10 uM Ludiomil	3.41	0.06	43	
3 uM Rotenone	0	0	0	

Table 5.2: The effect of sequential addition of antimitochochondrial agents upon the basal rate of oxygen uptake as expressed as percent of control in intact preparations of W256.

Cell preparations were characterised with classical antimitochochondrial agents DNP, oligomycin and rotenone as described.

The effect of anafranil and ludiomil upon the oxygen consumption rate in W256 was then determined. RCR values following characterisation of each preparation were recorded as shown.

DISCUSSION

The results described in this chapter suggest that lamprene behaves as a classic uncoupler of oxidative phosphorylation, stimulating oxygen uptake in the presence of an inhibitor of phosphorylation. In contrast the action of R6G is more complex. There appeared to be a biphasic response in oxygen consumption following incubation of cells with R6G. A transient stimulation of oxygen consumption, independent of phosphorylation and characteristic of uncoupling was observed initially. However, this was followed by a steady state inhibition of oxygen consumption also independent of phosphorylation indicating subsequent inhibition of electron transport.

2.4 Dinitrophenol stimulated oxygen uptake when incubated with fresh cell preparations as respiratory oxygen consumption was uncoupled from phosphorylation of ADP. Accelerated oxygen uptake was a result of decreased availability of ATP and NADH which regulate substrate cycling through glycolysis and the TCA cycle. Both R6G and lamprene behaved similarly. With an inhibitor of ADP phosphorylation, oligomycin, blocking phosphorylation had relatively little effect upon the rate of (uncoupled) oxygen uptake. However in all cell types, the rate of uncoupled oxygen consumption was reduced somewhat. Oligomycin did reduce oxygen consumption in coupled preparations. The electron transport chain inhibitors rotenone and antimycin did inhibit oxygen uptake in both coupled or uncoupled preparations. Although tumour cell respiration was completely inhibited with rotenone, full inhibition of respiration in intact hepatocytes was not achieved. Antimycin was more effective as an inhibitor of respiratory chain activity and associated oxygen consumption.

R6G showed a potent uncoupling activity. The increase in respiratory oxygen uptake by fresh preparations of hepatocytes, WIL and W256 tumour cells following exposure to R6G reflects an initial uncoupling of phosphorylation from electron transport and associated oxygen consumption rather than a stimulation of both phosphorylation hence oxygen uptake. Stimulation of respiratory activity was independent of any effect by oligomycin hence phosphorylation. This uncoupling

activity was transient and an associated steady state inhibition of cellular oxygen consumption following. This was observed in tumour cell preparations. Furthermore the effect was also independent of phosphorylation, occurring even in the presence of oligomycin. An important observation with the high concentration of R6G (10 μ M) was stimulation of hepatocyte respiratory function indicative of uncoupling of respiratory activity from phosphorylation. It is proposed however, that the magnitude of changes associated with the concentration of R6G used is less than the effect of this concentration of R6G upon tumour cell respiratory activity (Chapter 4). In view of this observation it may be more realistic to examine whether a concentration of R6G may be used which has an effect upon tumour cell oxidative function and little or no effect upon non-neoplastic host cells. The 3 μ M concentration cited by Gear (1974) at which inhibition of bioenergetic function in rat liver mitochondria is thought to occur may only pertain to the isolated organelle and may be of less importance to whole cells or intact tissue.

The effects of lamprene upon WIL and W256 tumour cells appear to be less complex than that of R6G. Lamprene showed clear uncoupling of respiratory control. The oxygen consumption rate in both cell lines was stimulated, even in the presence of oligomycin. Therefore, stimulation of oxygen uptake did not reflect an increase in associated phosphorylation of ADP. The electron transport inhibitor rotenone inhibited all respiratory activity in both cell types pretreated with lamprene.

Respiratory control indices for cell preparations examined in the studies described in this chapter varied between 2.4 and 6.2 for the tumour cell preparations and were adequately indicative of respiratory coupling. However, indices for hepatocytes were much lower and therefore imply that the degree of respiratory control in such preparations was limited.

The electron transport chain inhibitor rotenone is thought to act at a site close to the NADH-Dehydrogenase enzyme complex I. Treatment of cells with rotenone would not prevent channelling of electrons onto

ubiquinone via the succinate dehydrogenase enzyme complex II [succinate = (acceptor) oxidoreductase E.C. 1.3.99.1]. Therefore, some respiratory activity would be predicted even after cells were pretreated with rotenone. Since this was not observed with either tumour cell line examined the contribution of FAD+ linked electron transfer to net electron transport in tumour cells might be negligible. Oxygen uptake by hepatocytes continued after treatment of cells with rotenone. Antimycin, which acts at a site further along the respiratory chain, past ubiquinone, the crossover point for both complex I and complex II did fully inhibit oxygen consumption in hepatocytes.

The observed inhibitory action of R6G upon oxygen uptake by tumour cells that occurs even in the presence of an inhibitor of phosphorylation, is in agreement with observations by other workers. Results obtained with isolated rat liver mitochondria exposed to the analogous cationic dye rhodamine 123, suggest that rhodamine dyes may also have inhibitory activity at a site on the electron transport chain (Modica-Napolitano et al, 1984; Emaus et al, 1986).

R6G stimulated oxygen uptake was fully inhibited by rotenone in the tumour cell lines examined. As stated previously, following exposure to R6G, hepatocyte respiratory activity was not fully inhibited with rotenone, but was fully inhibited with antimycin. The studies with hepatocytes imply that any inhibitory effect that R6G has upon electron transport is exerted at a site further along the respiratory chain from ubiquinone, either near mitochondrial enzyme complex III or complex IV (cytochrome oxidase; ferricytochrome-C : Oxygen oxidoreductase E.C. 1.9.3.1). Studies with tumour cells indicate that such inhibition is certainly further along from mitochondrial enzyme complex I as shown by the ability of rotenone to abolish the response and assuming the respiratory chain is fully intact. Other workers have shown that in isolated rat liver mitochondria, R6G and cyanide based dyes have sites of action associated with NAD linked rather than FAD linked enzyme activity (Conover and Schneider, 1981). The studies described in this Chapter have not fully investigated the possible sites of action of R6G induced inhibition of electron transport,

therefore it may be possible that following uncoupling of phosphorylation from oxygen consumption and associated electron transport, the decrease in oxygen uptake and electron transport induced with R6G may be merely reflections of a cytotoxic action upon cell metabolism.

There is evidence to suggest other possible mechanisms of action of R6G on mitochondrial respiratory function. From studies with isolated rat liver mitochondrial preparations and submitochondrial particles obtained from rat liver Gear (1974) concluded that R6G could be exerting its action by inhibition of the adenine nucleotide translocase on the inner mitochondrial membrane, resulting in an inhibition of phosphorylation. However Higuti et al (1980) believed that inhibition of energy transduction by R6G was caused by a reduction of proton ejection during electron transport and a subsequent inhibition of the ATP synthase enzyme preventing phosphorylation. It is apparent that the inhibitory effects of R6G upon bioenergetic function may be contributed to by inhibition of more than one bioenergetic process.

Lamprene exhibited a clear uncoupling of respiratory activity. Oxygen consumption in tumour cell preparations was stimulated even in the presence of oligomycin. Rhodes and Wilkie (1973) suggested that Lamprene mediated inhibition of respiration in yeast was due to lamprene acting as an artificial electron acceptor rather than an uncoupler by passing the terminal cytochrome oxidase complex IV on the respiratory chain. This was because lamprene mediated stimulation of respiratory activity was not fully inhibited with the cytochrome oxidase inhibitor cyanide. The experiments with tumour cell preparations described in this chapter did not appear to support this theory. Lamprene was inhibited with rotenone in both tumour cell types examined. Rotenone induced inhibition reflects NAD^+ linked transport as described. FAD^+ linked electron transport does not appear to contribute to oxygen consumption in tumour cells. Rotenone sensitivity implies that lamprene mediated stimulation of respiratory activity, reflecting an enhancement of electron transport can still be inhibited with electron transport chain inhibitors. Therefore the

idea that lamprene accepts electrons in order to reduce molecular oxygen cannot be correct as such a process should not be sensitive to rotenone.

Addition of oligomycin to DNP treated preparations of cells resulted in small but significant reductions in oxygen consumption. This might have reflected the fact that the concentration of DNP causing uncoupling was submaximal and thus a reduction in the rate of oxygen consumption was characteristic of a subpopulation of coupled mitochondria within the preparation. It is necessary to emphasize that the degree of respiratory control achieved with intact cells is less than with preparations of isolated mitochondria. This is reflected in the respiratory control indices of the cell preparations, ranging between 1.2 and 6.2 in certain cases. Any addition of drug is dependent therefore, upon uptake which is assumed, but not necessarily to be exhibiting saturation kinetics. Concentrations of DNP, oligomycin, rotenone and antimycin were selected from studies with isolated mitochondria. In such studies not only was plasma membrane permeability not a feature, but organelle density within a preparation was far greater. Therefore any time delay to a particular response to drug was much less. If this were so, RCR values calculated would have been meaningless as subpopulations of mitochondria within each preparation would not have been exposed to drug. From work with hepatocytes it is clear that stimulation of uncoupled activity is maximal with DNP and could not be further stimulated by addition of lamprene or R6G. This occurred with tumour cell preparations incubated in the oxygen electrode in approximately similar concentrations to hepatocytes. Thus the drug concentrations selected did suffice for maximal responses not only with hepatocytes, but also tumour cells. Coarser responses to antimitochondrial agents incubated with tumour cells are more likely to be due to a reduction in respiratory control in whole cell preparations as compared to isolated tumour mitochondria.

Further evidence exists in the literature to show that maximal responses to antimitochondrial agents may be achieved at lower drug concentrations with preparations of intact cells as compared to

isolated mitochondria (Gregg et al, 1968; Gregg, 1972). In conclusion, with the drug concentrations used maximal cellular responses were observed in experiments with the oxygen electrode. Thus the studies described in this chapter may be summarised as follows.

From experiments with the oxygen electrode, intact rat hepatocytes and tumour cells respond to classic antimitochondrial agents as predicted from the organisation of components of their inner mitochondrial membrane. R6G incubated with tumour cells exerts a dual action upon respiratory activity. An initial uncoupling of oxygen consumption from phosphorylation occurs, followed by a phosphorylation independent inhibition of electron transport. It would appear that the effect of R6G upon tumour cell bioenergetic function is complex and there may be several sites at which this compound acts. R6G does appear to exert an inhibition upon hepatocyte oxidative metabolism which follows a similar mechanism of action. However at low drug concentrations R6G exerts an inhibition upon tumour cell mitochondrial respiratory function with little or no effect upon the metabolism of normal cells. Lamprene uncouples respiratory activity from phosphorylation in the tumour cell preparations examined in these studies. There is no evidence to suggest that ludiomil or anafranil at the 10 μ M concentrations selected had any effect upon coupled or uncoupled respiratory activity.

In conclusion, evidence presented in this chapter support the idea that R6G and lamprene exert a clear inhibition of tumour cell bioenergetic function in vitro.

CHAPTER 6

INHIBITION OF TUMOUR CELL MITOCHONDRIAL BIOGENESIS

INTRODUCTION

So far the approach to selective inhibition of tumour cell energy metabolism has concentrated on the inhibition of tumour mitochondrial function. An alternative approach to limiting energy production in tumour cells is to deplete cells of their mitochondrial content by interfering in the ability of tumour cell mitochondria to replicate.

In order to examine these concepts further, some of the aspects of mitochondrial genetics must be understood. These demonstrate how inhibitor titration has helped to elucidate differences between nuclear and mitochondrial contributions to mitochondrial replication. Mitochondria, not only play an important role in cellular metabolism, but they also possess their own unique genetic system. This system is responsible for the assembly of mitochondrial ribonucleic acids and a number of subunits of hydrophobic proteins of the inner mitochondrial membrane. Most of the mitochondrial DNA translation products are part of the enzyme complexes involved in ATP generation. Therefore inhibition of the function of the mitochondrial genetic system may have severe energetic and metabolic consequences for the cell (Van den Bogert, 1983).

All eukaryote mitochondria, examined to date contain double stranded circular molecules of DNA measuring about 5 microns and are equivalent to about 16,000 base pairs (Kroon and Saccone, 1980). The DNA contained constitutes the minimal amount of genetic information necessary for mitochondrial function. Much of this DNA is conserved. The DNA codes for 2 ribosomal RNA's and 22 transfer RNA's. All enzymes needed to transcribe mitochondrial DNA (mtDNA) and to translate messenger RNA (mRNA) are present (Van den Bogert, 1983; Clayton, 1984). Most of the reading frames of human mtDNA correspond to functional genes. Genes encoded for include the cytochrome C

oxidase subunits 1, 2 and 3, cytochromes b and c, a subunit of the ATP synthase complex and components of the NADH-ubiquinone reductase enzyme complex (Anderson et al, 1980; Attardi, 1981; Bibb, 1981; Chomyn et al, 1985).

Mitochondria are able to incorporate amino acids into protein and this process is sensitive to specific inhibitors (Galper and Darnell, 1971; Schatz and Mason, 1974). Thus animal mitochondria contain a functional genetic system, whose polypeptide products are part of enzyme complexes involved in oxidative phosphorylation (Kolarev et al, 1981). The majority of proteins found within mitochondria are encoded by nuclear DNA and are synthesized in the cytoplasm. These polypeptides once assembled must be transported, often as precursors to the sites of activity across the inner mitochondrial membrane. Examples of these proteins include the polypeptide subunits of succinate dehydrogenase which is involved in oxidative phosphorylation (Reid, 1985; Clarkson et al, 1987). The interaction between cytoplasmic and mitochondrial protein synthesis and mechanisms which regulate the processes are not clearly understood. However it is likely that mitochondrial protein synthesis will influence cytoplasmic transcription and translation. A severe lack of functional mitochondria would have fatal consequences for the strictly aerobic mammalian cell. Two models exist which attempt to explain how mitochondrial biogenesis may be regulated by the cell.

One model suggests that a protein synthesized in mitochondria represses the synthesis of enzymes coded for in the nucleus. If the number of functional mitochondria is lowered, the concentration of this repressor becomes reduced and inhibition is then abolished. Evidence for this model however is lacking. The second model proposes that impairment of oxidative phosphorylation serves indirectly as a signal for the synthesis of mitochondrial proteins. Data available, based upon evidence from non mammalian cells such as *saccharomyces* favours this model (Barath and Kuntzel, 1972; Van den Bogert, 1983).

Much of the evidence cited, referring to mtdna and subsequent transcription and translation in mammalian cells was obtained from

human HeLa and rat hepatoma cells. It is likely that some tumour cells will contain aberrant forms of mtDNA and protein synthesis in tumour mitochondria is likely to differ in certain tumours relative to normal cells (Pedersen, 1978).

Agents which interfere in the ability of mitochondria to undergo biogenesis were first studied in lower eukaryotes such as saccharomyces. Chloramphenicol was demonstrated to show a highly selective inhibition of mitochondrial protein synthesis (Clark-Walker and Linnane, 1967). Subsequent evidence indicated that mammalian cell mitochondrial protein synthesis was sensitive to chloramphenicol. There appeared to be two forms of protein synthesis occurring in mammalian cells, one form was susceptible to the effects of inhibition with cycloheximide, while another was unaffected by cycloheximide. The cycloheximide resistant process however showed a selective inhibition to chloramphenicol. From these studies it was concluded that the cycloheximide sensitive cytoplasmic protein synthesis was quite separate from mitochondrial protein synthesis resistant to cycloheximide. Chloramphenicol, however exerted a selective inhibition on cycloheximide resistant mitochondrial protein synthesis (Ennis and Lubin, 1964; Galper and Darnell, 1971).

Attempts were made to isolate chloramphenicol resistant mutant mammalian cells. Such cells were found to be deficient in respiratory chain components, requiring glycolytic substrates in order to maintain growth and proliferate. Cloning such respiratory deficient mutants gave rise to daughter cells, which were also respiratory deficient (Spolsky and Eisenstadt, 1972; Wiseman and Attardi, 1979; Howell and Sager 1979).

It was apparent that cytoplasmic genetic transfer between parent and daughter cells must be occurring, conserving the parent phenotype in daughter cells. Evidence with both these antibiotic agents indicated that protein synthesis in mitochondria could be selectively inhibited, furthermore biogenesis of mitochondria as a result of treatment with these agents was also reduced and thus a method was available for depleting cells of their mitochondria if treatment was prolonged.

Other antibiotics implicated in having inhibitory effects upon biogenesis were carbomycin and doxycycline (tetracycline) (De Vries et al, 1971; Van den Bogert et al, 1986(a); Van den Bogert et al, 1986(b)). Other agents described as having a selective inhibitory effect upon tumour mtDNA metabolism include ethidium bromide and methyl glyoxalbis (guanyl hydrazone) (MGBG) (Zylber et al, 1969; Feuerstein et al, 1979; Nass, 1984; Patel et al, 1984; Bugeja et al, 1987). Agents such as ethidium bromide or MGBG are thought to interfere with mitochondrial nucleic acid metabolism where as the antibiotics doxycycline or chloramphenicol are thought to interfere at the translational level following transcription of mRNA.

Any agent, which interferes in nucleic acid metabolism at the nuclear level can theoretically interfere in mitochondrial biogenesis. Many anticancer chemotherapeutic agents belong to this category. Examples include adriamycin, bleomycin and 5-fluorouracil (5FU). How selective this action is, is unclear. Early studies with inhibitors of the mitochondrial genetic system as stated previously were carried out on lower eukaryotic systems.

5FU was demonstrated to promote aberrant mitochondrial biogenesis in yeast (Oliver and Williamson, 1976(a); 1976(b)). This was demonstrated to occur as a result of incorporation of 5FU into certain species of RNA (Oliver and Williamson, 1977). The effect of 5FU upon mammalian cells is inhibitory. Growth inhibition of cells is thought to be as a result of inhibition of the enzyme thymidylate synthetase [Thymidylate Synthase E.C. 2.1.1.45]. DNA synthesis is inhibited indirectly as the source of deoxyuridine monophosphate, is removed. Since ultimately this is converted and phosphorylated to deoxy-thymidine triphosphate, an essential nucleotide required for DNA, DNA synthesis is inhibited. This inhibition of DNA synthesis by 5FU was considered to be the primary mechanism of achieving tumour cell cytostasis (Ardalan and Glazer, 1981; Schornagel et al, 1982; Heidelberger, 1975). However 5FU is also thought to have important effects upon RNA metabolism. Metabolism of 5FU by tumour cells gives rise to several nucleotide derivatives including Fluorodeoxyuridine monophosphate (FdUMP) and Fluorouridine Monophosphate (FUMP). FdUMP

is responsible for the inhibition of thymidylate synthetase and ultimately DNA synthesis. FUMP however, may be incorporated into RNA following phosphorylation to fluorouridine triphosphate. This important effect upon RNA synthesis in tumour cells has been stressed (Spiegelman et al, 1980; Cory et al, 1979; Mandel, 1981). Thus circumstantial evidence did exist with which to inhibit mitochondrial RNA metabolism with non cytotoxic concentrations of 5FU, hence reduce the expression of mitochondrial encoded proteins such as cytochrome oxidase. Recent evidence, provides more support for this theory that 5FU interferes with mitochondrial biogenesis in both saccharomyces and cultured mammalian guinea pig keratocytes (Bugeja et al, 1987).

The methods for assaying mitochondrial biogenesis were designed to examine whether a reduction in the synthesis of mitochondrial components following continuous exposure of tumour cells with 5FU in vitro occurred. A reduction in mitochondrial enzyme activity in response to long term incubation of cells with biogenetic inhibitors could be monitored. A reduction in mitochondrial activity would correspond to a decrease in mitochondrial enzyme content.

Enzyme activities measured, following long term exposure of cells to 5FU included cytochrome oxidase (COX) (ferrocytochrome C: oxygen oxidoreductase E.C. 1.9.3.1) which is encoded for on the mitochondrial genome. Also measured was succinate-cytochrome C reductase (SCR) activity. This enzyme system corresponds to a combination of the activities of succinate-dehydrogenase enzyme complex II (succinate-ubiquinone reductase E.C. 1.3.9.9.1) encoded in the nucleus, and ubiquinone-cytochrome C reductase enzyme complex III [E.C. 1.10.2.2]. Thus activity from a crude cell preparation was a reflection of electron transport in crude segments of the respiratory chain. Assaying individual respiratory chain enzymes would have necessitated specific purification steps including penetration of the inner mitochondrial membrane, not justifiable at this stage. As rotenone was omitted from the assay, entry of electrons via NADH dehydrogenase complex I could not be excluded. The main purpose of the assay was to see what effect growth of cells in 5FU achieved upon mitochondrial

enzyme content rather than on an individual enzyme (Takemori and King, 1964; King and Takemori, 1964; Singer, 1966).

These methods measure the products of mtDNA transcription and translation as well as the cytoplasmic contribution to mitochondrial biogenesis. A more direct method was thus made available as compared to the spectrophotometric methods which measured a reduction in the absorbance spectra of mitochondrial cytochromes resulting from treatment with biogenetic inhibitors (Morais, 1980; Bugeja et al, 1987). Furthermore it provided a more rapid answer as compared to more accurate gel electrophoretic methods for determining disappearance of protein bands following 5FU treatment. At any rate such methods would have involved mitochondrial isolation from cells grown up in tissue culture, a time consuming and expensive process.

The main aim of the studies presented in this chapter is to examine whether suitable chemotherapeutic agents may be selected which might inhibit tumour mitochondrial biogenesis. If mitochondrial biogenesis can be inhibited at least in vitro, preventing the ability of mitochondria to replicate, combining such agents with R6G may enable tumour cell energy metabolism to be inhibited at the expense of host cell metabolism.

The growth behaviour of WIL exposed to 5FU was examined in order to select non cytotoxic concentrations of drug for further experiments. An assay of cytotoxicity was designed to assess what concentration of drug inhibited growth. Growth rate experiments were also carried out to confirm what effect non toxic concentrations of 5FU had upon population growth rates of WIL. In comparison, similar experiments were carried out to determine the effects of functional inhibitors of tumour mitochondria R6G and lamprene on chemosensitivity and population growth rates. Mitochondrial enzyme activities were then investigated following continuous exposure of cells to 5FU.

METHODS

Assay of Cytotoxicity

Chemosensitivity was determined by an assay recently established within the Department of Medical Oncology. The assay is based on that described by Carmichael et al (1987). A full description of this assay is given in chapter 2.

Estimation of growth rates

Estimation of cell growth rates were carried out as described in chapter 2.

Mitochondrial Enzyme Activities

1. Cytochrome oxidase (ferrocytochrome C : oxygen oxidoreductase E.C. 1.9.3.1). This was determined by the method of Rafael (1983).

The basis of the assay was as follows:



The uptake per unit time of oxygen dissolved in the assay is used as a measure of the catalytic activity of COX. Cytochrome C is kept in the reduced form by ascorbate. TMPD (N,N,N,N; Tetramethyl-p-phenylene diamine) mediates electron transport. Oxygen uptake in the assay is measured polarographically with a Clark-type oxygen electrode calibrated as described previously (chapter 2). The assay blank which exhibits an oxygen uptake rate due to auto-oxidation of ascorbate and TMPD respectively is subtracted from the measured oxygen uptake.

Reagents were prepared as follows:

1. Phosphate-EDTA solution (phosphate buffer 50mM, EDTA 1mM adjusted to pH 7.2). Buffer was prepared by dissolving KH_2PO_4 , 4.288g and EDTA - $\text{Na}_2\text{H}_2\text{.2H}_2\text{O}$, 0.234g in distilled water, 450ml. pH was adjusted to 7.2 with KOH, 10M (KOH, 5.61g dissolved in distilled water, 10ml) added dropwise to the buffer. The buffer was made up to a final volume of 500ml.

2. Ascorbic Acid, 100mM. Ascorbate, 26.4mg was dissolved in phosphate-EDTA buffer, 1.5ml. pH was adjusted to 6 with 2 to 3 drops of KOH, 2M.
3. Lubrol, 10mg per ml. Lubrol, 100mg was dissolved in phosphate-EDTA buffer, 10ml at room temperature.
4. TMPD, 50mM. Repurified TMPD, 11.8mg was dissolved in distilled water 1ml.

Repurification of the general purpose grade reagent was achieved as follows:-

5 to 10 grams of TMPD was dissolved in 85% ethanol, 20 to 30ml containing 2 to 3 drops of concentrated HCl, in a pyrex beaker. The solution was heated on a hotplate in the fume cupboard to form a supersaturated solution. Recrystallisation was allowed to occur by cooling to room temperature. Crystals obtained were separated from solvent by filtration through a buchner funnel. Crystals were briefly rinsed in ethanol, 85%. If necessary the process was repeated. Purity was assessed from the colour of the solute. From impure gray coloured crystals, pure white crystals were obtained following two recrystallisation steps.

5. Cytochrome C 11.1mg per ml. Tris-acetate buffer.

The following purification steps were carried out to ensure that cytochrome C monomer was present in the assay.

Sephadex G100 was prepared as follows: Beads, 2g were mixed with Tris-acetate buffer, 40ml. Buffer was made up by dissolving Tris-base, 1.514g in acetic acid, 250ml (100mM). pH was adjusted to 7.5 before making up to volume. 50ml of this buffer was sterile filtered and a few milligrams of sodium azide were added to keep the solution sterile. Beads were allowed to take up buffer over a 72 hour period at room temperatures in a sealed buchner funnel. Prior to use, the mixture was degassed with a vacuum pump. Several short tipped paster pipettes were set up vertically on clamp stands. Pipettes were fitted with silicon tube ends and clamped. Glass wool, wetted in fresh buffer was inserted into each tube. Gel was carefully pipetted into each tube leaving a 1

to 2cm gap at the top. Azide-free buffer was washed through the columns to remove traces of sodium azide. Cytochrome C, 1ml made up in buffer, containing protein 100mg, and a few crystals of dithionate to keep the protein reduced was layered onto each column. Protein eluted from the column in a distinct band. Dilution was negligible. Polymer free preparations could be stored at -20°C and aliquots were used as required at a final concentration of 11.1mg per ml. Aliquots of cytochrome C were thawed out just before use.

Solutions 2 and 4 were freshly prepared each time. All solutions were kept at 4°C prior to the assay.

Preparation of Cells

WIL was seeded into flasks (75cm^2) at an approximate concentration of 5×10^5 cells per flask (1.25×10^4 cells per ml). Attachment and growth was allowed to take place over 48 hours. W256 cells were seeded into flasks (75cm^2) at an approximate concentration of 1×10^6 cells per flask (2.5×10^4 cells per ml). Growth was allowed to take place over a 48 hour period.

Cells were fed with fresh medium containing 5FU, 200uM. Control flasks were fed with fresh medium. Thereafter cells were fed with fresh medium or medium containing 5FU daily. 72 hours after initiating drug treatment, assays of cytochrome oxidase were carried out.

Cells were harvested as described in chapter 2. Harvested cells were counted and resuspended in Hanks Balanced Salts Solution (HBSS) containing phenol red, glucose 6mM and bicarbonate 5mM. Cells were washed with this medium and resuspended in fresh HBSS. Samples, 0.2ml were taken and placed in distilled water. 0.1ml of sodium deoxycholate, 50% was added to this and samples were stored at -20°C for subsequent analysis of protein (see chapter 2). Cells were broken up with a cell disruptor at pressures of between 600 to 800 psi for WIL and 500 to 700 psi for W256. Samples, 0.2ml were collected and stored at -20°C for subsequent analysis of protein as described

previously. Dilution of the cell preparation was estimated from differences in protein content of the preparation following cell disruption. Cells were diluted further by addition of lubrol (solution 3), 5ml.

Enzyme Reaction

Phosphate-EDTA, 1.06ml was equilibrated for 2 to 3 minutes in the oxygen electrode. Once a stable baseline was obtained on the chart recorder output ascorbate, 0.1ml, cytochrome C, 0.120ml and TMPD, 0.02ml were added sequentially to the electrode with a hamilton syringe. A net auto-oxidation rate was observed which was less than 8% per minute (full recorder range was 100%) and normally 4 to 5% per minute. Cell suspension, 0.1ml was added after 2 to 3 minutes. From each chart recorder trace, a blank rate was obtained which was subtracted from the sample rate and the net activity recorded as a change in oxygen consumption. Catalytic activity was calculated in nanomoles electrons per second per milligram of protein at 25°C from the equation:

$$\text{Net Slope} \times 960 \times \frac{\text{Total volume in electrode (1.4ml)}}{\text{Sample Volume (0.1ml)}} \times \text{Fraction of Full scale deflection}$$

$$\div \text{Number of milligrams protein per litre} \times 1000$$

where 960 = micromoles of electrons per litre transferable as oxygen dissolves at 25°C.

1000 = conversion factor to nanomoles.

Results were also expressed in terms of activity per 10^7 cells. To take into account the different buffers, cells were incubated in prior to assay, appropriate controls were run in the oxygen electrode.

Mitochondrial Enzyme Activities II

2. Succinate - Cytochrome C reductase (SCR)

Methods were essentially as described by King (1967).

The basis of the assay was as follows:



Reduction of the artificial electron acceptor 2, 6, - dichlorophenol indo-phenol (DCIP) is followed on a spectrophotometer at a wavelength of 600nm. The assay medium contained phosphate buffer, EDTA, succinate, DCIP and potassium cyanide (necessary to block competition with cytochrome oxidase). The reaction was initiated by addition of crude homogenate, 0.2ml

Reagents were prepared as follows:

1. Potassium cyanide 1mM. Solution prepared from dilution (1:7.69 v/v) of a stock solution of KCN, 0.5% in saline, 0.85%.
Dilution was carried out with saline, 0.85%.
2. Sodium Hydroxide 1M. NaOH, 4g was dissolved in distilled water, 100ml.
3. Sorensens phosphate buffer.
Solution A : KH_2PO_4 , 0.2M. KH_2PO_4 , 2.721g was dissolved in distilled water, 100ml. pH of the resulting solution was approximately 4.4.
Solution B : Na_2HPO_4 , 0.2M. Na_2HPO_4 , 2.84g was dissolved in distilled water, 100ml. pH of the resulting solution was approximately 9.2.
Buffer was made up by mixing A, 19.6ml with B, 80.4ml. pH was adjusted to 7.4 by dropwise addition of A or B depending on the initial pH of the mixture.
4. EDTA 3mM. EDTA- Na_2H_2 $2\text{H}_2\text{O}$, 11.8mg was dissolved in distilled water, 10ml.
5. Succinate 600mM. Na COO CH_2CH_2 COO H $6\text{H}_2\text{O}$, 0.97g was dissolved in distilled water, 10ml.
6. DCIP 0.53mM. DCIP, 1.54mg was dissolved in distilled water 10ml.

Solutions 4, 5 and 6 were freshly prepared. All solutions were kept on ice at 4°C.

Preparation of Cells

WIL and W256 were prepared as described previously (Chapter 2). Treatment of cells with 5FU was similar to methods described for assaying COX activity. However the incubation period with drug was 96 hours. Cell disruptor pressures were 750 psi for WIL and 600 psi for W256. In initial experiments cell aliquots were diluted with lubrol (solution 3 in COX assay) in order to penetrate mitochondrial permeability barriers to assay reagents. However mechanical disruption appeared to be sufficient and further treatment with lubrol did not significantly alter results. Dilution of cells was estimated from differences in protein content of the preparation following cell disruption. Protein was assayed as described in chapter 2.

Enzyme Reaction

Enzyme activity was measured in an assay volume of 3ml. To a 5ml cuvette was added phosphate-EDTA buffer 1.5ml, EDTA 0.3ml, DCIP 0.3ml, succinate 0.1ml, cyanide 0.3ml and distilled water 0.3ml. The absorbance of the assay mixture was determined in a spectrophotometer linked to a chart recorder at a wavelength of 600nm. The absorbance zero was set with distilled water. Basal absorbance values of the assay mixture ranged between 0.6 and 0.8 at a wavelength of 600nm. The reaction was initiated by addition of crude homogenate 0.2ml. This reaction was linear for up to 2 minutes. Recorder traces of the initial slope were obtained as a change in absorbance per minute. Enzyme activities were calculated as nanomoles succinate used per minute per milligram of protein at 25°C. From the equation:

$$\text{Activity} = \frac{\text{Slope} \times 2 \times 10^{-2}}{V \times C \times 7} \times 1000$$

where 2×10^{-2} is the absorbance change in units per chart recorded division

- V = Volume of homogenate in 3ml
C = Amount of protein in milligrams per ml homogenate
7 = Conversion factor obtained from absorbency index

Absorbency index for DCIP is $21\text{mM}^{-1}\text{ cm}^{-1}$ at 600nm at 25°C.

1000 = Conversion factor from micromoles to nanomoles succinate.

Enzyme activities were also calculated as activity per 10^7 cells. To check on the specificity of the reaction, aliquots of homogenate were preincubated with sodium malonate, 5 to 15mM, to determine effects upon enzyme activity.

Statistical analysis

Statistical differences between drug treatment cells and controls were determined by Students t-test for unpaired samples (Bailey, 1972).

RESULTS

Determination of Cytotoxicity

The ID₅₀ values for each drug screened against WIL is shown in table 6.1. R6G is observed to be the most toxic compound, with an ID₅₀ value ranging between 0.3 μ M to 0.5 μ M. Lamprene appears to be less toxic to WIL than R6G, exhibiting ID₅₀ values of 34 μ M and 36 μ M. However 5FU shows the least effect upon WIL. Absorbance at 570nm is reduced to 50% with a concentration of around 500 μ M

Effect of R6G, lamprene and 5FU upon the growth rate of WIL

The effect of a 24 hour exposure to R6G (10, 50 or 100 μ M) on the growth rate of WIL is shown in figure 6.1. R6G caused an inhibition of cell proliferation. However, after a lag phase cell proliferation was observed with a R6G concentration of 10 μ M. In contrast, no recovery was observed with R6G at a concentration of 50 or 100 μ M.

The effect of a 24 hour exposure of WIL to lamprene (10 and 50 μ M) is shown in figure 6.2. Lamprene solubilised in DMSO (4%) caused a significant inhibition of cell proliferation. However after a lag phase cell proliferation resumed when cells were treated with DMSO (4%) alone. Initially, cells treated with lamprene, 10 μ M resumed growth up to 4 days after treatment. However subsequent to this, cell growth did not continue and proliferation was inhibited. In contrast no recovery was observed at a lamprene concentration of 50 μ M.

The effect of 5FU (10 μ M, 50 μ M and 100 μ M) upon the growth rate of WIL, following a 24 hour exposure of cells to drug is shown in figure 6.3. Cell proliferation is not inhibited with 5FU. However a significant growth delay is observed in cells exposed to 5FU, 50 μ M and 100 μ M. Cell number reaches a plateau of 90 to 100 x 10⁴ cells per well at these drug concentrations. In contrast, cells exposed to 5FU, 10 μ M continue to grow although at a reduced rate when compared with controls.

DRUG	ID ₅₀ VALUE (Molar)	
Rhodamine 6G	5.0 x 10 ⁻⁷	3.0 x 10 ⁻⁷
Lamprene	3.6 x 10 ⁻⁵	3.4 x 10 ⁻⁵
5-Fluorouracil	7.0 x 10 ⁻⁴	3.0 x 10 ⁻⁴

Table 6.1: ID₅₀ values for WIL exposed to R6G, lamprene and 5FU. Duplicate values were recorded. Determinations of ID₅₀ were made from dose response curves following the MIT assay. MIT reduction was assayed 3 days after cells were exposed.

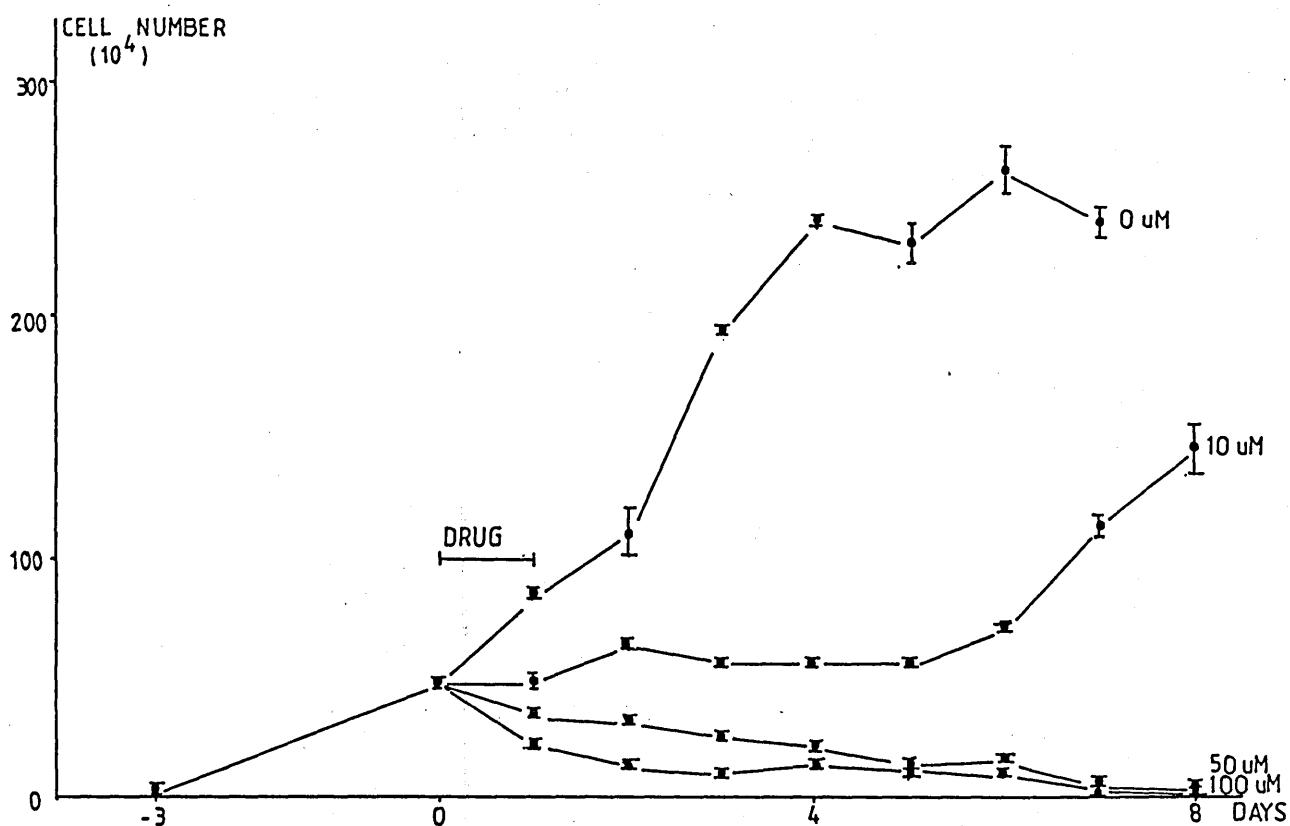


Figure 6.1: The growth rate of WIL following 24 hour exposure to R6G. Growth rate expressed as cell number per well versus time in days. $N = 3 \pm \text{SEM}$. Cells were seeded at day -3. Drug exposure as indicated was from day 0 to day 1. Recovery over a 7 to 8 day period was recorded.

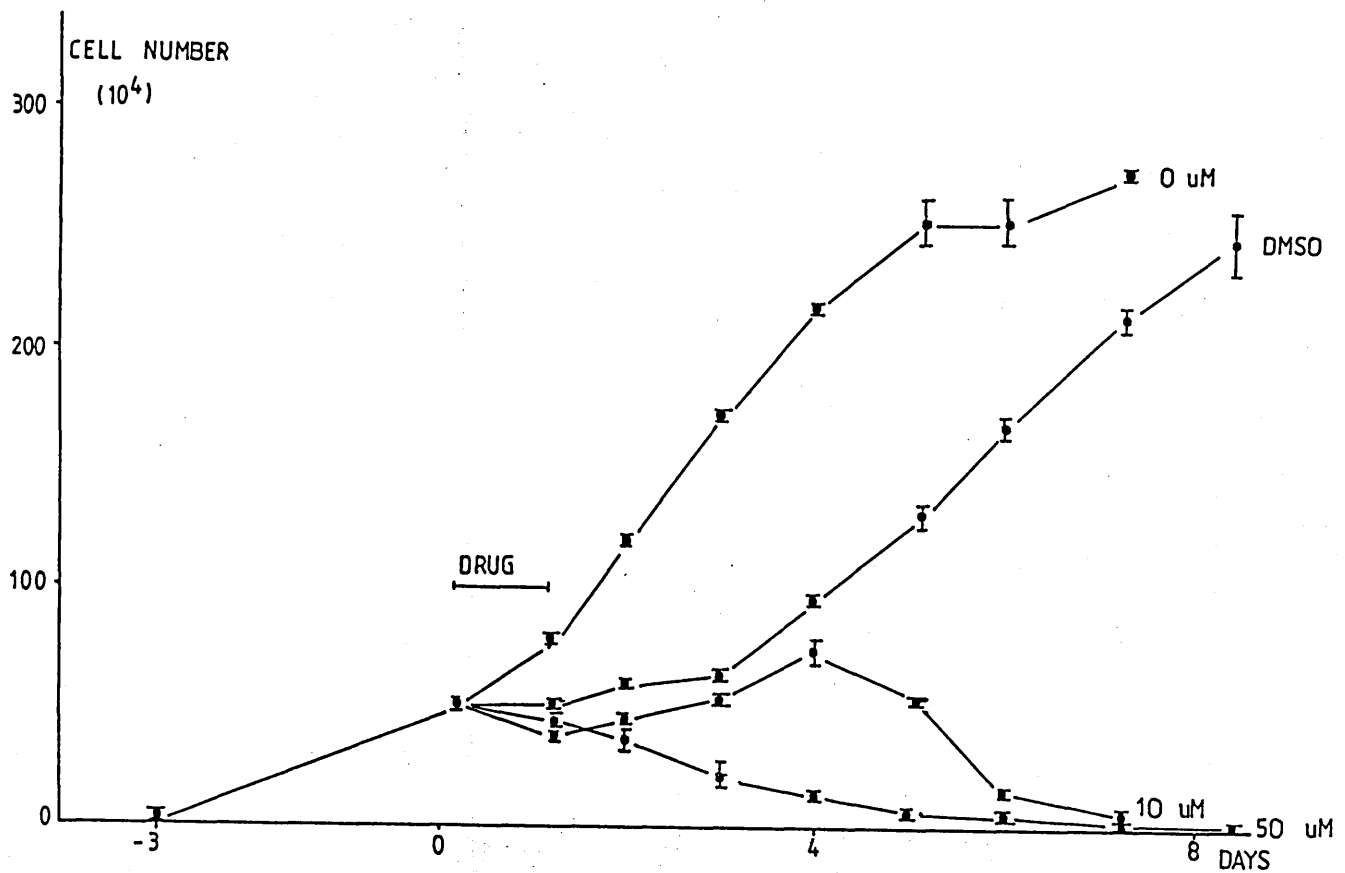


Figure 6.2: Growth rate of WIL following 24 hour exposure to lamprene. Growth rate expressed as cell number per well versus time in days . $N = 3 \pm SEM$. Cells were seeded at day -3. Drug exposure as indicated was from day 0 to day 1. As lamprene was solubilized in DMSO, an appropriate control was run. Recovery was recorded over a 7 to 8 day period.

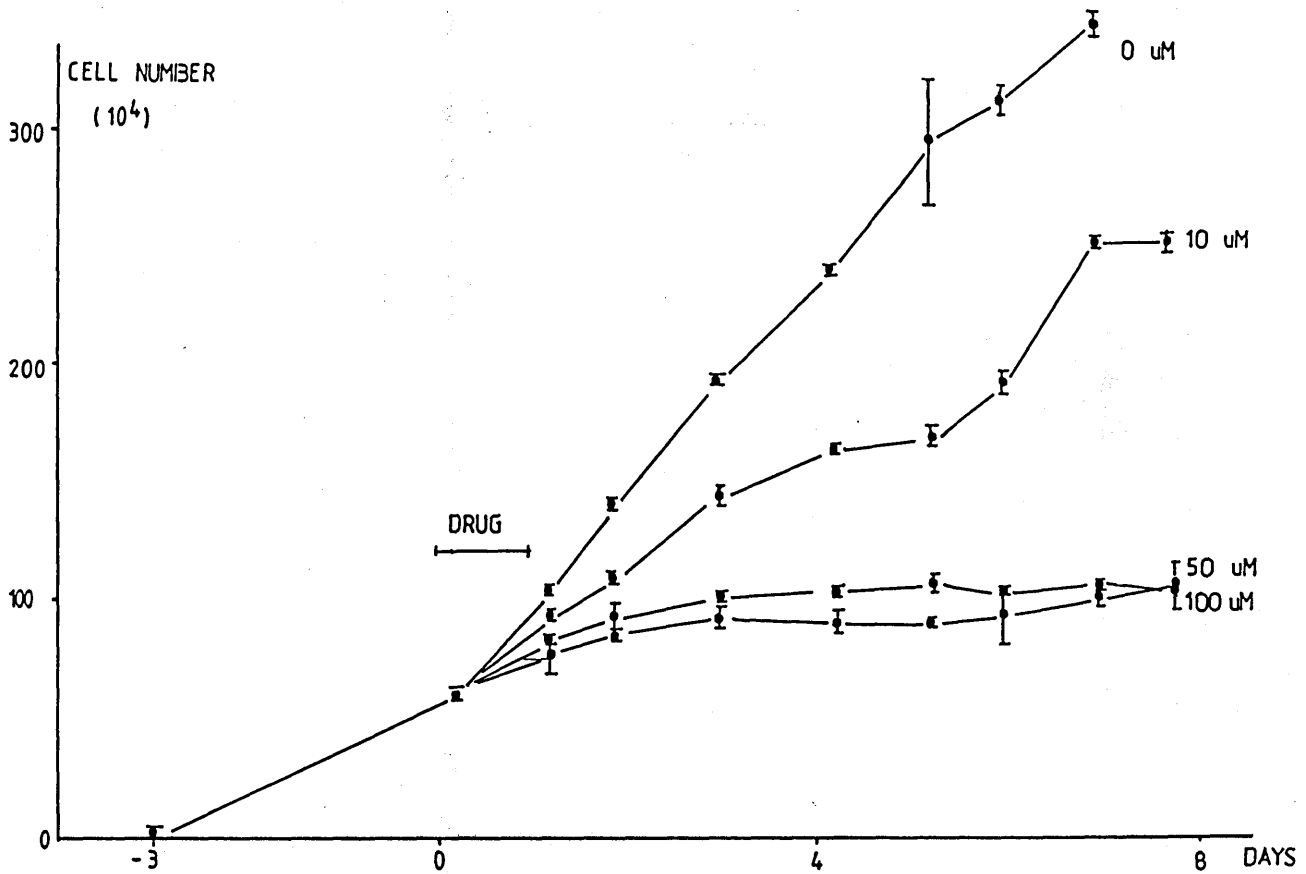


Figure 6.3: Growth rate of WIL following 24 hour exposure to 5FU. Growth rate expressed as cell number per well versus time in days. $N = 3 \pm \text{SEM}$. Cells were seeded at day -3. Drug exposure as indicated was from day 0 to day 1. Recovery was recorded over a 7 to 8 day period.

Effect of 5FU upon mitochondrial enzyme activity following prolonged exposure of cells to drug

Cytochrome oxidase activity determined after exposure of W256 to 5FU, 200uM, for 72 hours is shown in table 6.2. The activity of succinate-cytochrome C reductase determined after exposure of WIL and W256 to 5FU, 200uM for 96 hours is also shown in table 6.2.

Exposure to 5FU was continued for 96 hours prior to assay of SCR activity and not 72 hours as for COX activity in order to determine whether prolonging exposure had any further effect upon mitochondrial enzyme expression. Cytochrome oxidase activity could not be detected in WIL even though several variations in the methods described for enzyme were used. Treatment of W256 with 5FU does not show a reduction in enzyme activity. There was no significant difference between the activity of this enzyme in treated and untreated cells ($p < 0.1$). Succinate-cytochrome C reductase activity is shown for both cell lines WIL and W256. For WIL, exposure of cells to 5FU resulted in a 150% increase in enzyme activity relative to controls. This result was statistically significant ($p < 0.001$). The activity of SCR was reduced by 50% ($p < 0.001$) when W256 was exposed to 5FU (200uM) for 96 hours (table 6.2).

A 20 to 30% reduction of SCR activity (table 6.2) may be observed when concentrations of sodium malonate (5mM) were preincubated with preparations of cell homogenate prior to determinations of SCR activity.

ENZYME ACTIVITIES

COX Activity
nmoles electrons per 10^7 cells per sec.

		+/- SEM
W256 control	435	63
W256 + 5FU	1230	301

P < 0.1 Not Significant

SCR Activity
nmoles succinate per 10^7 cells per min.

		+/- SEM	
WIL control	361	18	
WIL + 5FU	550	14	*
W256 control	442	17	
W256 + 5FU	211	18	*
W256 control + 5mM Malonate	347		

* P < 0.001 Significant

Table 6.2: Mitochondrial enzyme activities of WIL and W256 following exposure to 50FU, 200uM. N = 3 \pm SEM. Cytochrome oxidase (COX) activity expressed as nmoles electrons per 10^7 cells per second. Values recorded for W256 cells exposed to 5FU and drug free controls. P values obtained from unpaired t-tests. Succinate cytochrome C reductase (SCR) activity expressed as nmoles succinate per 10^7 cells per minute. Values recorded for WIL and W256 cells exposed to 5FU and drug free controls. P values obtained from unpaired t-tests.

DISCUSSION

Results of experiments described in this chapter indicate that of the three agents R6G, lamprane and 5FU investigated, R6G was the most toxic to the cell line WIL. ID₅₀ values for this agent ranged between 0.3 and 0.5uM. Lamprane, solubilized in DMSO was less toxic with ID₅₀ values of between 34 and 36uM. 5FU had the least effect upon WIL. The drug concentrations required to decrease the absorbance at 570nm by 50% were between 300 and 700uM. The growth behaviour of WIL was examined over a 7 to 8 day period following a 24 hour exposure to each of the agents described R6G, lamprane and 5FU. R6G inhibited cell proliferation at all concentrations used. However, over the 8 day period during which recovery was measured cells recovered from the 10uM concentration of drug. No recovery was observed from higher concentrations of R6G. Lamprane also inhibited cell proliferation at concentrations of 10uM and 50uM. Although cells did appear to partially recover, 3 days after exposure to the 10uM concentration, further proliferation was subsequently inhibited and cell number decreased. Initially 5FU did not inhibit proliferation of WIL. Rates of cell growth did decrease after a lag phase. At concentrations of 5FU, 50 and 100uM cell number achieved a constant value 3 days after exposure of cells to drug. The 10-uM concentration, caused a delay in cell growth up to 2 days after exposure of cells to drug. Subsequently cell growth resumed following a similar growth pattern to that of controls.

Clear inhibition of mitochondrial enzyme activity of WIL and W256 following continuous exposure of cells to a concentration of 5FU, 200uM is not observed. Cytochrome oxidase activity in W256 was not significantly altered following treatment with 5FU. Succinate-cytochrome C reductase activity in preparations of WIL showed a stimulation of enzyme activity following treatment with 5FU. However W256 cells when exposed to 5FU, 200uM exhibited a significant reduction in enzyme activity compared to controls.

At first, a comparison of results obtained with the MIT assay do not appear to be consistent with results obtained from growth rate

experiments. However, although a response to a 24 hour exposure to drug was measured, the MTT assay monitored recovery after a 3 day period, whereas growth rate recovery from drug exposure is measured over an 8 day period. Thus for results with R6G, although cells appear to recover from a concentration of 10uM in the growth rate experiments, this concentration is equivalent to approximately 20-fold greater than ID₅₀. The growth rate of WIL exposed to R6G, 3 days after exposure is very low and could be interpreted within the context of a cytotoxic dose. From both experiments, it is clear that R6G does exert a prolonged inhibition of growth, although at low drug concentrations recovery may occur after a definite time delay.

From experiments with lamprane measured with the MTT assay, as the compound was insoluble in aqueous solution, a residue of drug remained in wells up until the point of addition of MTT. It is possible that the drug residue may have had a direct effect upon cell metabolism and ultimately reduction of MTT. The residue of the drug that did remain did not interfere with the absorbance of Formazan measured at 570nm, following addition of DMSO to the plate. DMSO was required to disperse lamprane prior to addition to cells for the MTT assays. It appeared that the concentration of DMSO used had little effect on cell growth rate, yet results indicated from growth rate experiments that the 4% concentration required to dissolve lamprane did have an inhibitory effect. 10uM lamprane solubilized in DMSO is inhibitory to growth, yet lamprane at this concentration in the MTT assay is not toxic. These consistent results might possibly indicate that the net effect of lamprane and DMSO on cell growth might make WIL more sensitive to the toxic effects of lamprane. This requires confirmation by treating cells with lamprane in the absence of DMSO and at this stage remains unproven.

Antimetabolites such as 5FU, are structural analogues of cell metabolites and interfere in metabolic pathways very often by incorporation into an enzyme active site. Further metabolism is prevented due to inactivation of the enzyme. 5FU and other conventional chemotherapeutic agents interfere with nucleic acid metabolism and are most effective at a single point or several points

along the cell cycle (Hill, 1978). The proportion of cells undergoing division is gradually reduced. Cells are reduced to a non proliferating state yet cell number is not reduced, a cytostatic effect is thus induced. With 50uM and 100uM concentrations of 5FU cell growth is inhibited, but cell number is not reduced. However, with higher concentrations of 5FU, as shown with the MTT assay, 600uM to 800uM, not only incurs non recovery, but also a reduction in cell number, hence cytotoxicity.

The effects of 5FU upon mitochondrial enzyme activity remains ambiguous. The activity of SCR in WIL and W256 was assessed in crude preparations of cells. Even if contamination with cytoplasmic dehydrogenases occurred and the activity of SCR was only part of the net dehydrogenase activity measured, interference in nucleic acid metabolism following treatment with 5FU should have resulted in reduced translation of both nuclear and mitochondrial polypeptides. All genomic translation should have been reduced with 5FU.

It is possible that during the 48 hour growth period prior to 5FU administration to cell cultures, enzyme synthesis per cell was optimal. The portion of cells undergoing subsequent mitosis may have been less than the proportion of cells already present in each flask. Biogenetic inhibition of mitochondria may only have been achieved thereafter. Net enzyme activity in either untreated controls or treated cells would have remained the same.

A remote possibility raised by the apparent stimulation of SCR activity in WIL with SCR, is that non-cytotoxic 5FU might stimulate mitochondrial enzymes by mechanisms as yet unclear.

Experiments conducted upon L1210 mitochondria indicate that bleomycin induced damage of mitochondrial DNA is less effective in intact mitochondria (Lim and Neims 1987).

The susceptibility of mitochondrial enzymes to inhibition with adriamycin is also reported to depend on lysis of the outer membrane of the mitochondrion with either digitonin or osmotic shock (Manella

et al, 1986). Therefore it may be possible that the experimental protocol here did not facilitate entry of 5FU into the mitochondrion. As a consequence enzyme activity detected, follow cell disruption and treatment with the detergent lubrol released intact enzyme activity.

Attempts to adequately inhibit mitochondrial biogenesis in cultured cells, such as chick embryo fibroblasts have not been wholly successful. It has been shown that the concentrations of carbomycin and mikamycin required to inhibit mitochondrial protein synthesis have failed, the drugs being cytotoxic at concentrations where protein synthesis on mitoribosomes is not suppressed (Morais, 1980). It has also been shown that in order to suppress COX activity with ethidium bromide in primary chick fibroblasts, long term drug treatment, exceeding 20 days was necessary (Desjardins et al, 1985). Therefore it is likely that either the concentration of 5FU required to inhibit mitochondrial protein synthesis was suboptimal, or treatment was not of long enough duration to adequately achieve inhibition of mitochondrial biogenesis.

All the possible reasons presented to explain the inability to inhibit mitochondrial biogenesis with 5FU assume relative sensitivity of the cells to 5FU. The high ID₅₀ values (300 to 700uM) obtained with 5FU from the MIT assay indicate that cells from the tumour may have developed resistance to the effects of 5FU. Resistance of cells to 5FU has been extensively studied. Early experiments reported that RNA synthesis in resistant cells was blocked by a reduction in pyrimidine incorporation into ribonucleotides. Reduced RNA synthesis was thought to be a feature of resistant cells and that 5FU metabolism to FdUMP could still proceed (Reichard et al, 1959; Heidelberger et al, 1960; Goldberg et al, 1966). It became apparent from later studies that 5FU had different effects upon both RNA and DNA metabolism. Cells resistant to 5FU were thought to lose the ability to synthesize FdUMP from ribonucleotides, but continued to do so through fluorodeoxyuridine (Wilkinson and Crumley, 1977; Cory et al, 1979; Wright et al, 1980). Subsequent evidence showed that resistant cells expressed elevated levels of thymidylate synthetase, the enzyme which FdUMP was supposed to inhibit. This implied that resistant cells lost

the ability to synthesize FdUMP (Cory et al, 1979; O'Byrne-Ring and Duke, 1980).

Recent theories concerning resistance to anticancer drugs are based upon the concept that a drug resistant phenotype in cells arises spontaneously prior to exposure to drug. Exposure of drug to this phenotype results in an increased frequency of mutation (De Vita, 1983; Carter, 1984; Riordan and Ling, 1985). Thus resistance to adriamycin for example would spontaneously lead to resistance developing to other drugs (Merry et al, 1986; Merry et al, 1987).

From early theories of resistance to 5FU which indicate the resistance to 5FU implies defective RNA metabolism taking place in cells, to the more recent concepts of a spontaneously generated mutation resulting in resistance to 5FU, it would appear that if cells were in any way resistant to the effects of 5FU and RNA metabolism was therefore altered in such cells, inhibition of mitochondrial biogenesis may not take place as a result of 5FU treatment. If, however, RNA metabolism was altered in such cells, mitochondrial biogenesis might be less under the control of RNA metabolism than first envisaged.

Studying expression of mitochondrial enzymes as a means of indicating the effectiveness of biogenetic inhibitors is a powerful and useful technique. The approach used here expressing mitochondrial enzyme activities as a function of inhibition is not the most sensitive method. With hindsight, gel electrophoretic techniques are more sensitive, although this would have required mitochondrial isolation from cells obtained from a tissue culture source. Well documented inhibitors of biogenesis could have been selected for these studies, but would not have had much clinical relevance with particular reference to anticancer chemotherapy.

The studies described in this chapter may be summarised as follows:
R6G exerts a clear inhibitory effect upon cell growth and at specified concentrations exerts a cytotoxic effect upon WIL. Lamprene is less toxic than R6G, but is also inhibitory upon the growth of WIL. 5FU appeared to be the least effective compound against the growth of WIL.

However, it did appear to exert a cytostatic effect upon the growth of WIL. Toxicity data indicate that cells might be resistant to the effects of the drug. Continuous exposure of WIL and W256 had little effect upon the induction of mitochondrial enzymes measured, although this might be a reflection of suboptimal duration of exposure to drug or a subinhibitory concentration of 5FU. No inhibition of tumour mitochondrial biogenesis was observed in vitro.

CHAPTER 7

ENHANCEMENT OF THE CYTOTOXIC EFFECT OF ANTIMITOCHONDRIAL AGENTS ON CELLS GROWN IN GLUTAMINE AS AN ENERGY SOURCE

INTRODUCTION

Cells in culture are thought to utilise glucose as a major energy source. Under anaerobic conditions cells obtained limited quantities of ATP from glucose. Glucose is oxidized to pyruvate and undergoes reduction to lactate by anaerobic glycolysis with the production of ATP (Eagle, 1955; Paul, 1965; Gregg, 1972; Weinhouse, 1982). For some time glutamine was described as an essential requirement of cells in culture, although early studies emphasised its importance in the biosynthesis of non-essential amino acids and incorporation into protein (Eagle, 1959; Patterson, 1972). More recent studies have indicated that if cells are provided with glutamine, ATP might be derived from mitochondrial activity. Studies with ^{14}C -labelled glucose and glutamine have shown that oxidation of glutamine provides a major source of carbon for energy production in a variety of cells. (Zielke *et al*, 1976; Donnelly and Scheffler, 1976; Pardridge *et al*, 1978; Reitzer *et al*, 1979; Wice *et al*, 1980; Moreadith and Lehninger, 1984; Patel *et al*, 1984; Zielke *et al*, 1984).

In normal intact tissues as well as cultured cells, glutamine entry into the TCA cycle is achieved through formation of α -ketoglutarate (Meister 1965). Recently it has been postulated that in tumour cells glutamine serves as a source of pyruvate. Glutamine is converted to α -ketoglutarate and via the TCA cycle forms Malate. Malate serves as a source of pyruvate which is then oxidized to acetyl CoA by the multienzyme pyruvate dehydrogenase complex. The acetyl CoA formed serves as the main carbon skeleton for subsequent oxidation. The participation of the mitochondrial Malic enzymes, in this process have been emphasised. In particular the NAD(P)^+ dependent Malic enzyme [Malate Dehydrogenase (oxaloacetate-decarboxylating) (NADP^+) EC

1.1.1.40], which is absent or low in activity in normal mitochondria has been identified in both hepatomas and mouse ascites [Sauer and Dauchy, 1978; Sauer et al, 1980]. Pyruvate formed by this enzyme is thought to stay within the mitochondria, thus the whole process is thought to occur within this organelle. However, Moreadith and Lehninger [1984] claim that the oxidation of glutamine by tumour mitochondria is accompanied by metabolic interactions with cytosolic Malate and/or pyruvate.

Oxidative breakdown of glutamine by cells in culture cannot proceed without molecular oxygen as the final electron acceptor. Under culture conditions, it was thought that cell metabolism was purely anaerobic. However, it has been demonstrated that oxygen is a necessary requisite for cell survival in culture (Brosemar and Rutter, 1961; Paul, 1965; Thomas, 1986). From estimates of the oxygen utilisation of Hela cells and the rate of depletion of glutamine by cultured Hela cells, a minimum of 9ATP molecules per molecule of glutamine utilised are thought to be generated (Reitzer et al, 1979).

In chapter 5, experimental evidence was presented which showed that R6G inhibited tumour cell mitochondrial function in vitro. The aim of the studies described in this chapter was to examine possible mechanisms by which the effects of R6G could be improved. Cells made dependent on mitochondrial energy metabolism show increased sensitivity to antimitochondrial agents (Patel et al, 1984). Thus the cytotoxic action of R6G might be enhanced in cells grown in glutamine as an energy source, since cellular ATP could be maintained only in the presence of functional mitochondria.

The cytotoxic action of R6G was investigated in tumour cells grown in medium containing either glucose and glutamine or glutamine alone as the energy source. Oligomycin, a specific inhibitor of the mitochondrial ATP synthase, was also examined in this system. Considerable evidence exists to suggest how oligomycin is thought to interact with a component of the ATP synthase. The oligomycin sensitive protein is a region of the enzyme complex thought to bridge the proton translocating element to the main ATP synthetase component,

the F_1 complex. Inhibition with oligomycin is therefore thought to stop energy transduction as proton translocation is uncoupled from ATP synthesis (Shaw, 1967; Senior, 1979; Prebble, 1981). The cytotoxic action of oligomycin upon tumour cells grown in medium containing glutamine as the energy source should confirm whether or not the approach is suitable for application to screening antimitochondrial agents as described.

Initially suitable media were selected for use in these studies. Once these were established, the toxicity of antimitochondrial agents was assessed in cells grown either in glucose and glutamine or glutamine alone. The tissue culture media selected for these experiments were developed from Patel et al (1984).

METHODS

Growth Media

Growth media for cells were as follows:-

1. Hams F10 : DMEM (50 : 50) supplemented with FCS (10%) and glutamine (2mM). This medium contained approximately 15mM glucose.
2. Minimal Essential Medium (MEM) with Hanks balanced salts solution (HBSS) (Flow Laboratories, Irvine) supplemented with FCS (10%) and glutamine (2mM). The medium contained approximately 5.5mM glucose.
3. MEM with glucose-free HBSS (Flow Laboratories, Irvine) supplemented with FCS (10%) and glutamine (2mM).
4. MEM with glucose-free HBSS supplemented with glutamine (2mM), and dialysed FCS (10%). 200ml serum was dialysed against 1 litre of physiological saline (NaCl, 0.9%) for 6 to 8 hours at 4°C. The saline was replaced with fresh NaCl four times during dialysis. Serum was sterilised by filtration (Sterivex G, 0.22um, Millipore, Harrow, Middlesex).

Estimation of growth rates

The effect of serum dialysis upon the growth of WIL was determined as follows. Cells were seeded into 25cm² flasks (Falcon, Becton Dickinson, Cowley, Oxford) at an initial concentration of 7×10^4 cells per flask (1.4×10^4 cells per ml). Cells were seeded and fed with either medium 2, 3 or 4. After allowing cells to attach and initiate growth for 2 days, cells were fed every 48 hours. Flasks were observed under phase contrast for growth characteristics. Estimation of growth rates was carried out as described in chapter 6.

Determination of the glucose content of the growth media

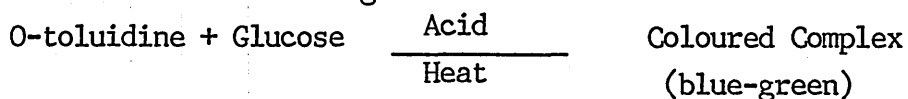
WIL was seeded into flasks (25cm²) at a concentration of 1.4×10^4 cells per ml. Cells were fed at 48 hour intervals with either medium

1, 2 or 3. Once the cells demonstrated exponential growth, they were fed and samples (0.2ml) of the medium were removed at intervals over the following 48 hour period. The samples were stored at -20°C prior to determination of the glucose content.

Estimation of the glucose content of growth media

Glucose was estimated by the Sigma-toluidine method (Hultman, 1959) with modifications by Hyvarinen and Nikkila (1962). A kit for assaying glucose by this method was purchased from Sigma, Poole, Dorset.

Sigma-toluidine reacts with glucose as follows:



The intensity of the coloured complex was measured with a spectrophotometer at a wavelength of 635nm. The reaction of glucose with toluidine is stoichiometric and the amount of coloured complex formed is directly related to the glucose concentration in the sample.

Assay Method

Reagents for the assay are as follows:

A. Sigma-toluidine solution 6% (v:v) Sigma-toluidine in glacial acetic acid with thiourea added as a stabiliser.

Reagent was stored at room temperature.

B. Glucose stock solution. Glucose, 10g per litre (55.5mM) in distilled water with benzoic acid added as preservative.

A standard curve was constructed with appropriate serial dilutions of solution B (glucose, 0 to 13.75 mM). The assay procedure was as follows. Sample or standard, 0.1ml was placed in pyrex test tubes, in duplicate. To each was added solution A (5ml). Tubes were agitated by vortex mixing. All tubes were placed in a boiling water bath for 10 minutes. Tubes were removed and cooled to room temperature by placing in tapwater for approximately 3 minutes. Tube contents were transferred to glass cuvettes and absorbance read at 635nm in a

spectrophotometer. Readings were stable for 30 minutes. Appropriate controls were set up to ensure non-interference of phenol red and other components in samples with the assay. A linear relationship between absorbance at 635nm and glucose concentration was obtained (figure 7.1). The line of best fit was determined by regression analysis. The glucose concentration of unknowns were obtained for each sample time point in different media. Results, expressed as mM glucose concentration, were plotted against time.

Assay of Cytotoxicity

Cytotoxicity was determined by an assay based on the reduction of a tetrazolium dye, MTT, as described in chapter 2 (page 31).

Uptake of Rhodamine 6G into Cells

Cells were plated onto petri dishes at a concentration of 1×10^4 cells per ml of either medium 1, 2 or 3. Cells were allowed to attach and grow for 96 hours. Cells were then fed with fresh medium containing R6G (20uM). At intervals of 1, 10, 30 and 60 minutes medium was removed from cells and dishes were rinsed with PBS. A coverslip was placed over cells and they were observed by fluorescence microscopy (Polyvar microscope, Reichert Jung, Vienna, Austria). An excitation wavelength of 546nm was selected to observe R6G autofluorescence.

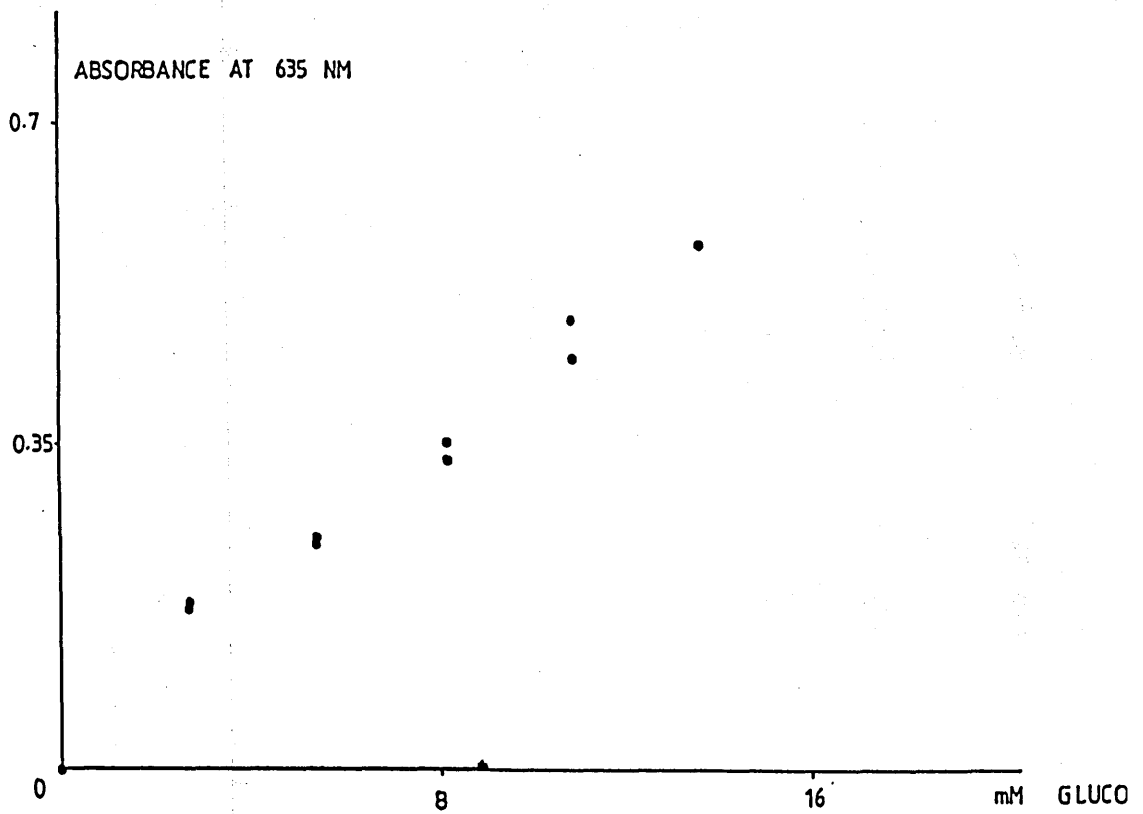


Figure 7.1: Absorbance at 635nm expressed against mM glucose concentration for a colorimetric method for assaying glucose concentration. A linear relationship between absorbance at 635nm and glucose concentration is obtained for the Sigma-toluidine method. The regression line fits the equation $y = 0.039x + 0.0322$ where the correlation coefficient $r = 0.994$.

Plate 7.1

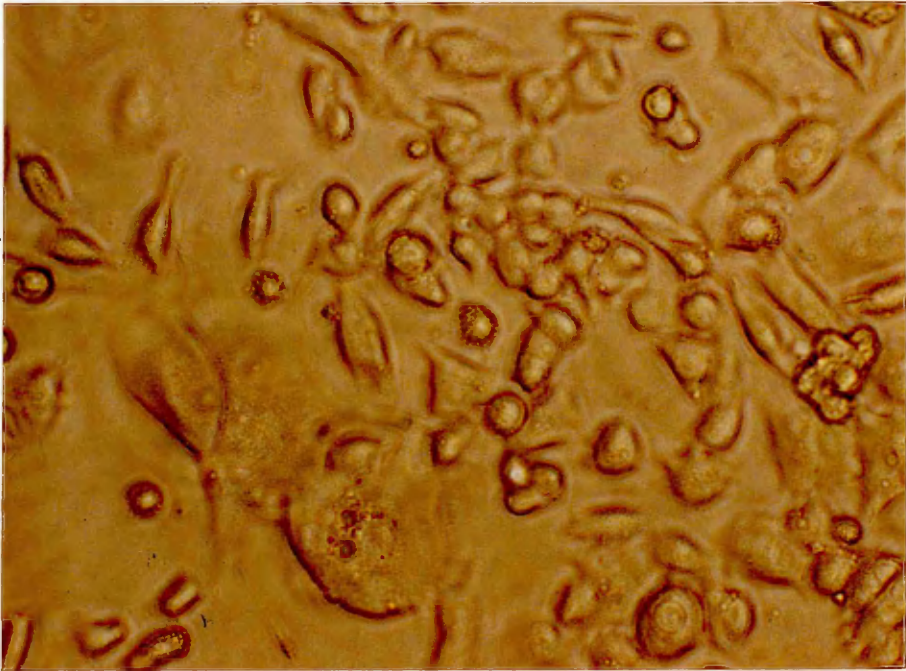


Plate 7.2

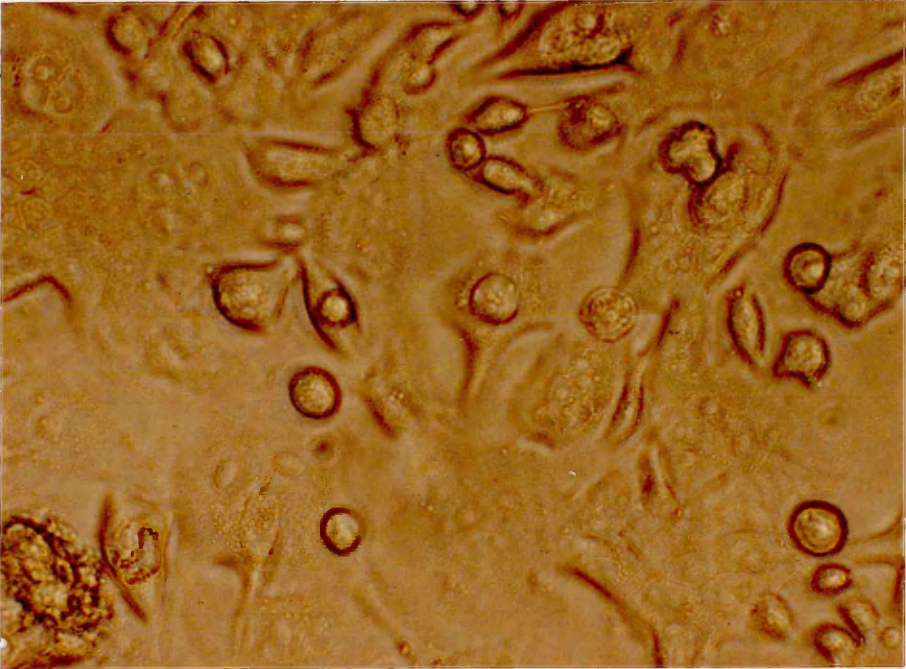


Plate 7.3



- Plate 7.1:** WIL, cells after 8 days in culture in MEM containing glucose and glutamine supplemented with undialysed FCS (10%). (X66 magnification).
- Plate 7.2:** WIL, cells after 8 days in culture in MEM containing glutamine supplemented with undialysed FCS (10%). (X66 magnification).
- Plate 7.3:** WIL, also after 8 days in culture in MEM containing glutamine supplemented with dialysed FCS (10%). (X66 magnification).

RESULTS

Effect of dialysed serum upon the growth of WIL in MEM containing glutamine

Plates 7.1, 7.2 and 7.3 show the appearance of cultures of WIL 8 days after seeding in various media. Growth and attachment of cells is observed in cells incubated with MEM containing glucose and glutamine or glutamine alone provided undialysed FCS (10%) is present (Plates 7.1 and 7.2). Cells incubated in medium containing dialysed serum showed very limited growth (Plate 7.3).

Growth of WIL medium containing either glucose and glutamine or glutamine

The growth of WIL in MEM containing either glucose and glutamine or glutamine alone is shown in figure 7.2. There is a slight increase in the growth rate of cells in glutamine compared to cells grown in medium containing both glucose and glutamine. Cells grown in glutamine reach saturation density in 6 to 7 days compared to 10 days in the presence of glucose.

Glucose content of the culture medium

Figure 7.3 shows the glucose content of the growth medium sampled at various times after addition to the cells. Initially the glucose concentration in F10:DMEM was about 15mM, the concentration decreased to 10mM, 48 hours after incubation with cells. The glucose concentration in MEM containing glucose and glutamine decreased from 6mM to 3.5mM over the same period. The glucose concentration in medium containing glutamine alone was negligible over the 48 hour period. Plates 7.4, 7.5 and 7.6 show cells in the respective media at the end of the incubation period. There was no significant difference in the appearance of the cells grown in MEM containing glucose and glutamine or glutamine. The cells grown in F10:DMEM did however appear to exhibit a higher cell density after 48 hours growth.

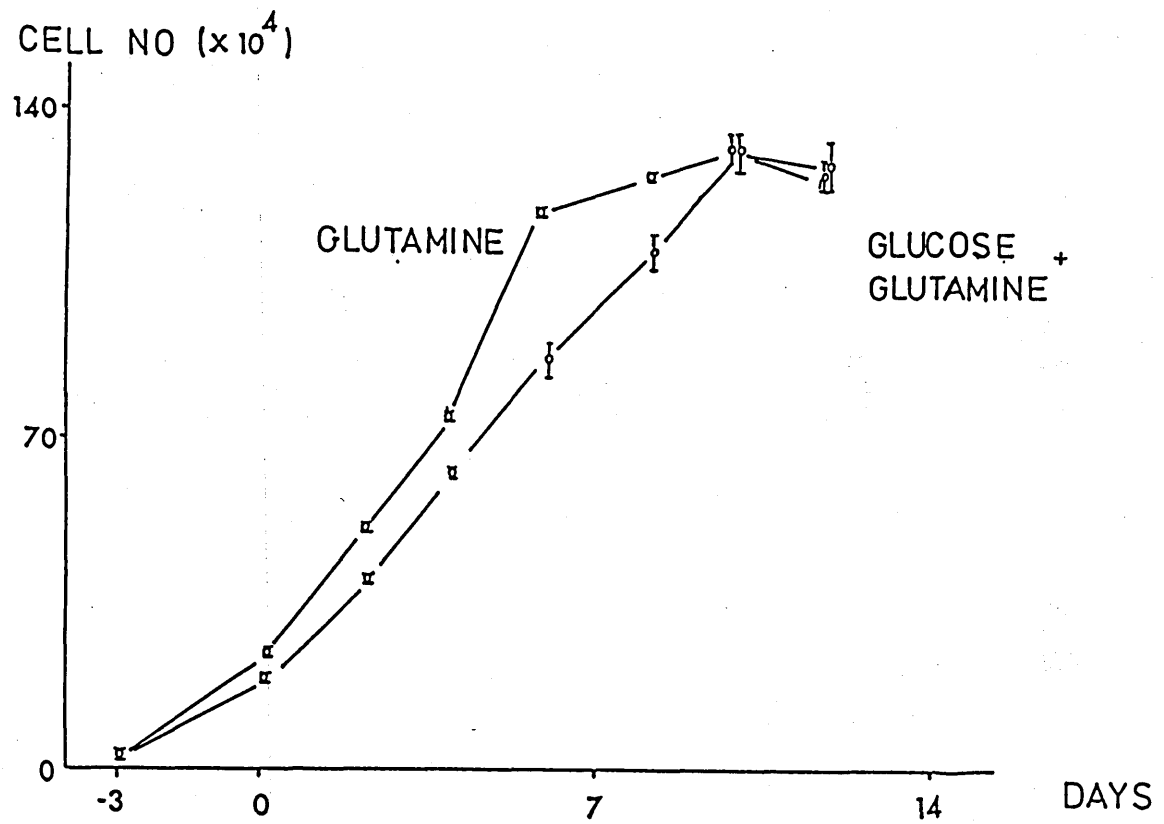


Figure 7.2: The growth of WIL in medium containing glucose and glutamine or glutamine alone. Results expressed as cell number per well against time in days. Cells were seeded at day - 3 and cell counts taken at 2 days intervals from day 0 onward.

Values are mean \pm SEM (n = 3).

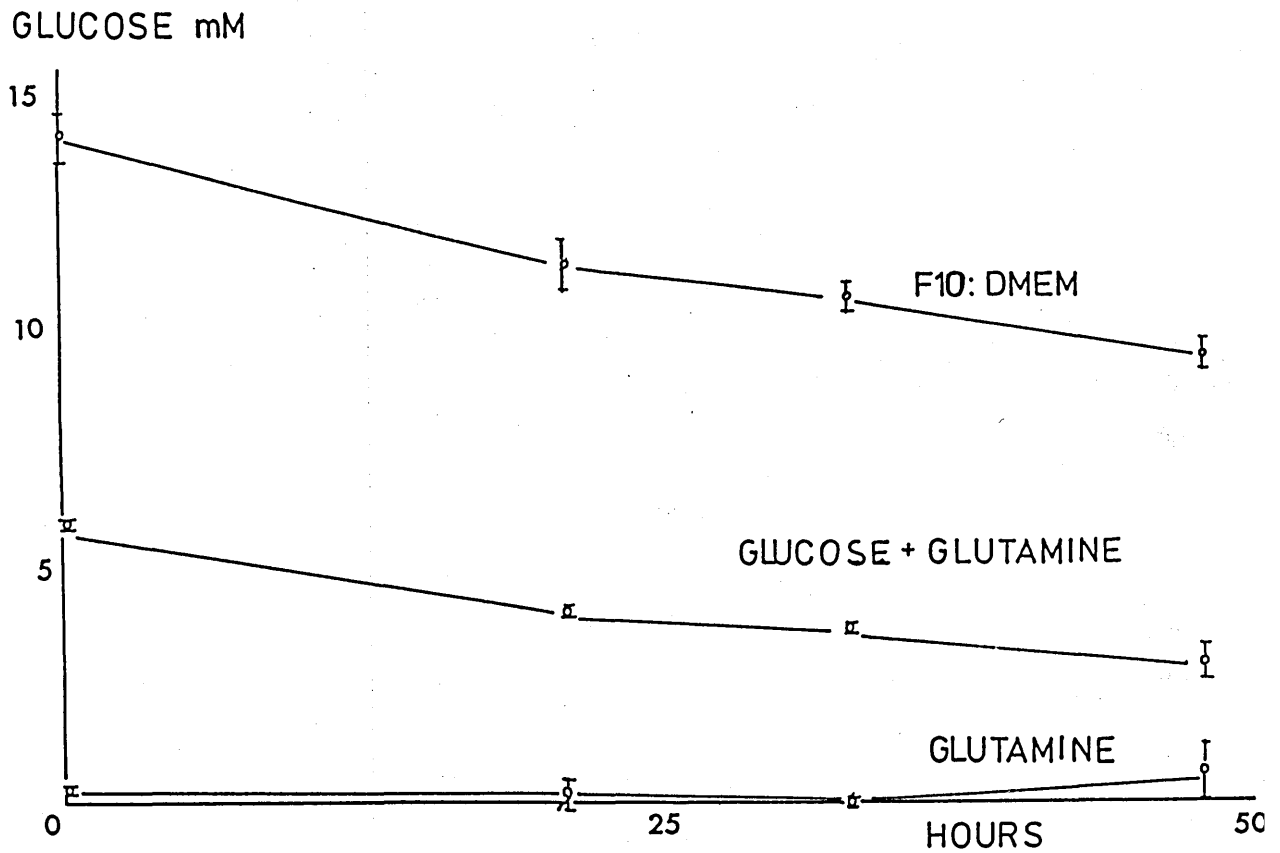


Figure 7.3: Glucose concentration of medium incubated with WIL over a 48 hour period.

Results expressed as mM glucose against time in hours. Media incubated with cells included F10:DMEM, MEM containing glucose and glutamine and MEM containing glutamine. Cells were fed at 0 hours and 48 hours.

Plate 7.4

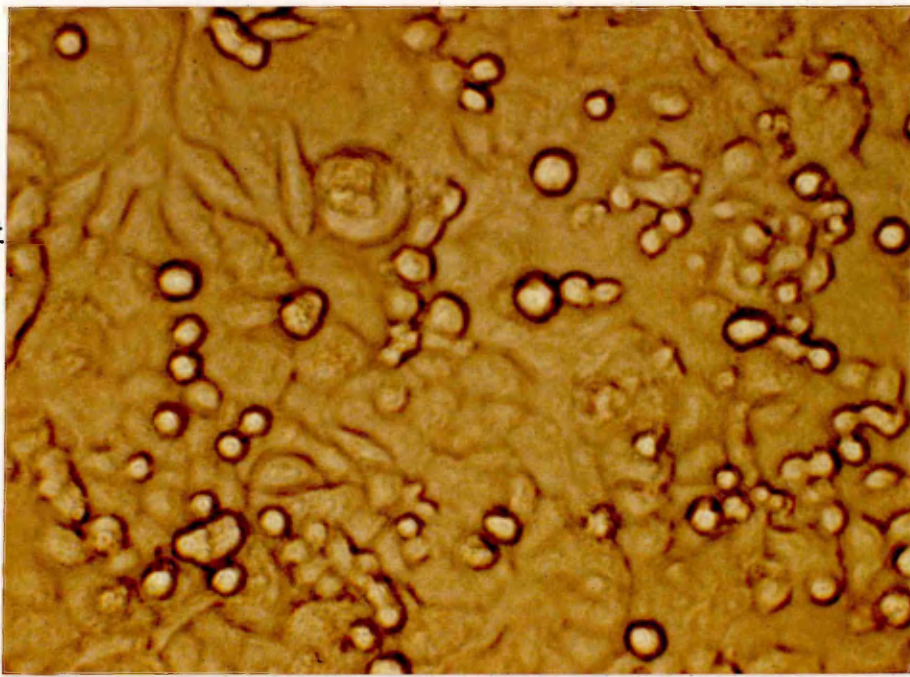


Plate 7.5

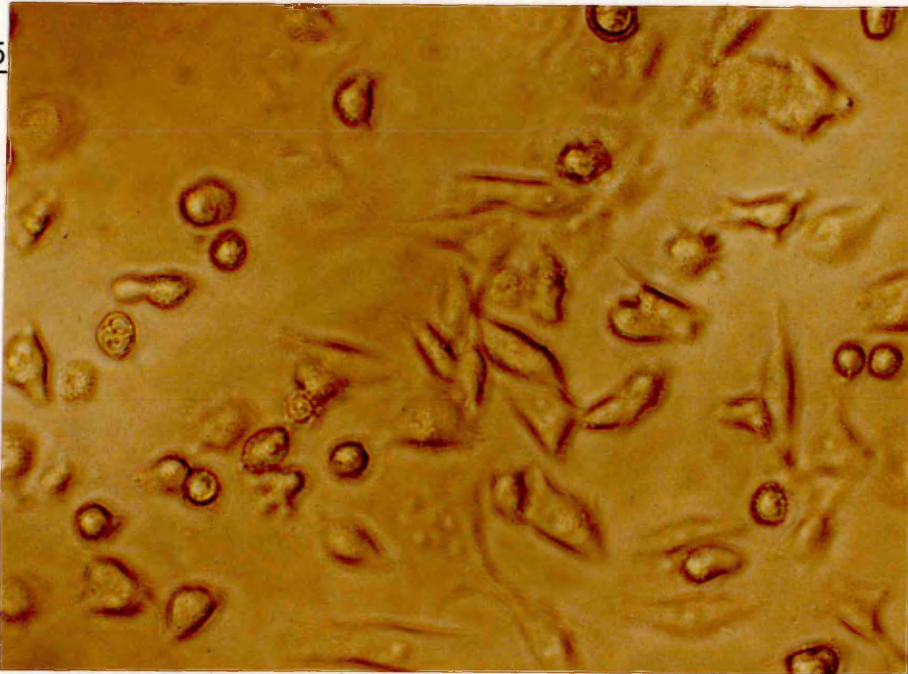


Plate 7.6

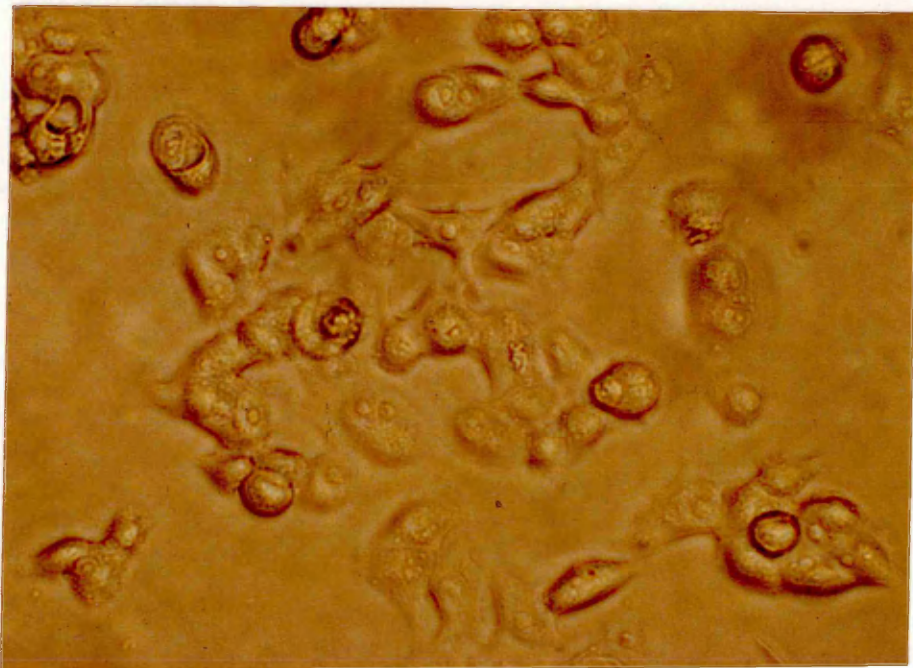


Plate 7.4: WIL, cells after 8 days in culture in F10:DMEM.
(X33 magnification).

Plate 7.5: WIL, cells after 8 days in culture in MEM containing both
glutamine and glucose. (X33 magnification).

Plate 7.6: WIL, also after 8 days in culture in MEM containing
glutamine. (X33 magnification).

Effect of Rhodamine 6G on the growth of WIL in medium
Containing either glutamine or glucose and glutamine

The effect of a 24 hour exposure to R6G (1, 10 or 50 μ M) upon the growth rate of WIL in medium containing glucose and glutamine is shown in Fig. 7.4. R6G caused a significant inhibition of cell proliferation. However, after a lag phase cell proliferation was observed with a R6G concentration of 1 μ M. In contrast, no recovery was observed with R6G at a concentration of 10 or 50 μ M. The effect of a 24 hour exposure of WIL to R6G (1, 10 or 50 μ M) in medium containing glutamine is shown in Fig. 7.5. Again R6G caused a significant inhibition of cell proliferation. After a lag phase, cell growth was observed with R6G concentrations of both 1 and 10 μ M. No recovery was observed at a R6G concentration of 50 μ M.

Cytotoxic action of R6G and Oligomycin

Table 7.1 shows the ID₅₀ of R6G and oligomycin for cells grown either in medium containing glutamine or glucose and glutamine. For R6G the ID₅₀ is similar regardless of the medium used. However, for oligomycin there is a 9-fold reduction in the ID₅₀ in medium containing glutamine when compared to that in medium containing glucose and glutamine.

Effect of Medium Composition on Rhodamine 6G uptake

Plate 7.7, shows the distribution of R6G 1 minute after addition to cells. Cells were grown in either F10:DMEM, MEM containing glutamine only or MEM containing glucose and glutamine. There appeared to be no difference in the distribution of dye in cells in any of the three media.

Plate 7.8 shows the distribution of R6G 30 minutes after addition to cells. Cells were grown in F10:DMEM, MEM containing glutamine or MEM containing glucose and glutamine respectively. There was no difference in the distribution of dye in cells in any of the three growth media. However, compared to the drug distribution in plate 7.7, fluorescence was much greater in the cytoplasm and appeared to be less specific in intracellular distribution.

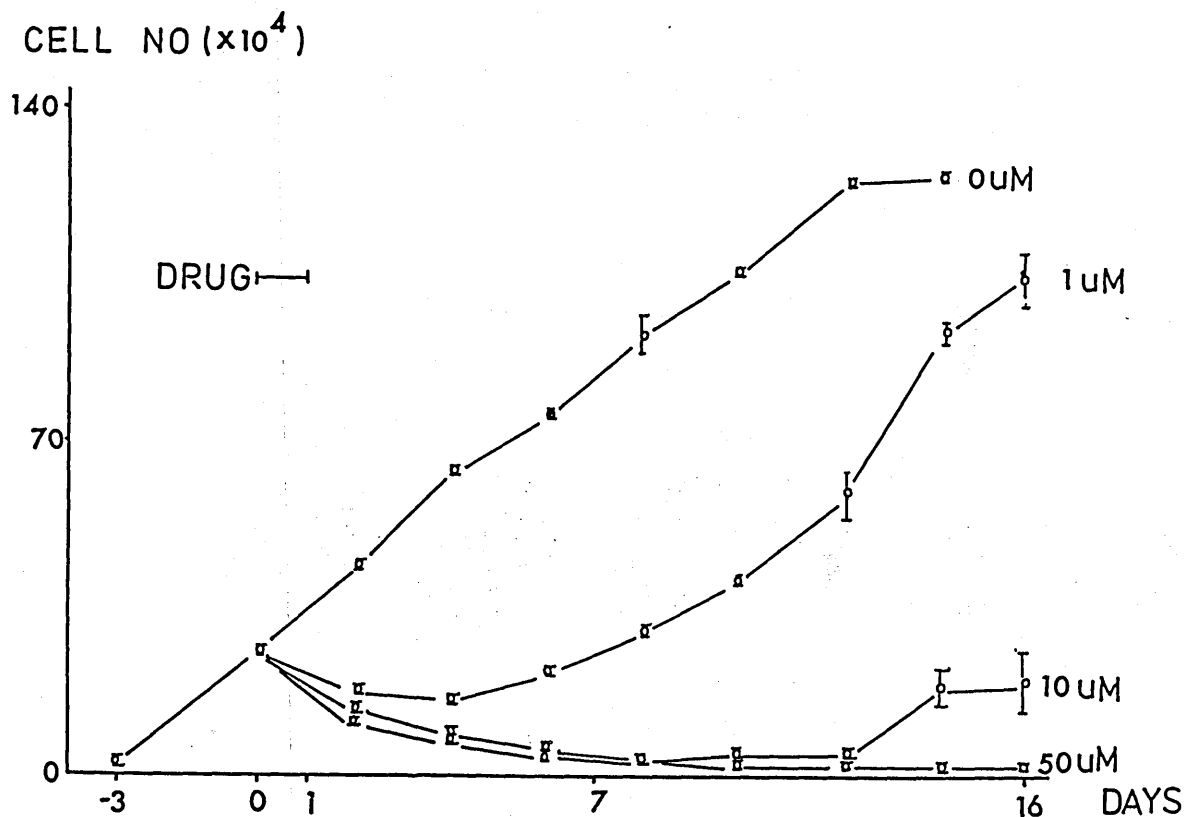


Figure 7.4: Growth of WIL in glucose and glutamine following exposure of cells to R6G for 24 hours.

Results are expressed as cell number per well against time in days. Cells were seeded at day -3 and cell counts taken at 2 day intervals from day 0 onward. Values are mean \pm SEM (n = 3).

Drug incubation (1uM, 10uM, 50uM) was carried out on day 0. Recovery was measured over a 14 to 16 day period.

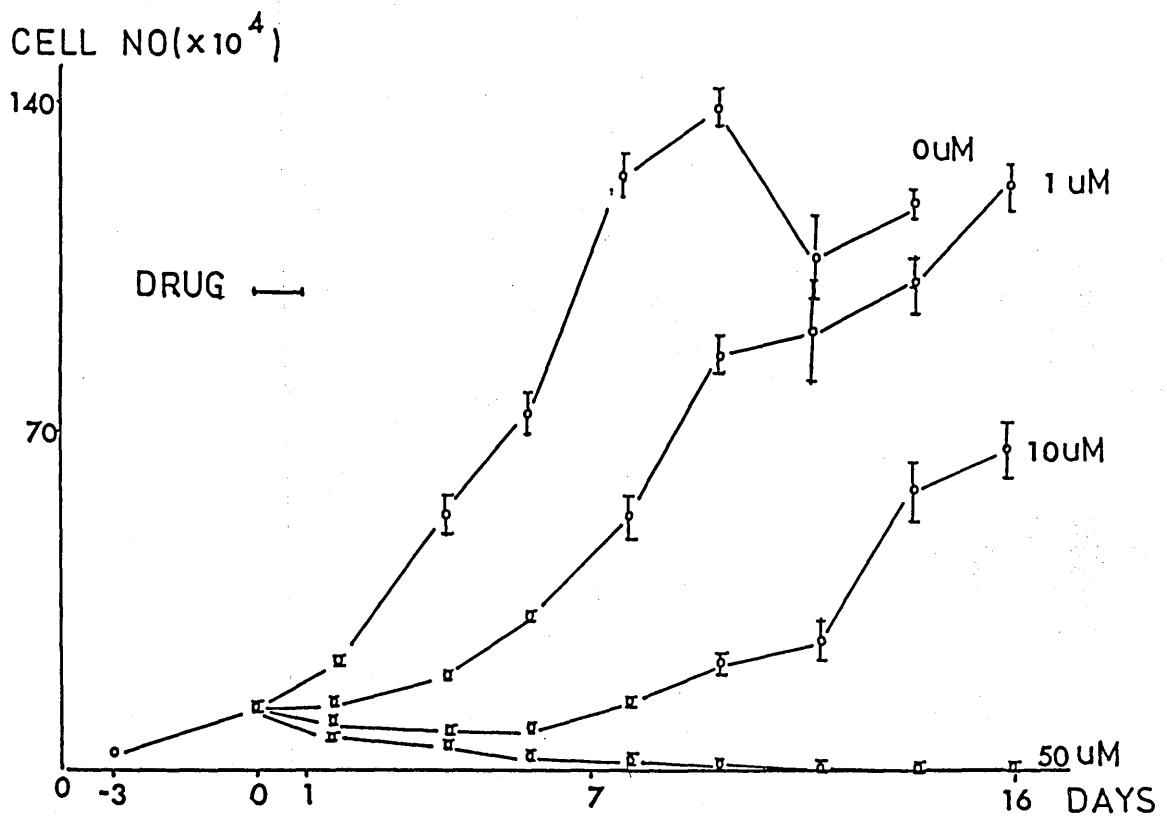


Figure 7.5: Growth of WIL in glutamine alone following exposure of cells to R6G for 24 hours.

Results expressed as cell number per well against time in days. Cells were seeded at day -3 and cell counts taken at 2 day intervals from day 0 onward. Values are mean \pm SEM (n = 3). Drug incubation (1uM, 10uM, 50uM) was carried out on day 0.

Recovery was measured over a 14 to 16 day period.

DRUG	ID ₅₀ (M)			
	MEM		MEM	
	GLUCOSE + GLUTAMINE	N	GLUTAMINE	N
Rhodamine 6G	1.05 ± 0.46 x 10 ⁻⁶	4	1.06 ± 0.5 x 10 ⁻⁶	4
Oligomycin	1.55 x 10 ⁻⁸	2	1.72 x 10 ⁻⁹	2

Table 7.1: The amount of drug required to kill 50% of the cells (ID₅₀) for each growth medium. Values are mean ± SEM.

Plate 7.7

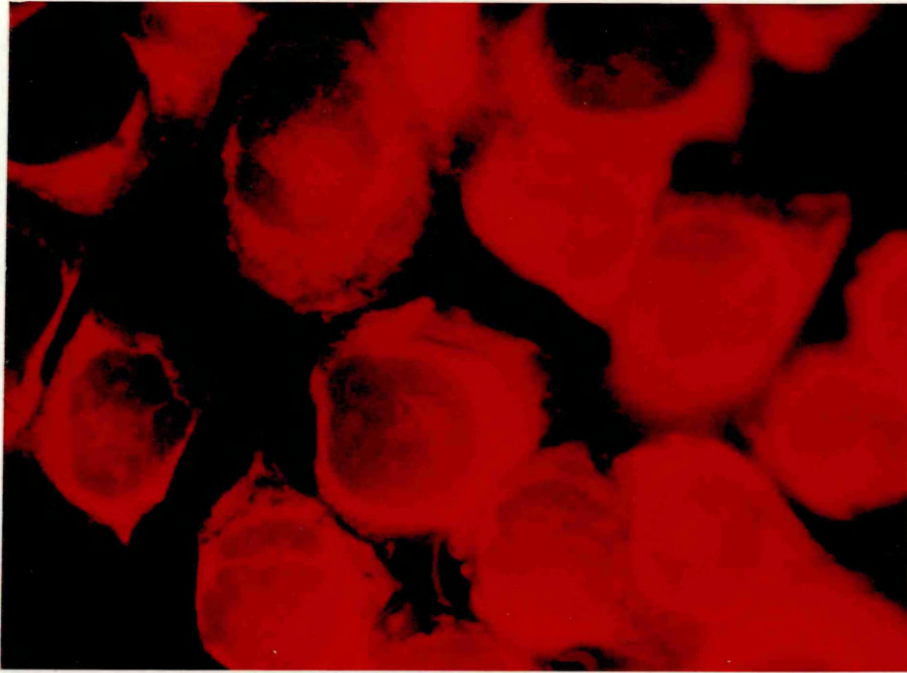


Plate 7.8

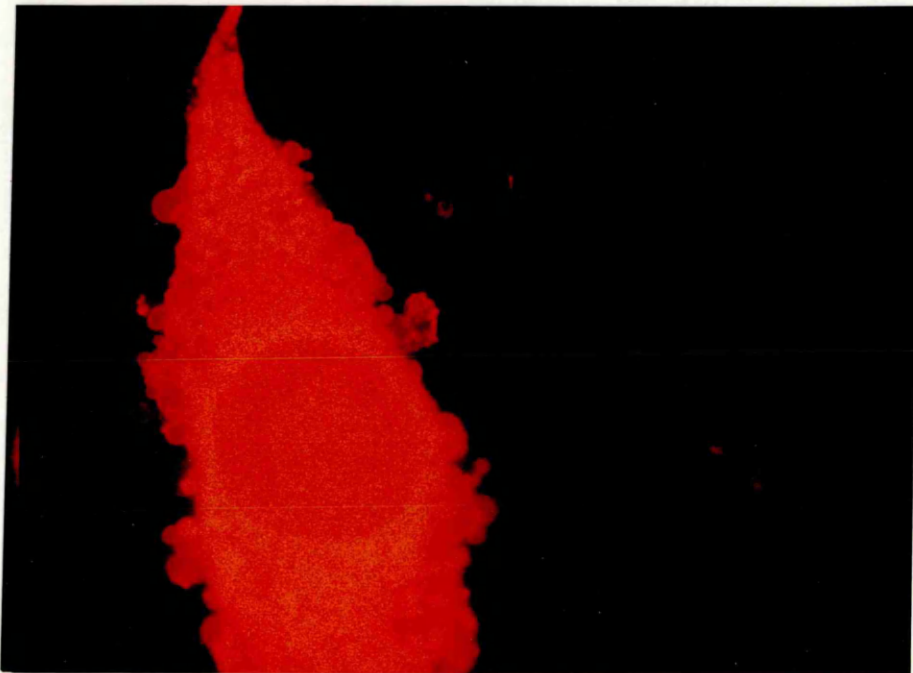


Plate 7.7: WIL grown in F10:DMEM following exposure to R6G, 20uM after 1 minute. (X225 magnification).
Fluorescence Excitation wavelength = 546nm.

Plate 7.8: WIL grown in MEM containing glucose and glutamine following exposure to R6G, 20uM after 30 minutes (X225 magnification).
Photographic conditions as for plate 7.7.

DISCUSSION

The results of experiments described in this chapter show that the human non-small cell lung cancer line WIL can maintain growth and proliferate when glutamine is supplied as a primary energy source in place of glucose. R6G inhibited cell proliferation in media containing either glucose and glutamine or glutamine. Growth in glucose-free medium allowed recovery from a higher concentration of R6G than was observed in medium containing glucose. From assays of cytotoxicity, ID₅₀ values obtained with R6G were identical for cells grown either in glucose and glutamine or glutamine alone. Thus there appeared to be no enhancement of the cytotoxic action of R6G in cells dependent on glutamine. Clearly, alteration of cellular energy metabolism has no effect upon the sensitivity of cells to R6G. The increased recovery from R6G (10 μ M) of cells grown in glutamine alone as an energy source may reflect the more rapid growth of cells in this medium. Alternatively cells grown in glutamine may exhibit alterations in their handling of R6G. However, studies which examined the uptake of R6G in media containing either glutamine or glucose and glutamine did not show any difference in the uptake or handling of R6G as determined by fluorescence microscopy.

Results with the classic mitochondrial inhibitor oligomycin showed a decrease in the ID₅₀ from 15.5nM to 1.7nM. Thus a 9-fold enhancement of cytotoxicity to oligomycin in cells grown in glutamine was observed. These results suggest that when cells are dependent upon their mitochondria for an energy supply, agents such as oligomycin are indeed more cytotoxic. However, this was not the case for R6G. This supports the observations (discussed in chapter 5) that R6G is not simply an antimitochondrial agent, but may have other sites of action which are responsible for cytotoxicity.

Rhodamine 1, 2, 3 an analogue of R6G has been described as exerting its cytotoxic action by some mechanism other than by inhibition of mitochondrial function, possibly inhibiting mitochondrial protein synthesis (Abou-Khalil *et al*, 1986). More recently R6G has been shown to exhibit inhibitory activity upon protein phosphorylation in rat

brain extracts (O'Brian and Weinstein, 1987). Therefore, although R6G does have antimitochondrial activity in vitro, this may be only one of its cytotoxic actions.

An essential requirement for studies of this nature is the adequate removal of glucose from cells. The use of dialysis to remove glucose from serum in the culture media resulted in an inability of the medium to support cell growth. This may have been due to loss of essential small molecules such as growth factors, as well as glucose during dialysis. The glucose content of the glutamine medium containing undialysed FCS did appear to be negligible. However, glucose requirements for cells in culture can be as little as 0.8 to 0.3mM (Graff, et al, 1965; Racker, 1965). The technique selected for determination of glucose content of the culture medium was a fairly insensitive method. However, this method was chosen rather than the more specific and sensitive glucose oxidase based assays capable of detecting nanomole quantities of glucose in serum. The method selected is not specific and detects other α -D-glucopyranose molecules such as fructose, galactose or mannose which are favoured glycolytic substrates for cells in culture, and would also have been detected if present in large enough concentrations (Cristofalo and Kritchevsky, 1965; Reitzer, et al, 1979). It is known that tumour cells in culture have access to glycogen stores, although the amount of stored glycogen in cells is rapidly depleted if the glucose concentration of the medium is reduced (Wu, 1959; Paul, 1965; Racker, 1965). It is not clear whether glucose is primarily a glycolytic substrate as there is evidence to suggest that glucose derived pyruvate may fuel significant mitochondrial metabolism (Pardridge, et al, 1978). If glutamine is more important as an energy substrate, the residual glucose present in tissue culture medium as a result of supplementing media with undialysed serum may be of less importance as an energy source.

In order to confirm that cells are more sensitive to antimitochondrial agents when glutamine is supplied as the major carbon source, glutamine must be shown to be metabolised by mitochondrial pathways. Other workers have indicated that up to 60% of glutamine is utilised by mitochondrial oxidation. However, 10 to 20% of the glutamine

supplied, may undergo transamination to generate pyruvate for lactate fermentation. Primary metabolism is therefore mitochondrial, but significant amounts of ATP may be derived from non-mitochondrial utilisation (Windmueller and Spaeth, 1974; 1975; Reitzer, et al, 1979; McKeehan, 1982). Lactate production through oxidation of glutamine is not necessarily indicative of glycolysis however. Some workers have stated that lactate production may be a way of generating acetyl CoA for auto-oxidation via the TCA Cycle (Zielke, et al, 1980). Others have suggested that glutamine oxidation may merely serve as a stimulus for glycolytic cleavage of glucose when supplied exogenously (Lanks, 1986). More recently it has been suggested that glutamine utilisation is primarily required for end product biosynthesis of amino acids or TCA cycle intermediates, with energy production as a secondary feature. Thus the TCA cycle is essentially used as a linear pathway to generate aspartate or citrate for example, from glutamine carbon, though some ATP is generated through oxidative phosphorylation (Lanks, 1987). An assessment of lactate production and ammonia production would have been useful in confirming to what extent mitochondrial utilisation of glutamine had occurred. There are other substrates present in tissue culture medium which may have been utilised as a carbon source, such as branched chain amino acids or free fatty acids. The extent to which these substrates are utilised might be a feature of the cell type under study. For example, mouse macrophages may utilise some of these substrates as a carbon source (Newsholme, et al, 1987; Thomas, 1986).

In order to maintain cells in glutamine, cells had to be fed at not more than 48 hour intervals, although the protocol for some experiments necessitated a 24 hour period between feeding. This might have reflected the lability of glutamine which is known to be unstable in culture medium at 37°C, although some workers claim that the compound is stable for 5 to 6 days. (Tritsch and Moore, 1962; Griffiths and Pirt, 1967). Alternatively it is possible that rapid utilisation of glutamine or a build up of toxic ammonia might have inhibited growth in flasks where feeding periods exceeded 48 hours. It has been recognised that cells in culture have a carbohydrate requirement for utilisation in the pentose phosphate pathway,

essential for the manufacture of deoxyribose and thus nucleic acid synthesis (Reitzer, et al, 1979; Zielke, et al, 1984; Thomas, 1986). This requirement would only have been met by residual glucose present in serum or endogenous glucose derived from stored glycogen. Depletion would have effectively halted cell growth, though the time course within which this would have occurred is uncertain. Some hexoses formed as a result, such as Fructose-6-phosphate would have been generated as glycolytic substrates, though in very small amounts (Lehninger, 1975; Partridge, et al, 1978).

Some workers have shown that cell growth can take place in the presence of mitochondrial inhibitors and have reasoned that energy production by glycolytic means has greater significance for the cell than mitochondrial pathways (Donnelly and Scheffler, 1976; Wiseman and Attardi, 1979; Morais, 1980). Results obtained in these studies show that the increased sensitivity to oligomycin by cells grown in glutamine confirm that the energy contribution by glycolysis was limited.

Cells may have a preference for other substrates and therefore, any enhancement of cytotoxicity might reflect metabolic instability if access to a preferred substrate is denied (Donnelly and Scheffler, 1976; Morais, 1980; Newsholme, et al, 1986). Furthermore it might not necessarily be possible to show that an antimicrobial agent shows a selective cytotoxic effect over general cytotoxic agents with cells grown on non-glycolytic substrates. Any general cytotoxic agent would inhibit cellular energy metabolism by a secondary or tertiary action. However, the results obtained with oligomycin and R6G show that a general cytotoxic agent whose toxicity is not determined by a specific antimicrobial action does not show any enhanced toxic effect against cells grown in glutamine.

The experiments described in this chapter may be summarised as follows:-

A system was established to determine whether a mitochondrial energy source made cells more sensitive to antimicrobial agents. Cells were able to grow in culture medium containing glutamine as the major

energy source. This medium was shown to be essentially free of glucose. An enhanced sensitivity to the specific antimitochondrial agent oligomycin was observed with cells grown in glutamine as an energy source. R6G did not appear to show an enhanced cytotoxic action upon cells grown in glutamine. Although this compound has antimitochondrial activity, this may not be its major cytotoxic property.

CHAPTER 8

SELECTIVE MANIPULATION OF TUMOUR CELL ENERGY METABOLISM: STUDIES IN VIVO

INTRODUCTION

The studies described in this chapter were aimed at evaluating how effective novel combination chemotherapies directed at tumour cell energy metabolism would be in retarding growth of tumours which were resistant to conventional therapy in vivo.

Studies carried out with fluorescent cationic dyes such as R123 indicate that rhodamine has a highly specific affinity for tumour cells in vitro. Uptake of R123 appeared to be specific for neoplastic and transformed cell lines and resulted in selective toxicity toward tumour cells at concentrations which were non toxic to non-neoplastic cells (Bernal et al, 1982(b); Lampidis et al, 1983; Nadakavukaren et al, 1985). R123 and an analogue R6G were observed to have a highly specific affinity for mitochondria and thus selective toxicity was attributed to inhibition of tumour cell bioenergetic function in vitro (Gear, 1974; Higuti et al, 1980; Davis et al, 1985; Modica-Napolitano and Aprille, 1987). Experiments described in chapter 4 confirmed that R6G does exert an inhibitory action upon tumour cell respiration, hence energy metabolism in vitro at concentrations which appear not to alter respiratory activity in normal cells.

R123 when given in combination with 2-deoxyglucose, an inhibitor of glycolysis, was effective in increasing the survival times of mice bearing Ehrlich ascites tumours. This treatment however was of limited value when applied to a variety of other tumours or leukaemias (Bernal et al, 1983). 2-deoxyglucose is a non metabolisable analogue of glucose. It is thought to prevent metabolism along the glycolytic pathway at the reaction catalysed by hexokinase (E.C. 2.7.1.1) to form 2-deoxyglucose-6-phosphate from 2-deoxyglucose. Glycolytic inhibition is thought to result from competition between 2-deoxyglucose and

glucose for hexokinase. A consequence of this is that phosphoglucoisomerase (E.C. 5.3.1.9) which normally catalyses conversion of glucose-6-phosphate to fructose-6-phosphate is inhibited by 2-deoxyglucose-6-phosphate (Wick et al, 1957; Barban and Schulze, 1961). Studies with 2-deoxyglucose have had some success in reducing growth of animal tumours. It is thought that the effects upon respiration of 2-deoxyglucose are responsible for the antitumour effect (Woodward and Hudson, 1954; Ball et al, 1957).

Initial studies carried out in this Department were designed to evaluate to what extent tumour growth could be reduced with inhibitors of mitochondrial function, R123 and R6G, in combination with the antiglycolytic agent 2-deoxyglucose. Preliminary work with R123 was carried out on the Walker 256 (W256) rat carcinosarcoma. R123 injected alone intraperitoneally (I.P.) did reduce the rate of growth of the tumour. When administered in combination with the glycolytic inhibitor 2-deoxyglucose, the growth rate was reduced by more than 75% provided that the drugs were administered 24 hours after implantation. When given 48 hours after implantation no such inhibition was observed. If however, a related compound R6G was administered 48 hours after implantation, tumour growth inhibition was apparent (Fearon et al, 1985(a)). Since drug therapy was initiated immediately after inoculation of animals with tumour, it is possible that inhibition of angiogenesis rather than tumour growth might have been observed. Thus a more rigorous approach was required for these studies.

The antileprosy agent lamprene (clofazamine hydrochloride) is known to inhibit respiratory function hence energy metabolism in both yeast and isolated rat liver mitochondria (Rhodes and Wilkie, 1973). A possible role in cancer chemotherapy has been implicated for this compound following similar results obtained with transformed fibroblasts (Wilkie, 1979). Experiments described in chapter 4 confirmed that lamprene inhibited tumour cell bioenergetic function in vitro. As yet no studies have been carried out to ascertain the potential of lamprene in experimental cancer chemotherapy in vivo.

The earlier work with the W256 tumour allowed rapid accumulation of preliminary results since this tumour has a rapid growth rate. However, in order to examine whether manipulation of tumour cell energy metabolism is a valid approach to chemotherapy, a model more closely related to human tumours was required. Studies were therefore initiated to investigate a human non-small cell bronchial carcinoma, WIL which grows subcutaneously in immuno-incompetent athymic mice. This tumour has been shown to be resistant to conventional chemotherapeutic agents such as adriamycin and etoposide (Merry et al, 1986).

From previous studies with rhodamine, the compound appears to remain in tumour cells for some time (3 to 5 days) after administration both in vivo and in vitro (Summerhayes et al, 1982; Beckman Jr et al, 1987). This suggested that repeated injections were likely to exceed toxicity threshold. As described in chapters 4 and 5, lamprene required solubilisation in dimethylsulphoxide (DMSO). This suggested that insolubility might pose a major barrier to metabolic breakdown and excretion by tumour cells and thus drug accumulation was likely to occur in aqueous cell compartments over several days. 5-Fluorouracil (5FU), an antimetabolite of uracil used as a conventional anticancer agent has been shown to inhibit mitochondrial biogenesis in both lower eukaryotes and cultured mammalian cells (Oliver and Williamson, 1977; Bugeja et al, 1987). Preliminary work in this Department indicated that at a concentration well below that used in conventional chemotherapy, 5FU inhibited the growth rate of the W256 tumour by about 50%. When combined with a dose of R6G, which on its own caused a 10% reduction in tumour growth, a synergistic effect was observed, such that tumour growth was almost completely inhibited provided that rhodamine 6G was given within 8 hours of 5FU administration. 5FU is retained for up to 72 hours, throughout a variety of tissues in mice (Chadwick and Rodgers, 1972; Liss and Chadwick, 1974). Thus it would appear that all the agents selected for the study had prolonged retention times and single administration was likely to suffice for experimental purposes, at least initially.

The effectiveness of any therapy directed against solid malignancies implanted subcutaneously in athymic mice must be accurately assessed. Methods used previously are based upon estimating tumour growth from caliper measurements of the tumour and estimating tumour volume from the equation:

$$\text{Tumour Volume} = \frac{\pi}{6} \times (\text{diameter})^3 \quad (\text{Steel, 1977; Poulsen et al, 1982}).$$

This method assumes a uniform shape of the tumour and lacks precision. A method was required which was both accurate and precise. In these studies a novel method has been developed with which to estimate tumour volume. It is based upon casting moulds of each tumour in dental alginate and determining the volume of water required to fill each impression cast. Thus precise estimates of tumour volume could be made. This method was sufficiently sensitive to be able to detect small changes in growth rates.

METHODS

Animals

Batches of newly weaned female athymic mice of the MF-1 strain were obtained from commercial sources (OLAC, Bicester, Oxon) or from animals bred in sterile isolators in the CRC Department of Medical Oncology. Animals were kept in sterile cages in vertical laminar flow hoods during the course of experiments. Pre-sterilised food (Special Diet Services, Essex) and water were given to mice ad libitum. Cages, food and water were sterilised by autoclaving with steam at 120°C for 30 minutes. Groups of 32 to 40 mice, of the same age were used in each experiment. Experimental animals were implanted with WIL tumour from animals passaged routinely in the animal house.

Tumours

The tumour was the same as that used for the studies in vitro. This was the lung adenocarcinoma WIL. It was maintained by routine passage every 4 to 6 weeks in athymic nude mice. Tumour passage was carried out under sterile conditions within the laminar flow hood which housed the mice. All instruments were sterilised by autoclaving. A tumour bearing animal was killed by cervical dislocation and the tumour removed to a sterile petri-dish. Viable tumour was dissected into pieces, 2 - 3mm³. Animals for implantation were anaesthetised with ether in a sterile ether jar. Anaesthesia was maintained with a sterile cotton wool pad, soaked in ether, placed over the nose. An incision was made in the skin, 3 - 4mm long above one of the hind limbs with fine tipped scissors. The scissor tips were inserted subcutaneously and widened slightly to open the incision. With a pair of fine forceps the incision was held open and with a second pair of forceps, a piece of tumour was inserted into the wound. Once in place the skin was secured by sterile clips (Vicary Davidson, Glasgow). Animals were placed in fresh sterile cages in the laminar flow hood.

Determination of Tumour Volume

Tumour volumes were estimated from moulds of each tumour cast in dental impression material. All procedures were carried out in a laminar flow hood as aseptically as possible. Tumour bearing mice

were anaesthetised with ether. Whilst the animal was being anaesthetised, 2 to 5g of elastigel (Wrights Dental Suppliers, Dundee, Scotland) was mixed with an equal quantity of tap water and poured into a plastic bottle top (30mm diameter x 20mm height). The anaesthetised animal was held over the cast for 2 - 3 minutes, such that, the tumour formed an impression in the elastigel. Anaesthesia was maintained by a nose cone containing cotton wool steeped in ether. The animal was replaced in its cage. The set mould was removed from the cast by means of a microspatula. The number assigned to each animal by means of ear tags was recorded on a strip of card. This number was then clipped on to each mould with rocket clips (Vicary Davidson, Glasgow). Moulds were stored at 4°C for between 24 and 48 hours in plastic sandwich boxes. Storage beyond this resulted in deterioration of the cast. Tumour volume was estimated by determining the weight of water required to fill each impression.

Experimental Protocols

Mice were implanted with the tumour. After 4 to 6 weeks, when the tumour volume was about 500mm³, the mice were randomised into four groups of 8 to 10. Animals were identified by means of numbered ear tags. Tumour volumes were measured for each mouse and the total body weight recorded. Drugs were then administered as a single I.P. injection at a dose related to the body weight of each mouse. Control animals received an equal volume of phosphate buffered saline (PBS). Drugs were administered either alone or in combination. R6G was administered at a concentration well below the toxic dose as assessed from previous experiments ($\leq 2\text{mg/kg}$). A single non cytotoxic dose of 5FU (50 mg/kg⁻¹) was administered as determined by the LD₅₀ in normal mice (LD₅₀ = 50mg kg⁻¹ day⁻¹ for 10 days, Roche, Personal Communication). Lamprene was administered at a concentration that was also well below the LD₅₀ in normal mice (LD₅₀ > 4000mg kg⁻¹ Merck Index, 1983). Because of problems with solubility, lamprene was given orally. The mouse diet was ground to a fine powder, sterilised, mixed with lamprene (0.05% of the diet by weight) and moistened to a mash with sterile water. Mice were given this diet for 15 days. Tumour volumes were measured at intervals of 7 days following drug administration.

Estimation of Tumour doubling times

Growth of subcutaneously implanted tumours proceeds according to a pattern that deviates from exponential growth and is described as gompertzian (Laird, 1964; Laird, 1965; Steel, 1977; Poulsen et al, 1982). Tumour growth over the period of study however was almost linear. Tumour doubling times were therefore estimated over a 7 day period or over the duration of the experiment. Tumour doubling times were estimated as follows:-

V_t = Volume at any given time t

x = Time in days of duration of experiment

V_o = Volume in ml on day of drug administration (week 1)

V_x = Volume in ml at end of experiment (week 4).

where growth per day = $\frac{V_x - V_o}{x}$

Tumour doubling time in days = $\frac{2 \times V_t}{\text{Growth per day}}$

Statistical Analysis

The significance of any difference was determined by Students 't' test.

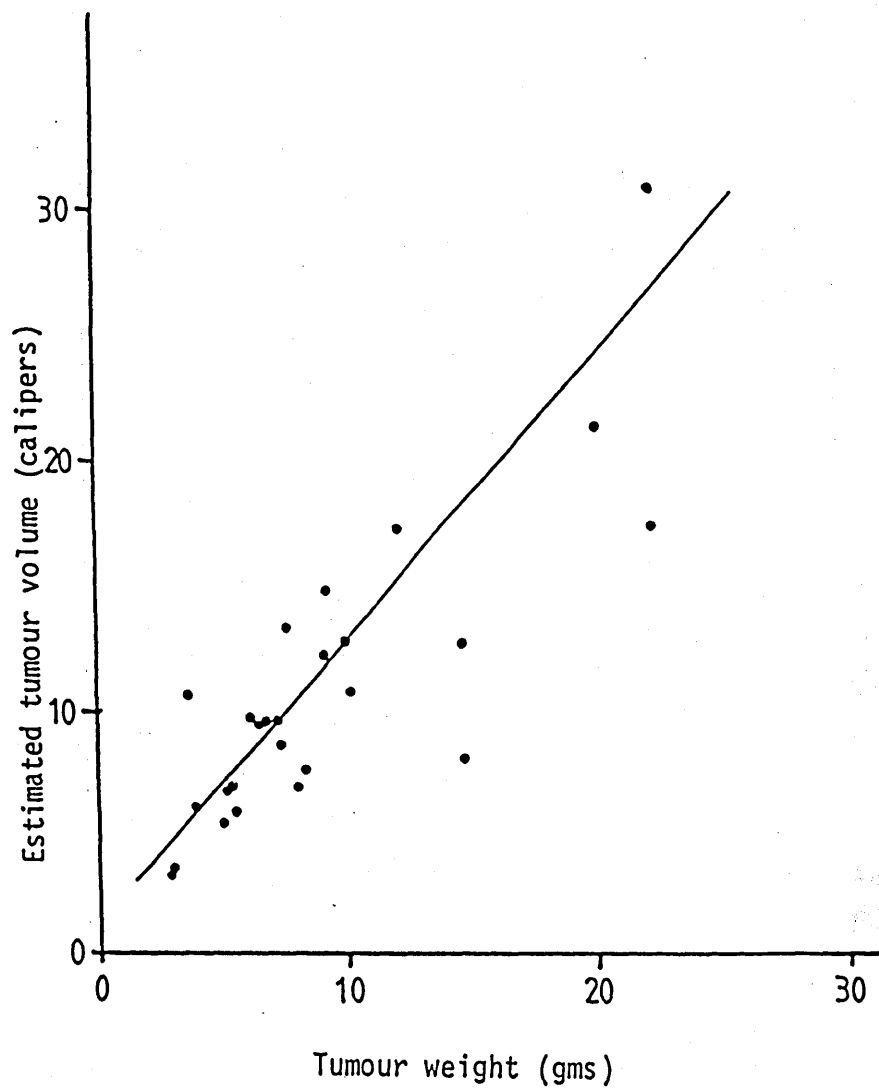


Figure 8.1: Estimated tumour volume expressed against weight of excised tumour in grams. Tumour volume in mm^3 , determined from caliper measurements. Individual determinations shown about the regression line.

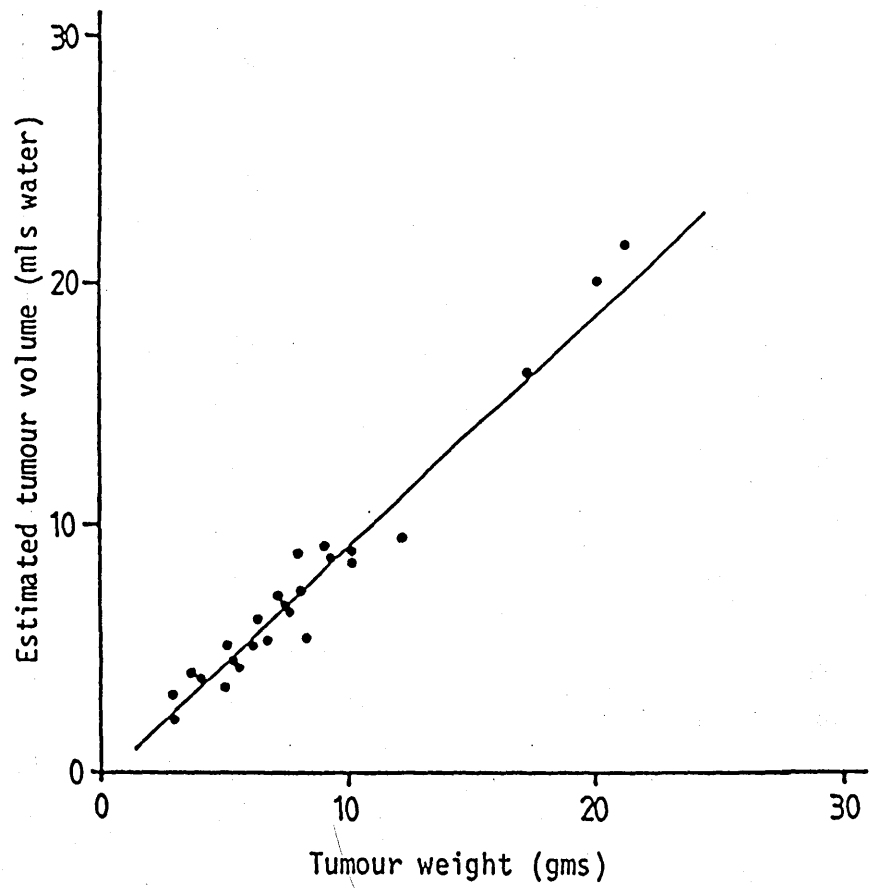


Figure 8.2: Estimated tumour volume expressed against weight of excised tumour in grams. Tumour volume in ml determined from volume of water required to fill moulds cast of tumour. Individual determinations shown about the regression line.

Treatment Dose	Time After Treatment Days	Doubling Time Days	SEM	N	
Control	17	39.1	2.9	6	
2 Deoxyglucose 0.5g/Kg	15	55.3	3.0	6	*1
Lamprene 0.05% Diet	16	61.8	3.1	6	*2
2 Deoxyglucose + Lamprene 0.5g/Kg 0.05% Diet	16	52.4	4.2	6	*3

P<0.005 *1

P<0.0005 *2

P<0.025 *3

Table 8.1: Effect of 2-deoxyglucose and lamprene, administered individually and in combination upon the estimated tumour doubling time of WIL xenografts implanted subcutaneously in athymic mice. Results are expressed as mean doubling times in days \pm SEM, where n = 6. Statistical analyses performed with unpaired t-tests.

RESULTS

Estimation of Tumour Volume

Figure 8.1 shows the relationship between tumour volume estimated from caliper measurements and the actual weight of the tumour after excision from the animal. Figure 8.2 shows the relationship obtained when tumour volume is estimated by a mould cast from the same tumours. Both estimates correlate well with the actual tumour weight. However, when tumour volume is estimated from moulds there is far less scatter about the regression line than when calipers are used.

Effect of Lamprene and 2-deoxyglucose on the growth rate of WIL

Table 8.1 shows the effect of 2-deoxyglucose and lamprene on the growth of WIL implanted subcutaneously in athymic mice. Both lamprene and 2-deoxyglucose administered alone, produced a significant increase in the tumour doubling time ($P < 0.005$; $P < 0.0005$ respectively). No synergistic effect on tumour doubling times was observed when the compounds were administered together. However, significant tumour growth delay was induced with this combination ($P < 0.025$).

Effect of R6G and 2-deoxyglucose on the growth rate of WIL

Table 8.2 shows the effects of R6G and 2-deoxyglucose on the growth of WIL implanted in nude mice. There was no increase in tumour doubling times regardless of whether the agents are given alone or in combination. Moreover it is clear that tumour doubling times for animals treated with R6G or 2-deoxyglucose in combination with R6G are significantly decreased when compared with controls ($P < 0.025$).

Effect of R6G and 5FU on the growth rate of WIL

The effect of 5FU and R6G on the growth of WIL implanted subcutaneously in athymic mice is shown in Table 8.3. Each agent when administered alone did not significantly alter tumour doubling time relative to controls. However in combination, an almost 2-fold increase in doubling time was obtained ($P < 0.05$).

Treatment Dose	Time After Treatment Days	Doubling Time Days	SEM	N	
Control	14	41.3	1.6	6	
2 Deoxyglucose 0.5g/Kg	14	41.5	2.2	6	
Rhodamine 6G 1.6mg/Kg	14	34.4	2.0	5	*
2 Deoxyglucose + Rhodamine 6G 0.5g/Kg 1.6mg/Kg	14	33.7	2.3	5	*

P<0.025 *

Table 8.2: Effect of 2-deoxyglucose and R6G, administered individually and in combination upon the estimated tumour doubling time of WIL xenografts implanted subcutaneously in athymic mice. Results are expressed as mean doubling times in days \pm SEM, where n = 5 to 6. Statistical analyses performed with unpaired t-tests.

Treatment Dose	Time After Treatment Days	Doubling Time Days	SEM	N	
Control	7	19.5	1.7	6	
Rhodamine 6G 2mg/Kg	7	23.0	2.7	7	
5 Fluorouracil 50mg/Kg	7	18.7	1.7	7	
Rhodamine 6G + 5 Fluorouracil 2mg/Kg 50mg/Kg	7	34.3	5.3	7	*

P<0.05 *

Table 8.3: Effect of R6G and 5FU, administered individually and in combination upon the estimated tumour doubling time of WIL xenografts implanted subcutaneously in athymic mice. Results are expressed as mean doubling times in days \pm SEM, where n = 6 to 7. Statistical analyses performed with unpaired t-tests.

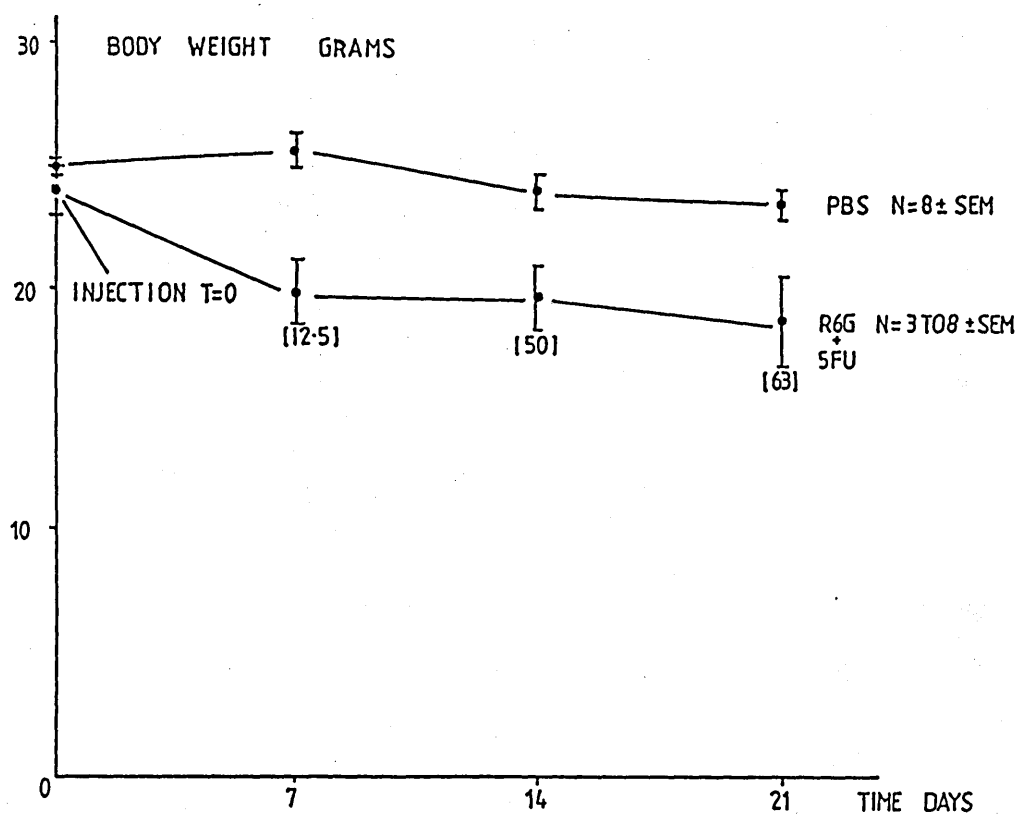


Figure 8.3: Effect of R6G (3.6mg/Kg^{-1}) and 5FU (50mg/Kg^{-1}) in combination upon mean body weight of athymic mice implanted with WIL tumour xenografts. Figures in parenthesis indicate percentage mortality in experimental group following single drug administration at $T = 0$. Controls received equivalent injection volumes of phosphate buffered saline.

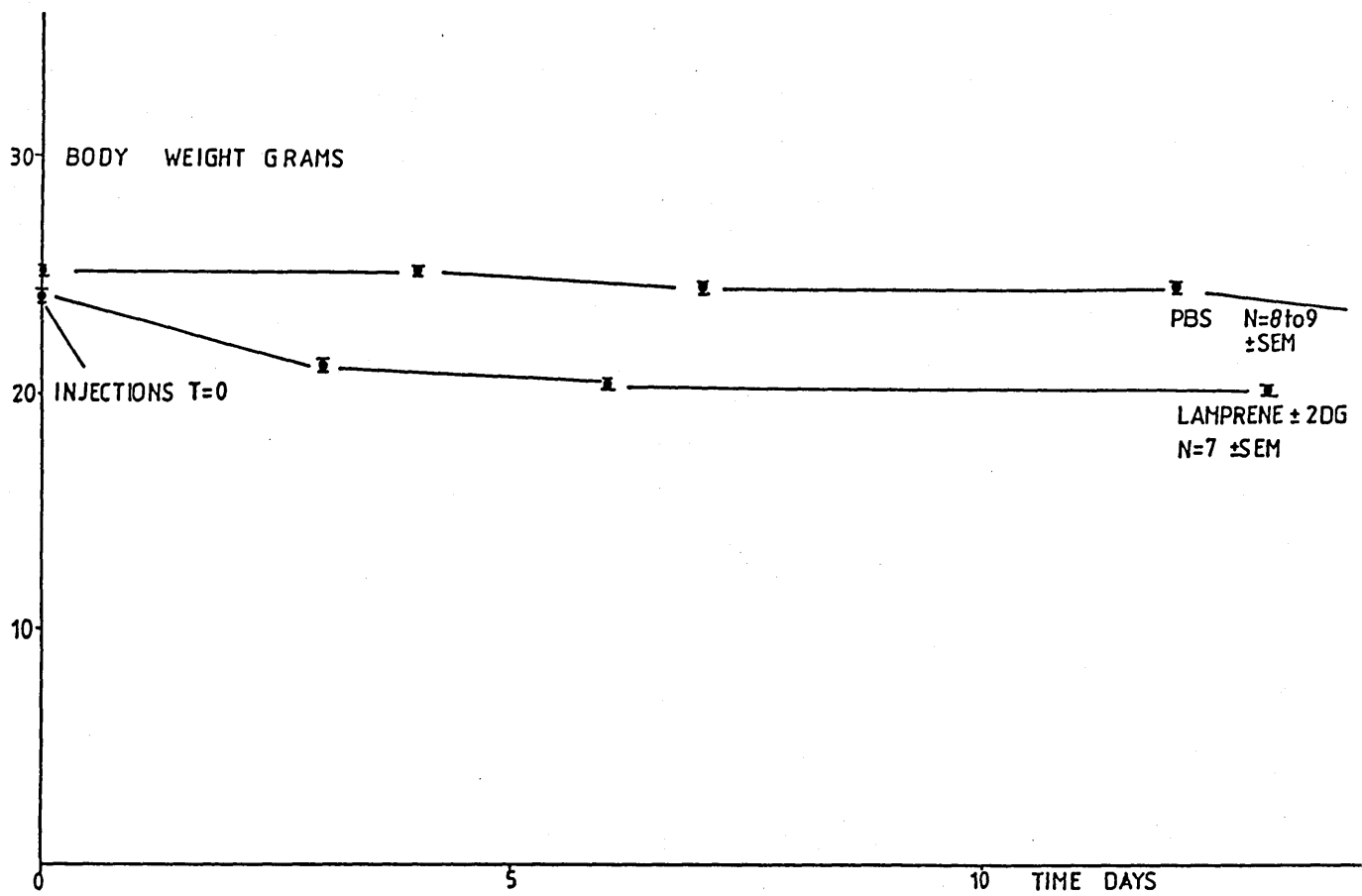


Figure 8.4: Effect of lamprene (0.05% of diet) and 2-deoxyglucose (0.5g/Kg⁻¹) in combination upon mean body weight of athymic mice implanted with WIL tumour xenografts. Controls received equivalent injection volumes of phosphate buffered saline.

Effect of R6G and 5FU upon whole body weight

The effect of combined administration of 5FU and R6G upon whole body weight of athymic mice implanted with WIL tumour subcutaneously is shown in Figure 8.3. When compared to animals given saline injections (PBS) there is a clear reduction in body weight in animals given combination of R6G (3.6mg/Kg) and 5FU (50mg/KG). It is also apparent that the combination is toxic over a period of several weeks after drug administration, with mortality at 12.5% at 7 days after drug administration increasing to 63% at 21 days after administration.

Effect of Lamprene and 2-deoxyglucose upon whole body weight

The effect of combined administration of lamprene and 2-deoxyglucose upon the whole body weight of nude mice implanted subcutaneously with WIL tumour is shown in Figure 8.4. When compared to animals given saline injections (PBS), a clear reduction in body weights is observed in animals given combinations of lamprene (0.05% by weight mixed with diet) and 2-deoxyglucose (0.5g/Kg^{-1}). There was however, no enhanced toxicity in this experimental group when compared to controls administered with saline.

DISCUSSION

From the results obtained in this study it is clear that both R6G and 5FU in combination and lamprene and 2-deoxyglucose in combination delay the growth of WIL tumour implanted subcutaneously in athymic mice. With an accurate and precise method established for estimating changes in tumour volume it is also clear that R6G or 5FU administered individually have little effect upon tumour growth. However a clear synergism is observed when each drug is given in combination with a second agent. Lamprene and 2-deoxyglucose individually delay tumour growth and although synergism is not observed when each compound is administered in combination, a significant delay in tumour growth is still achieved. It is possible that this drug combination resulted in a reduced food intake, as indicated by a reduction in body weight in animals treated with lamprene and 2-deoxyglucose.

Mice administered with R6G and 2-deoxyglucose showed a significant reduction in tumour doubling time. This drug combination was in fact toxic and therefore weight loss associated with drug treatment probably accounted for a reduced tumour doubling time.

Weight loss induced in host animals bearing subcutaneous tumours does occur without drug treatment. Such a condition described as cachexia is defined as "the sum of those effects produced by neoplasms in the host, which are not the immediate result of mechanical interference with recognisable structures" (Costa, 1977). A major symptom of this condition, weight loss, is not simply related to caloric intake, tumour burden or tumour cell type. Among controls, the WIL tumour does not induce cachetic weight loss although individual animals, sometimes exhibit cachexia like symptoms. This is not widespread. However, drug induced toxicity such as that caused by R6G and 2-deoxyglucose also causes animals to become sickly and lose weight, but this is more likely due to a reduced food intake.

The main aim of the work described in this chapter was to determine whether indeed selective manipulation of tumour cell energy metabolism could be achieved to inhibit or delay tumour growth without associated

effects upon host metabolism. Lamprene and 2-deoxyglucose as antimitochondrial agent and antiglycolytic agent respectively, did appear to achieve delays in tumour growth, but clearly an effect upon host metabolism was observed following severe weight loss. R6G and 2-deoxyglucose as antimitochondrial and antiglycolytic agent respectively had no significant benefit in delaying tumour growth. Results indicate that although antimitochondrial therapy may prove to be of benefit in tackling growth of tumours resistant to conventional forms of chemotherapy, the choice of agents available may prove to be limiting.

2-deoxyglucose as an inhibitor of glycolysis might not be ideal. It was selected because earlier studies showed some success as a tumour specific antiglycolytic agent in studies with animal tumours (Ball et al, 1957; Bernal et al, 1983). Its mode of action is thought to occur via competitive inhibition of glycolytic enzymes (Wick et al, 1957; Barban and Schulze, 1961). It is conceivable therefore that prolonged contact of tumour areas supplied with blood glucose could eventually remove the effects of inhibition. Other workers have described other possible selective inhibitors of tumour cell glycolysis which have yet to be assessed (Floridi et al, 1981(a); Floridi et al, 1981(b); Guminska et al, 1986). Thus a thorough study of how best to maintain inhibition of glycolysis in vivo, so that effects upon tumour growth could be shown is required. Similarly single bolus injections of antimitochondrial agents are also likely to be of limited value unless it could be shown that the active component of each drug is exposed to the tumour for long enough, as observed with R6G.

A major drawback in utilising R6G, apart from the severe general toxic effects this agent has, even at very low doses, is the non specific nature of its tumour selective toxicity as described in Chapter 7. Recent studies having indicated possible modes of action of this compound other than inhibition of mitochondrial activity, which might explain toxicity. Both Abou-Khalil et al (1986) and Kuzela et al (1986) imply that rhodamine may exert biogenetic inhibition of mitochondria in normal mammalian cells. Furthermore, O'Brien and

Weinstein (1987) indicate that R6G may exert a cytotoxic action upon cells following inhibition of protein kinase C.

The synergistic effects of 5FU and R6G upon tumour growth delay cannot be critically discussed in terms of biogenetic inhibition of tumour mitochondria being induced with 5FU and functional inhibition of tumour mitochondria being induced with R6G. From results obtained in Chapter 6, it was established that a clear inhibition of mitochondrial enzyme activity indicative of reduced expression of mitochondrial enzymes was not obtained with non-cytotoxic concentrations of 5FU in vitro. It can be assumed therefore that some mechanism other than inhibition of tumour cell mitochondrial activity must be responsible for the delay in tumour growth that is observed. It is well known that 5FU is both an inhibitor of RNA metabolism and inhibitor of DNA synthesis in tumour cells (Heidelberger, 1975; Mandel, 1981). It is possible therefore that R6G merely facilitated permeation of 5FU at non-cytotoxic concentrations into the tumour. Thus accumulation of 5FU may have enabled inhibition of tumour growth by this agent alone. This disappointing possibility does detract from the main aim of this thesis, to effect selective manipulation of tumour cell energy metabolism and thus reduce tumour growth by this means.

Work referred to, which showed reduced tumour growth following delivery of combinations of antimitochondrial agents and antiglycolytic agents were successful with both the W256 rat tumour and the Ehrlich ascites murine tumour (Fearon et al, 1985a; Bernal et al, 1983). Both studies had one feature in common. That is, chemotherapy was initiated immediately after inoculation of animals with tumour. The studies described in this chapter, as a consequence were aimed at a tumour of human origin which had been implanted and allowed to grow for several weeks before therapy was initiated. If it is possible to make use of drugs whose main function is to alter tumour energy metabolism, then methods for prolonging contact of these drugs with tumour in vivo must be developed. Inhibition of energy metabolism may thus be maintained over several weeks in an animal model. Furthermore, such studies would require improvements in site specific delivery of a compound or combination of compounds, but also

maintenance of the therapeutic effect via slow release of drug(s) for example.

Recent work in this Department has shown that inhibitors of gluconeogenesis such as 3-mercaptopycolinic acid or hydrazine sulphate can potentiate the reduction in growth of W256 tumours implanted in rats when given in conjunction with the antimitochondrial agent Rhodamine-6-G. Furthermore this effect was dependent on the metabolic state of the animals in the study. Rats which had been starved, i.e. made hypoglycaemic prior to advent of drug therapy were found to show greater responses in tumour growth inhibition with combinations of antimitochondrial and antigluconeogenic agents. The effects of these drugs correlated well with a reduction in blood glucose concentration over the same time period (Fearon et al, 1985(a); Fearon et al, 1987).

Therefore, a more stringent approach is required to evaluate the potential of inhibitors of tumour cell energy metabolism in vivo, particularly if such agents can selectively inhibit growth of those tumours of human origin which are resistant to conventional forms of cancer chemotherapy. When such agents are incorporated into novel regimens, directed at solid human malignancies, unless attempts are made to prolong contact of such agents with the neoplasm, significant growth inhibition of tumour will not be achieved. Thus the potential of using inhibitors of tumour energy metabolism is likely to be missed.

Studies described in this chapter may be summarised as follows:

A precise method for assessing the growth of tumours in athymic mice implanted subcutaneously with solid malignancies has been developed. Thus a more accurate appraisal of the effects of how novel chemotherapeutic regimens exert their effects in such models can be made. Preliminary work with combinations of antiglycolytic and antimitochondrial agents do not produce significant delays in tumour growth that are characteristic of an inhibition of tumour cell energy metabolism. However, this may reflect the choice of agents selected for the study as clear evidence exists in the literature which supports the hypothesis that manipulation of tumour energy metabolism

may provide a way of inhibiting tumour growth in vivo. R6G and 5FU did produce a significant delay in tumour growth in athymic mice implanted with the WIL tumour. Although this effect was reproducible, the mechanism by which growth inhibition was achieved remains to be established.

CHAPTER 9

GENERAL DISCUSSION

The main purpose of these studies was to identify antimitochondrial agents which could be used to inhibit tumour cell energy metabolism without adversely altering the energy metabolism of host cells. Of the compounds examined, Rhodamine 6G (R6G) and lamprene showed most promise in altering cellular energy metabolism of tumour cells in vitro, as well as appearing to retard growth of tumour in vivo.

In order to select appropriate antimitochondrial agents a screening system was required so that such compounds might be compared in their relative ability to specifically block tumour cell energy metabolism without adversely affecting energy metabolism in non tumour cells.

Chapter 1, the introduction to this work described the limitations of conventional cancer chemotherapy, in particular its non-selectivity affecting both neoplastic and also rapidly growing normal tissues such as bone marrow and GI tract. A major assumption behind the development of agents in use is that all tumour cells are more rapidly growing than normal cells. Not only are there normal tissues which exhibit comparable growth rates and are thus susceptible to the toxic effects of anti-cancer chemotherapy, but slowly growing well differentiated tumours exist which are hardly affected by conventional drugs. Thus the aim of this project was to assess whether differences which might exist between the energy metabolism of tumour cells and normal tissues could be exploited so that novel, tumour selective compounds might be developed for cancer chemotherapy in the future.

A review of the literature indicated that tumour energy metabolism could be broadly classified according to tumour cell growth characteristics. Rapidly growing tumours were more often highly glycolytic and poorly differentiated, containing structurally abnormal mitochondria. Slowly growing tumours existed however with comparably normal growth rates being well differentiated and containing

mitochondria similar in morphology to normal tissues from which the tumour was derived. The aim of this work was to develop a suitable therapy against both tumour types.

Experiments described in Chapter 3 were thus designed not only to test whether the oxidative capacity of a poorly differentiated, glycolytic tumour was indeed impaired relative to normal tissue, but also whether such an experimental system might be used as the basis of a screen in vitro for monitoring the effectiveness of antimitochondrial agents against tumour cell energy metabolism. Studies were initiated to look at the respiratory properties of isolated mitochondrial preparations obtained from the highly glycolytic, rapidly growing rat W256 tumour carcinoma and from normal rat liver. Respiratory activity was monitored with a Clark-type oxygen electrode. Results indicated that not only were preparations of tumour mitochondria structurally intact, oxygen consumption was coupled to phosphorylation of ADP, but tumour mitochondria were also able to metabolise oxidizable substrates succinate, malate and pyruvate. Oxygen consumption ^{rates} were somewhat less than liver mitochondria, but this was attributed to a lower mitochondrial content in tumour tissue relative to liver. A major deficiency in W256 mitochondria was the inability to metabolize B-OH butyrate. The non-utilisation of B-OH butyrate was attributed to reduced activity of succinyl-CoA : 3-oxo acid CoA transferase (E.C. 2.8.3.5) and Acetyl-CoA transferase (E.C. 2.3.1.9), enzymes which have been found to be either reduced or absent in other tumour tissues (Tisdale and Brennan, 1983). In the longterm, isolated mitochondrial preparations proved to be unsuitable for screening compounds which might inhibit tumour cell energy metabolism. Such a study could not reveal much information about the integration of mitochondrial pathways of energy metabolism into the metabolism of the intact cell. Furthermore, practical difficulties in obtaining isolated mitochondrial preparations from either animal tumours or xenografts implanted in athymic mice were envisaged. As tumours are made up of several cell types it could not be established whether the mitochondrial preparation obtained was solely tumour in origin. For screening purposes therefore, a second model was required to establish

which antimitochondrial agents had potential as anti-cancer agents in vitro.

Whole cells obtained from a tissue culture source were not only of a single cell type, but correspond most probably to a pure, cloned subpopulation of cells from the tumour. Furthermore a better indication of cell viability could be made with a variety of tests designed for continuous cell lines in culture, thus doubts as to the viability of the preparation observed with mitochondrial preparations, following contamination with tumour necrosis, were not present.

Experiments in Chapter 4 were designed to examine whether cultured tumour cells could be used to screen potential antimitochondrial agents. Two tumour cell lines, the rat carcinosarcoma W256 and human bronchial tumour WIL, were compared with freshly isolated rat hepatocytes, a source of non-malignant cells. The W256 tumour is characteristic of a poorly differentiated, rapidly growing glycolytic tumour, whereas the WIL tumour is representative of a more differentiated tumour cell type. Thus agents investigated in this study were directed at both types of tumour cell which would leave the energy metabolism of the non-malignant cell type unaltered.

Of the compounds studied, R6G, lamprene, anafranil and ludiomil all had inhibitory effects upon tumour cell energy metabolism. Cellular oxygen consumption was altered and the glycolytic response measured by lactate production rates increased as a result of treatment with these agents. However, both lamprene and R6G exerted their action at concentrations which were lower than anafranil and ludiomil. At concentrations of 10 μ M and 15 μ M, R6G had a biphasic action upon tumour cellular oxygen consumption. A transient stimulation was followed by a steady state inhibition of oxygen uptake in all three cell types examined. The dose dependent inhibition of oxygen consumption was more pronounced in the tumour cell lines and R6G, 10 μ M had little effect upon steady state oxygen consumption by hepatocytes. Lamprene stimulated oxygen consumption in both tumour cell lines. Examining the glycolytic response, both drugs stimulated lactate production in the tumour cell lines examined, although hepatocyte lactate production was

unaltered. Data obtained in these studies was not adequate to draw any firm conclusions at this stage, but results obtained with R6G and lamprene did warrant further investigation.

Clear evidence of the potential antimitochondrial activity of R6G and lamprene could only be obtained by comparing the inhibitory action such agents might have on mitochondrial function relative to classic inhibitors of mammalian mitochondrial function. Experiments described in Chapter 5 examined the effects of the classical mitochondrial inhibitors DNP, an uncoupler; oligomycin, a phosphorylation inhibitor; and electron transport chain inhibitors rotenone and antimycin, when incubated with WIL, W256 or rat hepatocytes in the oxygen electrode. Fresh cell preparations, incubated in the polarographic oxygen sensor responded to DNP, oligomycin, rotenone and antimycin as predicted from the organisation of the electron transport chain in each cell type. R6G, 10 μ M stimulated oxygen uptake in tumour cell preparations, independently of phosphorylation, characteristic of uncouplers. This transient effect was followed by a steady state inhibition of oxygen uptake, also independently of phosphorylation indicating possible inhibition of electron transport. Lamprene, which stimulated oxygen uptake independently of phosphorylation, exhibited behaviour characteristic of an uncoupler. Thus a clear demonstration of the inhibitory effects of both R6G and lamprene on mitochondrial function could be obtained.

Results obtained did lead to speculation that the site of inhibition of electron transport induced by R6G in tumour cells occurred either at mitochondrial enzyme complex III or IV. Recent evidence with an analogue of R6G, R123 indicated that primary antimitochondrial activity is directed at the F1FO complex of the ATP synthase and that any associated inhibition of electron transport was thought to occur at mitochondrial enzyme complex I (Modica-Napolitano and Aprille, 1987).

Results obtained in Chapter 5, while not specifically investigating the ATP synthase as a site of action indicated that R6G did exhibit uncoupler like behaviour and implicated the enzyme complex III or IV

as a site of inhibition of electron transport. The stimulatory activity of R6G on oxygen uptake in isolated mitochondria under specific conditions has been confirmed by some groups (Higuti et al, 1980), but not others (Mai and Allison, 1983). Experiments which utilise a whole cell model for examining tumour energy metabolism, while not suitable for examining the mechanism of action of inhibitors is admirably suited to confirming whether respiratory inhibition is induced by antimitochondrial agents. As rat hepatocytes did respond to the concentration of R6G used in this study, it was postulated that concentrations of R6G might exist which altered tumour cell mitochondrial activity without affecting non tumour tissue (Lampidis et al, 1983; Nadakavukaren et al, 1985).

Experimental methods for inhibiting tumour cell energy metabolism were not confined to functional inhibitors of tumour mitochondria in vitro. In Chapter 6 agents which interfered in the ability of mitochondria to replicate were examined for use with functional inhibitors to block tumour energy metabolism. Inhibition of mitochondrial biogenesis in a variety of eukaryotic cells has been achieved with compounds such as chloramphenicol and tetracycline both in vitro and in vivo (Galper and Darnell, 1971; Van den Bogert et al, 1986(b)). Circumstantial evidence existed which indicated that tumour mitochondrial biogenesis might be inhibited with non cytotoxic concentrations of the conventional anti-cancer agent 5FU (Oliver and Williamson, 1976(a); 1976(b); Bugeja et al, 1987). Subtoxic concentrations of 5FU were employed to try and deplete mitochondrial protein content. Activity of specific mitochondrial enzymes were assayed as a function of a reduction in biogenesis.

The cytochrome oxidase enzyme complex IV, which is coded for by mitochondrial DNA (Pfanner and Neupert, 1987; Nelson, 1987), was assayed by a polarographic method following exposure of tumour cells to 5FU for 72 hours. Succinate-cytochrome C reductase activity, a combination of the activities of succinate dehydrogenase, enzyme complex II and ubiquinone-cytochrome C reductase, enzyme complex III was monitored by a spectrophotometric method following exposure of tumour cells to 5FU for 96 hours. Of these enzymes, complex II is

thought to be coded for by the nucleus and imported into mitochondria in precursor form, while complex III is coded for by mitochondrial DNA (Pfanner and Neupert, 1987; Nelson, 1987).

Results from these experiments were not conclusive. Possible reasons for why inhibition of biogenesis was not observed following treatment of cells with 5FU included too short a duration of drug exposure to cells or that higher concentrations of 5FU were required to inhibit biogenesis and these concentrations might have been cytotoxic.

The results presented in Chapter 5 suggest that NAD-linked respiration is of more importance in tumour cells than FAD-linked respiration. However, results obtained in Chapter 3 showing that succinate could stimulate respiration in tumour cell preparations and results in Chapter 6 which showed that succinate dehydrogenase was intact and fully functional contradicted results obtained in Chapter 5. Clearly, use of rotenone, a potent inhibitor of electron transport at complex I gave misleading results implying that succinate-linked respiration was not operating optimally in tumour cells. Titration of rotenone with rat hepatocytes in the oxygen electrode failed to block oxygen uptake completely, although antimycin fully inhibited oxygen consumption. Perhaps therefore, the specificity of rotenone in tumour cells is open to question.

Experiments described in Chapter 7 were designed to examine whether the cytotoxic action of the antimitochondrial agents under study were due to the inhibitory effects these compounds had upon tumour cell energy metabolism. The effects of R6G and oligomycin, a specific inhibitor of mitochondrial phosphorylation, were examined on the growth of tumour cells grown in either glucose and glutamine or glutamine alone as the major carbon source. Glutamine utilisation by tumour cells in culture requires mitochondrial participation, in the absence of a glycolytic substrate. The inhibitory effects of R6G on the growth of cells on either glucose and glutamine or glutamine alone was contrary to expected results. Sensitivity to R6G was equal with cells grown under either conditions. In fact, growth delay of cells in glutamine was less than cells supplied with glucose. However,

there did not appear to be any difference in the uptake of the drug as indicated by photomicrographic evidence of cells incubated with R6G in each medium.

Cytotoxicity assays based on the reduction of MIT as an indicator of cellular viability, indicated that cells supplied with glutamine were more sensitive to oligomycin. Cells grown in glutamine exhibited a 9-fold reduction in the ID₅₀ to oligomycin as compared to cells grown in the presence of glucose. ID₅₀ values for R6G were equal for cells grown in either glucose or glucose depleted medium. Although R6G has antimitochondrial activity, this might not be its major cytotoxic property. This series of experiments has shown that cytotoxic compounds whose toxic effect is determined by antimitochondrial action such as oligomycin, might thus be identified by the experimental techniques described in this chapter.

Any evaluation of compounds which selectively inhibit tumour cell energy metabolism in vitro must be studied further and it is therefore necessary to study the effectiveness of such agents in an experimental model of cancer which reflects the situation in vivo. Thus the experiments in Chapter 8 monitored the ability of the agents R6G, lamprone and 5FU to inhibit the growth of a human tumour xenograft implanted subcutaneously in colonies of immune-incompetent athymic mice.

The non-small cell lung tumour WIL used for these studies has previously shown to be resistant to conventional forms of chemotherapy (Merry et al, 1986; 1987). The growth of tumours implanted subcutaneously above the hind limbs of female athymic mice was monitored as a function of the time course of tumour growth and tumour doubling times could be calculated as a consequence.

A novel method for accurately and precisely estimating tumour volume was developed. Moulds of growing tumours were cast in dental impression material. Tumour volumes estimated from the volume of water required to fill the cast were found to more accurately predict

growth of tumours as compared to conventional caliper measurements and subsequent estimation of tumour volume.

Experiments were designed to show the possible effects of antimitchondrial agents upon an established tumour growing in vivo and moreover, therapy was directed at a tumour which was human in origin. This approach represented an advance on previous experiments which utilised animal tumour models and furthermore, only showed an enhancement of anti-tumour action if treatment commenced soon after inoculation of animals with tumour (Bernal et al, 1983; Fearon et al, 1985(a)).

The effects of different experimental chemotherapies was examined using the delay in tumour doubling time as a parameter of therapeutic efficacy. Combining the antiglycolytic agent 2-deoxyglucose with the antimitchondrial agents R6G or lamprene gave results which were in part encouraging. 2-deoxyglucose and R6G did not potentiate tumour growth delay. In combination, severe toxicity was observed and this resulted in a reduction in tumour doubling time. However when 2-deoxyglucose was combined with lamprene, growth delay of tumours was observed by each drug alone and in combination. No synergism between the two was noted. The delay in growth with these drugs was not however, necessarily valid as animals did not remain weight stable throughout the course of treatment as a probable result of reduced food intake. Clearly from studies reported in the literature, combining antiglycolytic and antimitchondrial agents to alter tumour energy metabolism in vivo is a valid approach, although the choice of drugs has proved to be limiting (Bernal et al, 1983; Fearon et al, 1987). R6G and 5FU in combination did produce a significant delay in tumour growth although the mechanism by which this was achieved remains to be established. The original hypothesis of utilising a functional inhibitor of mitochondria with a proposed inhibitor of mitochondrial biogenesis was found to be invalid following the inconclusive results obtained in Chapter 6. It is clear that in order to explain any of the effects upon tumour growth observed with the drugs used, assays in vitro were essential in attempting to assign

possible mechanisms of action to explain how these agents delayed tumour growth.

From the work presented in this thesis, two compounds of those studied have shown to behave as antimitochondrial agents. The antimitochondrial activity of R6G however does not appear to define its cytotoxicity. More work is required to determine whether or not the action of lamprene upon tumour cells is selective. Unless a definite mechanism of action can be attributed to a particular experimental anti-cancer agent cited in the literature then basic studies are required at the subcellular level to determine the mechanism of action. Such studies might involve experiments at the mitochondrial level, submitochondrial level or enzyme level. Only when this has been established will studies at the level of the whole cell or intact animal remain unambiguous. Such an approach is not only valid for antimitochondrial agents, but also for potential antiglycolytic agents (Floridi *et al*, 1981(b); Guminska *et al*, 1986) and biogenetic inhibitors of tumour mitochondria (Van den Bogert, 1983).

The animal model chosen represents the least controlled system with which an experimental chemotherapy can be investigated. The procedures adopted for work described here represent a more rigorous approach as compared to previous studies (Bernal *et al*, 1983). However, more work is required to ensure that a cytotoxic drug concentration is maintained at the tumour site. Methods of drug administration and site delivery must be developed further. The delay in tumour growth obtained with R6G and 5FU requires explanation. It is just conceivable that evidence obtained for a drug's mechanism of action in vitro might not be applicable in vivo. However, until proven otherwise, only further work carried out with models in vitro will indicate how R6G and 5FU potentiate delay of tumour growth. The toxic effects of R6G upon tumour cell metabolism might be related to an inhibition of mitochondrial biogenesis (Abou-Khalil *et al*, 1986; Kuzela *et al*, 1986) or metabolism that is not primarily related (O'Brien and Weinstein, 1987). Other mitochondrial probes exist with

similar properties and might be evaluated further (Conover and Schneider, 1981; Mai and Allison, 1983).

It is hoped that differences in cellular energy metabolism between tumour and non tumour cells represent a promising path for selectively inhibiting the growth of tumour relative to host cells in cancer patients. This approach has found favour with other groups (Bernal et al, 1983; Fearon et al, 1987).

Rhodamine-6G has served as a laboratory based reference for the selective manipulation of tumour cell energy metabolism. However, real benefit may be derived from an investigation of agents such as lamprene which in the future might be used clinically.

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