STUDIES ON THE PROTECTION MECHANISMS AGAINST DOXORUBICIN TOXICITY IN RESISTANT AND SENSITIVE HUMAN TUMOUR CELLS

MUZAHIM M. AL-KABBAN

DEPARTMENT OF PATHOLOGICAL BIOCHEMISTRY

UNIVERSITY OF GLASGOW (ROYAL INFIRMARY)

THESIS SUBMITED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE UNIVERSITY OF GLASGOW, SCOTLAND

SEPTEMBER 1989

CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEDICATION

To those whom I love, my father and in memory of my beloved mother.

To my wife, Kifah
whose love, support and understanding made this undertaking possible.

DECLARATION

The work presented in this thesis was performed solely by the author except for areas of acknowledged collaboration.

MUZAHIM AL-KABBAN

ACKNOWLEDGEMENTS

I am indebted to my supervisors, Drs. M. J. Stewart and I. D. Watson for their expertise and continued encouragement and direction throughout these studies and during the preparation of this thesis.

As this project was carried out at the Department of Pathological Biochemistry, Glasgow Royal Infirmary, I would like to extend my thanks to Prof. J. Shepherd and his predecessor Prof. H.G. Morgan for allowing me access to the excellent facilities in their department.

During the course of this project I have collaborated with the NMR user group from the Department of Pure and Applied Chemistry, Strathclyde University, who were always very helpful, and to Dr. J. Reglinski and Profs. W.E. Smith and C.J. Suckling I extend my thanks.

Thanks also due to Drs. R.I. Freshney, J.A. Plumb and S. Merry from the Department of Medical Oncology, University of Glasgow for allowing me facilities for chemosensitivity assays and for providing lung tumour lines.

My thanks go also to Mr. S. McIntosh for the practical help given to me during my studies.

I am most grateful to my Government, REPUBLIC of IRAQ for financial support.

Finally I thank my wife Kifah for her continuous support and encouragement throughout.

ABBREVIATIONS

BSO	Buthionine-S,R-Sulfoximine
CDNB	1-Chloro-2,4-dinitrobenzene
CV	Coefficient of variation
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DOX	Doxorubicin , Adriamycin
FBS	Foetal bovine serum
FT	Fourier transform
GSH	Reduced glutathione
GST	Glutathione-S-transferase
HPLC	High performance liquid chromatography
I.U	International unit
k'	Capacity ratio
LD_{50}	Lethal dose to kill 50 % of population
MDA	Malondialdehyde
MHz	Megahertz
MTT	[3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide: Thiazolyl blue].
NAC	N-acetylcysteine
NMR	Nuclear magnetic resonance
ODS	Octadecyl sulphate
PBS	Phosphate buffered saline
RNA	Ribonucleic acid

rpm	Revolutions per minute
SE	Standard error
SH	Thiol
T	Delay time
Tris	Tris (hydroxymethyl) methylamine
t-test	Student's t-test
μ	Micron
UV	Ultraviolet (light)

SUMMARY :

A rapid, sensitive and selective HPLC method for the quantitation of glutathione (GSH) at the cellular level was developed by the author. Glutathione is resolved isocratically by ion-pair high-performance liquid chromatography and detected by UV at 200 – 210 nm. The mobile phase consisted of an aqueous buffer of methanol containing 0.1 % tetrabutyl ammonium hydroxide adjusted to pH 3.5 by 10 % v/v orthophosphoric acid. This method is able to detect GSH in a small amount of cells and can be adapted for quantitative determination of biological thiols and some other cellular compounds of special interest such as amino acids at small tissue volumes.

The non invasive nuclear magnetic resonance (NMR) technique which was developed for this study (^{1}H spin echo NMR) is capable of detecting certain small molecules and structural entities in intact tumour cells. This method is specific and selective, providing information on the concentration and conformation of such molecules as glutathione, phosphorylcholine, lactate, mobile triglyceride, acetyl choline etc in the living cells. The technique has the advantage that it is non-invasive, providing detailed structural information on individual species present in the cell matrix. It has been used in this case to study the rate of energy consumption following the activation of the glycolytic pathway with glucose. The signals and patterns observed have been used in a preliminary way to study changes in glutathione metabolism and in lactate production when challenged by therapeutic agents.

The effect of doxorubicin on the cellular biochemistry of Hela tumour cells using ^{1}H spin echo NMR of the intact and viable cell in conjunction with the dual wave length HPLC of cell lysate is reported here. Dose-related changes were observed in lactate and reduced glutathione concentration. Doxorubicin induces a time-dependent depletion of the cytosolic pool of glutathione and a change in the glycolytic pattern of

the cells. The glutathione depletion could be partially reversed by controlled pre-treatment of the cells with N-acetylcysteine and cysteine, the protection being linked to the intracellular concentration of the thiol. Glutathione was also measured in other doxorubicin-sensitive cells from small cell carcinoma of lung (GLC_4 210), and the levels compared with those in cells with acquired resistance and a line of resistant non-small-cell adenocarcinoma of lung A549 (alveolar type 2). The effect of different doxorubicin concentrations on GSH was measured using the HPLC method which has been shown to correlate with the NMR studies in live cells.

^1H spin echo NMR of the leukaemia cell line (J111) is also reported here for studying cellular glycolysis. The concentration of cells in the NMR tube is high (approx. 10^9 cells in 0.4 ml) and as a result the available oxygen is restricted, making the NMR experiment a plausible in vitro tumour model in which kinetics in the living cell can be probed in a non-invasive manner. Treatment with pharmacological concentrations of doxorubicin produced immediate enhanced anaerobic glycolysis and eventual cell death.

Glutathione-S-transferase (GST) activity in the different lung tumour cells was linked with their content of glutathione and the effect of doxorubicin on such activity was studied as well.

High levels of lipid peroxidation were noticed in the two sensitive cell lines (Hela and GLC_4 210 [S]) as doxorubicin readily passes into these cells and interacts with glutathione. N-acetylcysteine pre-treatment of Hela cells showed little protection from the effect of doxorubicin.

The effect of doxorubicin on the viability of the different tumour cells was studied using MTT dye reduction by living but not dead cells. An increase in A549 sensitivity to doxorubicin was produced using Buthionine-S,R- Sulfuximine at a non-toxic concentration.

The inherent resistant of A549 tumour cells toward doxorubicin was circumvented by using one of the antiarrythmic drugs (amiodarone) which trap the drug in the cells by decreasing its efflux. This was indicated by measuring intracellular

doxorubicin and amiodarone after harvesting of the cells in two different ways (scraping and trypsinization). These results were confirmed by the HPLC measurement of GSH in these cells after amiodarone and doxorubicin treatment and by using a chemosensitivity assay .

I. INTRODUCTION

1.1. Overview:

Carcinoma is a Greek word meaning crab (Latin : Cancer). It was introduced by the Greek physician Galen who recognized that breast cancer had veins arising from the tumour, giving it the appearance of a crab (Bett , 1957).

The oldest recorded case of malignancy in man was a tumour of a mandible which was discovered in Kenya by Leakey in 1932. It is estimated to be half to one million years old (Stathopoulos, 1975). Evidence of many cancers, such as skull osteosarcoma, squamous papilloma of hand and ovarian cystadenoma have been observed in the mummified remains of ancient Egyptians (Ghalioungui, 1984).

Later on the early Greeks were interested in the malignant nature and the anatomical distribution of various types of tumours, especially breast cancer. During that time Hippocrates and his school described the external manifestations of malignancy. Galen followed them from 130 – 202 A.D. His hypothesis about Cancer suggested that the body contained four humours responsible for health; blood, phlegm, choler (yellow bile) and melancholy (black bile). According to this hypothesis, people with predominantly black bile were more likely to develop tumours in their organs in which bile thickened and solidified (Levitt et al., 1979). This was the first suggestion of the possibility of carcinogenic substances. The first modern report of a carcinogenic substance was made by Percival Pott in 1760 when he discovered that chimney-sweeps had a very high risk of scrotal cancer due to exposure to cancer-causing oils in soot with which their clothes were impregnated (Williams, 1983). In 1912 Bayon produced cancer experimentally for the first time by injecting tar into rabbits ears and this led Kennaway in 1922 to make the first attempt to identify cancer producing compounds in coal tar (Bett, 1957).

Cancer is an abnormal accumulation of cells originating from a single cell and transformed, such that growth and spread do not respond to normal growth mechanisms or regulatory systems within the body. The process by which the normal

cells are transformed to tumour cells is called carcinogenesis. Many substances are thought to be carcinogenic. Some example of these are :-

(1) Chemicals , e.g. Cigarette smoke, Arsenic , Asbestos, Nickel, Chromium, Petroleum, Coke, Coal soot, Coal tar, Benzene, Wax, etc..... .

(2) Radiation , e.g. X-rays, Radioactivity and Ultraviolet rays (sun) .

(3) Dietary factors , e.g. High fat and protein diet with low roughage.

(4) Viruses, e.g. Genital herpes virus and polyoma virus which cause salivary gland tumours.

(Benjamin, 1981 ; Pitot, 1981 ; Williams, 1983).

There are two types of tumour : benign, in which cells are encapsulated and do not invade or spread, and malignant in which cells have the capacity to invade and spread, i.e. metastasise. Metastatic spread involves normal tissues in addition to the tumour.

Tumour cells have special characteristics by which they differ from the normal, these include pleomorphism (changes in size and shape), increased mitosis and potential for direct invasion. They also have the ability to form clones and to continue growth and division in culture. Loss of growth control may be due to either alteration of the physical or chemical structure of the cell membrane, particularly of the glycoprotein components which make cells unresponsive to the normal growth limiting factors, to intracellular changes such as nuclear / cytoplasmic size ratio, abnormalities during cell division causing unbalanced growth, or to deficient regulatory factors such as hormones e.g. tumours in post-menopausal patients may be related to low oestrogen levels during cell division (Moore, 1975 ; Calman et al., 1980 ; Pitot, 1981)

Unlike normal cells in which growth is characterized by equal balance between cell division and death, there is in cancer tissue an inequality between the number of dividing and dead cells. Furthermore within the tumour bulk there are connective

tissue elements, blood vessels and other cells such as lymphocytes and macrophages in addition to the tumour cells. These non-tumour tissues are important both for tumour growth and in cancer chemotherapy, as they help in nutrition and blood supply and also support the tumour tissues (Souhami and Tobias, 1986).

1.1.1. Cell Cycle and Tumour Growth :

The cell cycle is characterized by different phases of growth and reproduction of cell constituents (Figure 1). The principal phases in cancer cells do not differ from those of normal cells and involve replication and distribution of DNA among the progeny. Two important stages which occur during the cell cycle :-

(1) Interphase : Consisting of three specific parts or intervals.

a. Gap 1 (G 1) : The interval between conclusion of mitosis and onset of S phase. During this interval RNA and protein synthesis occur and both accumulate in the cytoplasm.

b. S phase (synthesis phase) : In this phase DNA replication occurs ; protein synthesis in this phase is predominantly in the nucleus.

c. Gap 2 (G2) : The interval between the end of S phase and the onset of the next mitosis. In this interval cells complete their growth.

(2) M phase (mitosis) :

This phase involves four stages, the prophase, metaphase, anaphase and telophase. During these the nuclear membrane is broken up and the chromosomes

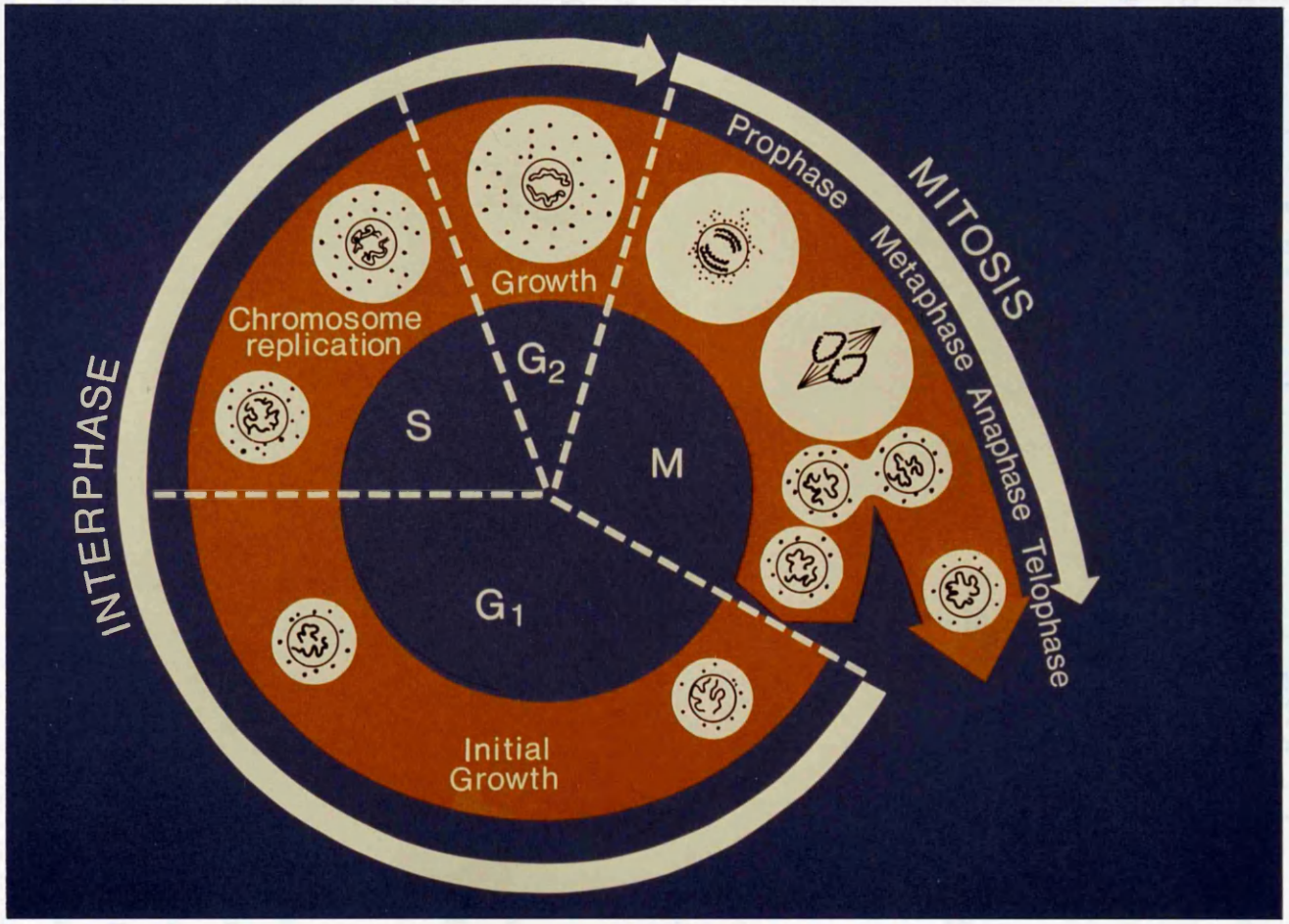


Figure 1 : Cell cycle.

condense, line up and sort themselves into two identical sets at either end of the cell. Later the cell divides into two daughter cells. The entire cell cycle takes about 10 – 24 hours of which only one hour is required for mitosis.

In a mass of tumour cells we can see different phases of the cell cycle in different growth stages. This is important in cancer chemotherapy because some antineoplastic drugs act specifically against actively dividing cells. For this reason chemotherapy is more useful in the early stages of disease than in the later stages.

Tumour cells grown in culture show Gompertzian growth characteristics, i.e. rapid in the beginning, becoming slow due to decrease in nutrition (Figure 2). In vivo the diagnosis of certain tumours may be helped using tumour markers, for example a patient with lung cancer may develop Cushing's syndrome due to secretion of ACTH and hence cortisol, or hyponatraemia due to increased antidiuretic hormone secretion. Monitoring of these markers can be important in case of follow up of patients, since a decrease in hormone levels may indicate a response to the therapy (Calman et al. , 1980 ; Moore, 1975). A number of proteins, eg. Alpha fetoprotein and Carcinoembryonic antigen are also produced by certain malignant tissues and may be used to plot the progress or remission of disease.

1.1.2. Treatment of Cancer :

In an Egyptian Papyrus (3,000 to 2,500 B.C.) there is a record of eight cases of breast cancer which were treated by cauterization. Ancient Egyptian people tried to cure cancer using drugs, but with no recorded successes. No more substantial progress was made until 1900 when x-rays were used successfully for leukaemia and other cancers by inducing remission (Bett, 1957). In a famous accident in World War II, a bomb from an American ship released Nitrogen mustard gas as it sank. The

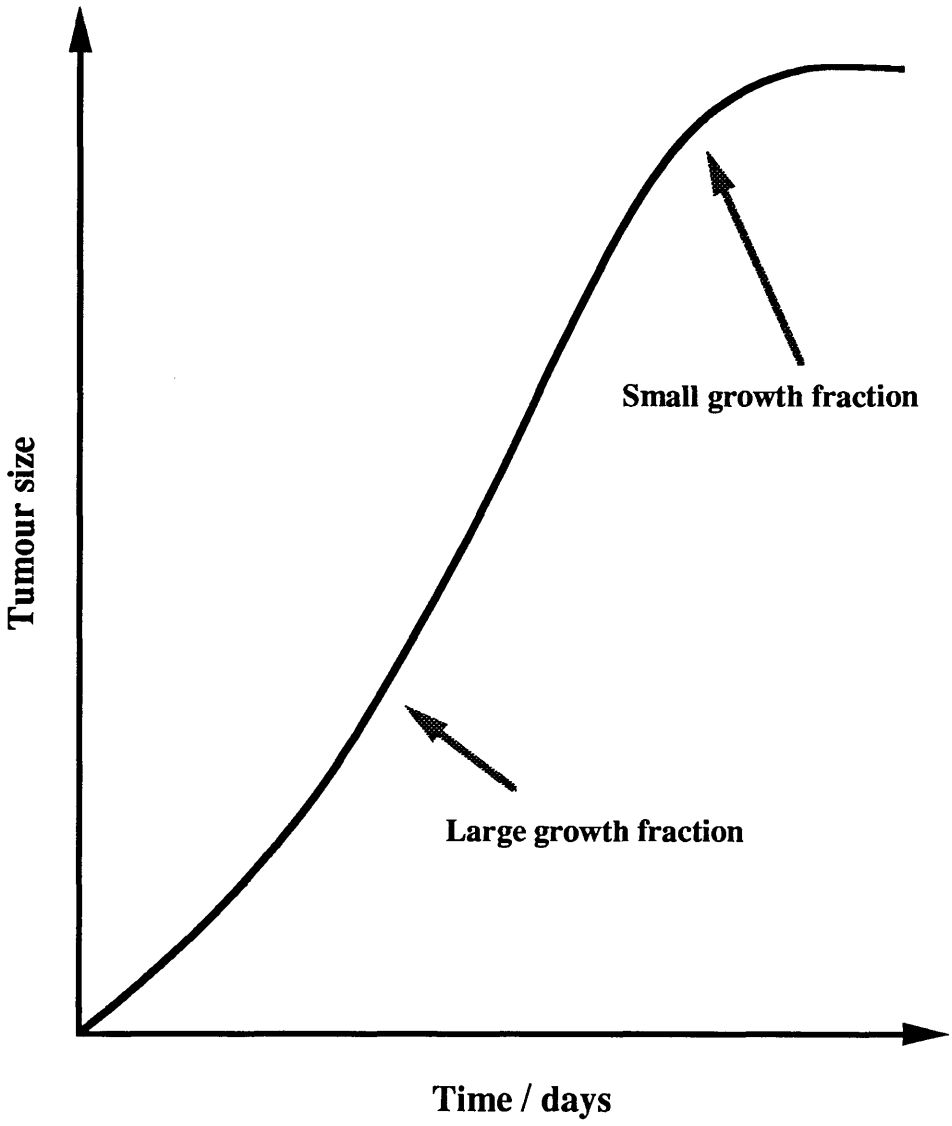


Figure 2 : Tumour growth pattern (Gompertzian growth).

dead and the surviving sailors were found to have developed marked leucopaenia (Levitt et al., 1979). It was surmised that this compound could be used to treat leukaemia, and by 1946 Nitrogen mustard had been introduced into clinical practice.

Previously the main methods for cancer treatment had been surgery and radiotherapy. In 1947 the antifolate aminopterin was used to treat leukaemia, followed by the production of methotrexate in 1949, 6-mercaptopurine in 1952 and the antitumour antibiotic actinomycin D in 1954 . From 1955 until the present numerous cytotoxic agents such as antimetabolites, alkylating agents and antitumour antibiotics with significant antitumour activities have been discovered (Souhami and Tobias , 1986).

The main pharmacological characteristics of cytotoxic drugs in the treatment of malignant disease are the narrow therapeutic index. i.e. there is very narrow gap between an effective and a lethal dose, the very small doses required, the fact that they are often unstable, with a wide variety of metabolic pathways, and finally that most of them are effective only at certain stage of the cell cycle. The routes of cytotoxic drug administration are determined by their stability, solubility and toxic effect (Calman et al., 1980).

1.1.3. Cancer Chemotherapy :

The anticancer drugs can be classified into five main groups with each group being subdivided into sub-groups of several drugs used for the treatment of different cancers. These groups are antimetabolites, alkylating agents, natural products, hormones and miscellaneous agents (Table 1). The anticancer antibiotics are a sub-group of the natural products which involve different cytotoxic agents (Table 2).

Table 1:

Grouping of Anticancer Drugs :

Group	Sub-group
I. Antimetabolites	**a). Nucleic acid antagonists.** **1. Purine antagonists.** **2. Pyrimidine antagonists.** **b). Folic acid antagonists.**
II. Alkylating agents	**a). Nitrogen mustards.** **b). Nitrosourea.**
III. Natural products	**a). Mitotic inhibitors (Vinca alkaloids)** **b). Antitumour antibiotics.** **c). Enzymes.**
IV. Hormones	**a). Steroid hormones.** **b). Polypeptide hormones.** **c). Anti–oestrogens.** **d). Anti–androgens.**
V. Miscellaneous agents.	**a). Platinum complexes.** **b). Hydrazine derivatives.** **c). Chelating agents.**

Table 2 :
Antitumour Antibiotics :

Name (of drug)	Mode of action	Clinical use
(I) Actinomycin D	Inhibits RNA synthesis by intercalating between guanine and cytosine bases of DNA leading to blocking of transcription.	Childhood cancers such as Rhabdomyosarcoma, Wilms' tumour and Ewings sarcoma.
(II) Anthracycline		
1. Doxorubicin	Inhibits DNA synthesis by intercalation between the base pairs leading to strand breaks. It is more effective in active dividing cells especially during the S phase while they are concentrated in the nucleus after absorption.	A wide spectrum of activity in childhood and adult solid tumours : lymphomas ; small cell bronchogenic carcinoma ; adenocarcinoma of ovary; breast and soft tissue sarcoma.
2. Daunorubicin	" " "	Acute lymphatic and myeloblastic leukaemia.
3. Mithramycin	Inhibits RNA synthesis in the same manner as Actinomycin D and DNA as in the case of Doxorubicin.	Malignant hypercalcaemia and germ cell tumours.
4. Bleomycin	Inhibits DNA synthesis by breaking single strands. It is active during G_2 and M phase.	Lymphomas and testicular tumours, squamous cell carcinoma of the head, neck, skin and cervix.

The anthracycline antibiotic doxorubicin is the most useful of these, because of its wide spectrum of activity which includes tumours of childhood, adult solid tumours and lymphomas. Small cell bronchogenic carcinoma, adenocarcinoma of the ovary and breast and soft tissue sarcomae have all been treated using this drug.

1.2. DOXORUBICIN :

1.2.1. Discovery :

The antineoplastic agent doxorubicin (DOX) $C_{27}H_{29}O_{11}N$ also known as adriamycin, is one member of more than 500 of the anthracycline group of antibiotics. Arcamone et al., (1969) first synthesized it from the fermentation process of the fungus Streptomyces peucetius var caesius. They described it as the 14-hydroxyl derivative of the anthracycline antibiotic daunorubicin.

1.2.2. Structure and Chemistry :

The structure of doxorubicin is shown in figure 3. It has a molecular weight of 579.9 daltons. The molecule is composed of three distinct sections which interact with each other. The first three rings comprise a substituted anthraquinone structure attached to a substituted cyclohexenyl ring forming a tetracycline ring structure. The remaining part of the molecule is the unusual amino sugar daunosamine which is attached to C7 of the cyclohexenyl ring through a β-glycosidic linkage.

The molecular structure of the anthraquinone nucleus indicates that the first and third ring structure of this molecule could be involved in most of its chemical activities due to the methoxy group at C4 of the first ring and the two hydroxyl groups on C6 and C11 of the third ring. Hydroxyl substitution may be of the most importance in the case of one electron reduction producing a semiquinone. The hydroxyl protons of the third ring form hydrogen bonds with the quinoid oxygen on

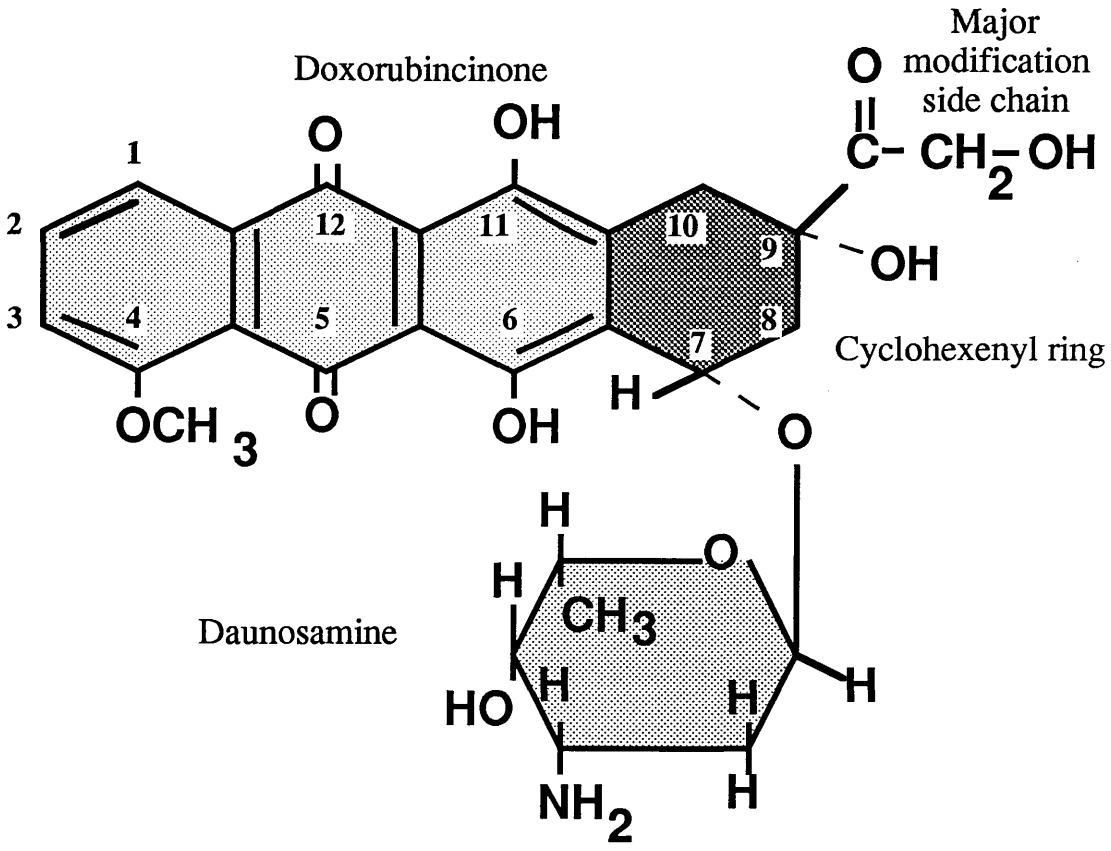


Figure 3 : Molecular structure of doxorubicin

the second ring. The molecular and electronic structure of the doxorubicin anthraquinone nucleus are changed by this hydroxyl substitution (Gianni et al., 1983), (see 1.2.3.4.).

The fluorescence emission spectrum of doxorubicin peaks at 520 – 620 nm at the two excitation wave lengths, 253 and 485 nm. The ultraviolet spectrum maxima of doxorubicin in methanol are at 233, 253, 290, 477, 495, and 530 nm.

1.2.3. Biochemical Effects of Doxorubicin :

The quinone and hydroquinone moietes on adjacent ring structures in the doxorubicin molecule are available for a number of molecular interactions which may have biochemical and pharmacological relevance. Of interest and great importance is the ability of doxorubicin to interact with cellular macromolecules such as DNA and cellular membranes. Those and other activities are due to the special ability of doxorubicin molecules in biological systems to act as metal ion chelators, undergo redox cycling and act as bioalkylating agents.

1.2.3.1. DNA Interaction :

DiMarco and Arcamone, (1975) noticed that the doxorubicin chromophore concentrated in the nuclei and stained the chromosomes of cell cultures forming a DOX-DNA complex, with a number of changes in the physico-chemical properties of both reactants seen. The most important changes are the progressive hypochromicity and a bathochromic shift of doxorubicin (Porumb, 1978). Due to intermolecular

charge transfer between the base pairs and the quinone chromophore, a new absorption maximum at 505 nm appears (Figure 4), and there is also fluorescence quenching (Figure 5) (Manfait et al., 1982). As a result, the doxorubicin chromophore is removed from solution and the hydroxyl and quinone groups of the two adjacent rings are not available for reaction (Patel et al., 1981).

For DNA the intercalation process decreases its sedimentation coefficient, increases its solution viscosity and its melting temperature and reduces its coiling properties (DiMarco and Arcamone, 1975). The drug causes unwinding of supercoiled closed circular DNA (Bauer and Vinograd, 1970).

1.2.3.1.1. DNA Intercalation Process (Figure 6) :

Pigram et al., (1972) suggested that the antibiotic chromophore is inserted between the adjacent base pairs with extensive reciprocal overlap. The amino sugar moiety sits in the major groove of DNA double helix with its charged amino group close to the second phosphate anion from the intercalation site. In (1980) Quigley et al., produced evidence that the location of daunosamine sugar was in the minor groove of the DNA double helix. Nakata and Hopfinger, (1980 a&b) ; Patel et al., (1981) confirmed the orientation of the drug perpendicular to the plane of the base pairs with the location of sugar ring in the minor groove of double helix. They proposed that in the major groove intercalation the anthraquinone ring is nearly parallel to the base pairs.

Phillips and Roberts (1980) using NMR found that the two middle rings of the drug overlap with the adjacent base pairs of the DNA double helix, while the first ring projects through the intercalation site. The cyclohexenyl ring also protrudes out of the intercalation site but in the opposite direction to the first ring. These authors also

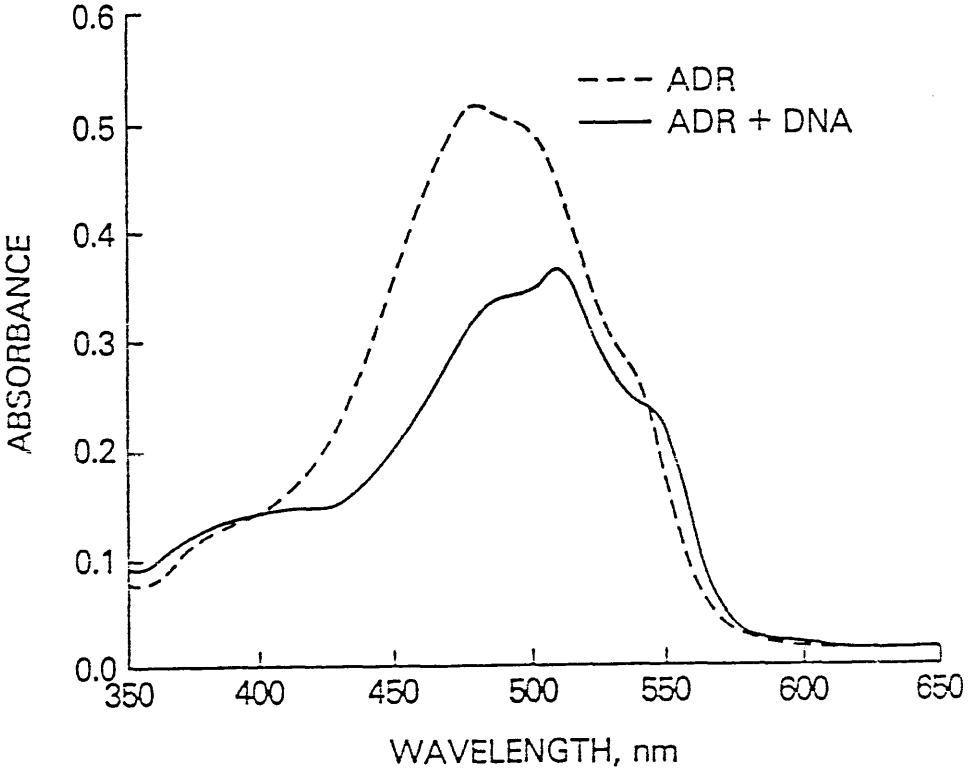


Figure 4 : Visible spectra of doxorubicin, 50 μM, (dashed line) ; and doxorubicin + DNA, 800 μM (solid line) in phosphate buffered saline at pH 7.4. (Gianni et al., 1983).

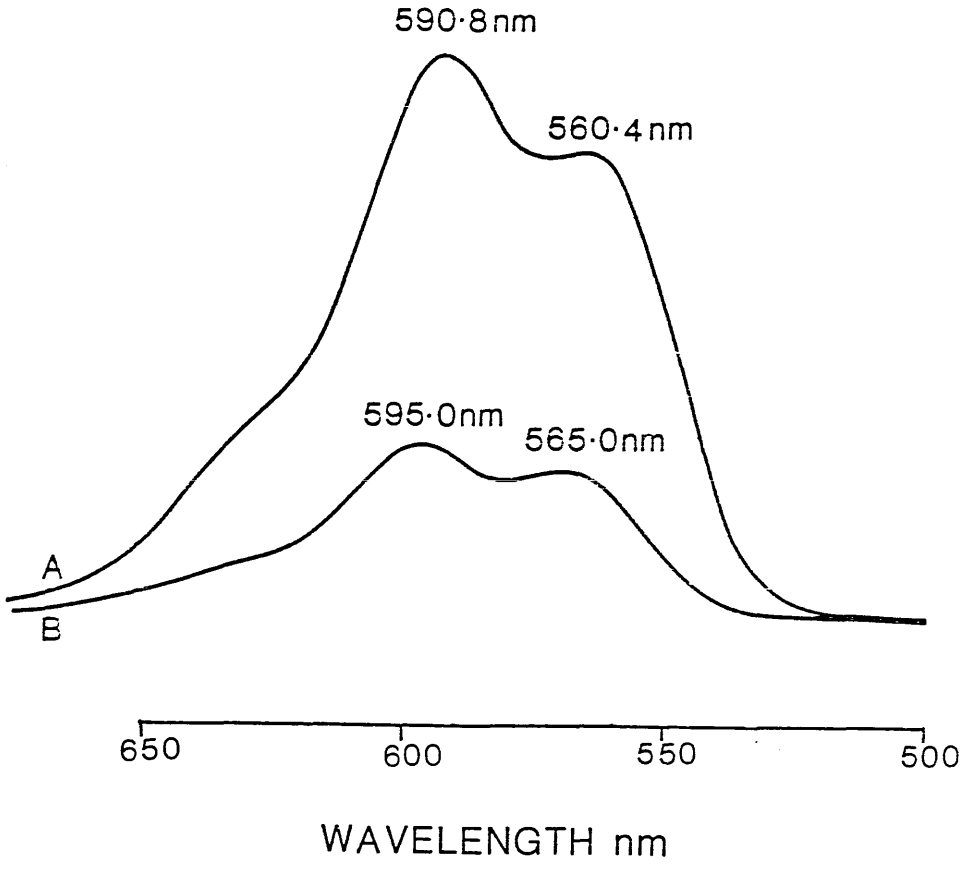


Figure 5 : Fluorescence emission spectra of A) Free doxorubicin, 2.6 x 10^{-4} M, pH 6.48 ; B) DNA-DOX complex at doxorubicin concentration of 2.6 x 10^{-5} M. All spectra are taken in aqueous solution in the presence of 10^{-2} M $NaClO_4$ (Manfait et al., 1982).

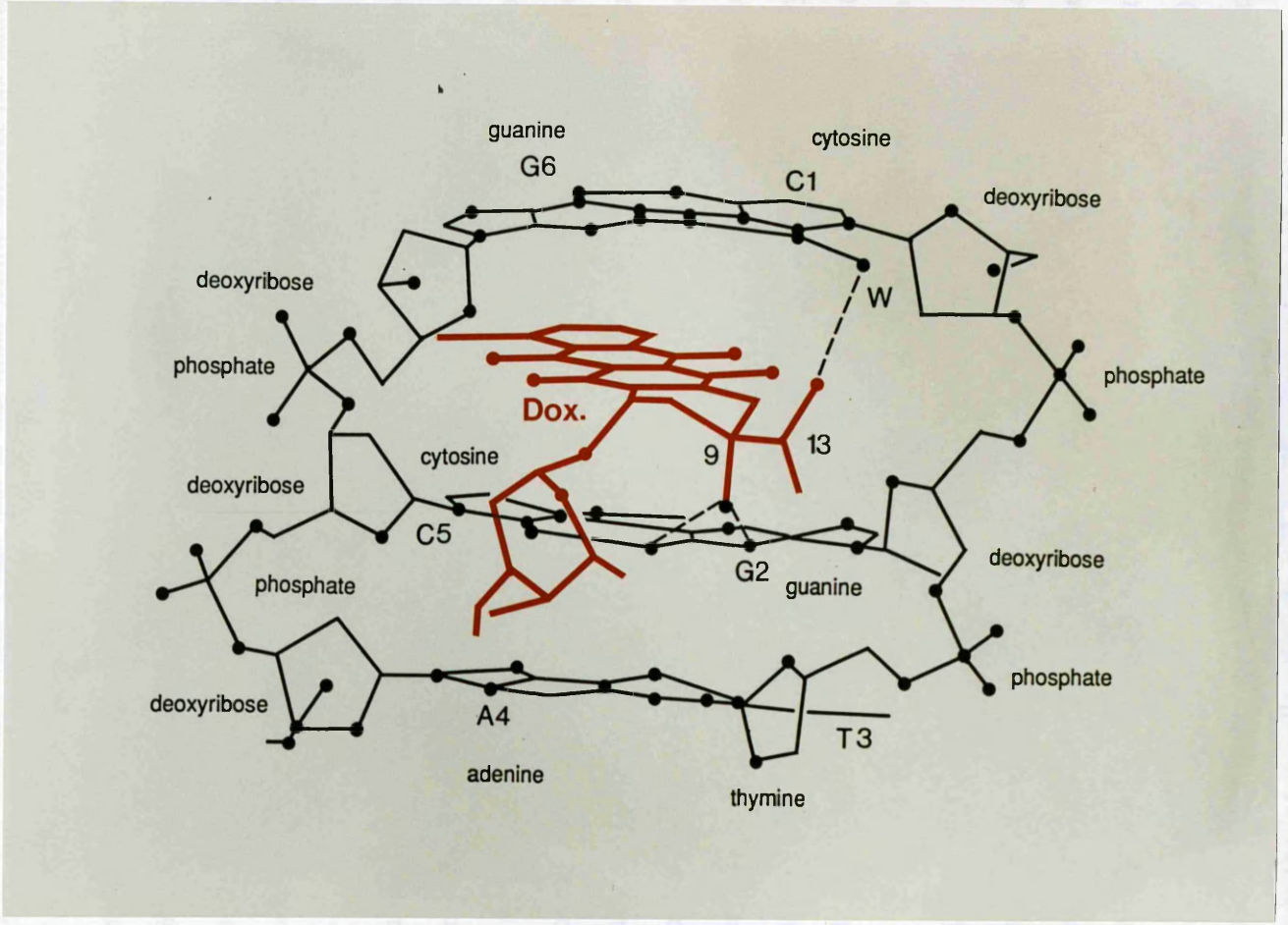


Figure 6 : Intercalation of doxorubicin into DNA.

proved that the major and minor groove models of intercalation differ only in the orientation of the cyclohexenyl ring to the hydrogen bond of the base pairs and the extent of the interaction between the charged amino sugar and the DNA phosphate group. Dorr and Alberts, (1982) said that in the intercalation process the amino sugar of doxorubicin interacts either with the nearest phosphate group, or with the one from the DNA nucleotide removed aside from the site of intercalation by the doxorubicin-chromophore, causing separation of stacked base and uncoiling of the DNA double helix. Moreover in the intercalation process the helix becomes bent, slightly elongated and stiffened at the site of doxorubicin intercalation (Reinert, 1983).

Stabilization of intercalation depends on the amino group of the daunosamine sugar and is decided by both the charge on amino sugar moiety and the position of the charged amino group (Neidle, 1978 ; 1979). This was supported by the finding of DiMarco (1975) ; Capranico et al., (1989) that there are three interactions shared in the stabilization of drug-DNA complex. First, the electrostatic interaction between the protonated amino group of daunosamine and the ionized phosphate group of the base pairs. Second, the hydrogen bonds between the cyclohexenyl ring of the anthraquinone and the base pairs. Third, the intercalation processes represented by the weak hydrophobic stabilization between the intercalating molecule and the adjacent base pairs. While Manfait et al., (1982) added that the phenolic groups of doxorubicin were also involved in drug-DNA intercalation interaction stabilisation.

The uptake of cytotoxic drugs that inhibit DNA function occurs throughout the regenerative cell cycle and reaches its maximum during DNA duplication, i.e. the maximum doxorubicin toxicity occurs during the S phase of the cell cycle (Kim and Kim, 1972 ; Barranco et al., 1973 ; Barranco, 1975 ; Kimler and Cheng, 1982). DiMarco, (1975) believed that in the case of tumours, the inhibition of nucleic acid synthesis in the intact cells prevents cell proliferation and provides the permanent antitumour activity of doxorubicin while inhibition of DNA synthesis in different mouse tissues occured within one hour of treatment (Formelli et al., 1978). Hixon

et al., (1981) showed that mitochondrial DNA synthesis is more sensitive to doxorubicin inhibition than that of the nucleus and suggested that this is one of the causes of its cardiotoxicity.

Ross and Smith, (1982) ; Bellamy et al., (1988 b) suggested that doxorubicin can easily produce single and double strand breaks in DNA in addition to DNA - protein cross-links, and these may decide the doxorubicin cytotoxicity. DNA repair is inhibited by a high intracellular concentration of this drug even when the external doxorubicin concentration is reduced by changing the medium. Durand and Olive, (1981) proposed that doxorubicin could easily produce significant inhibition of DNA synthesis but that significant DNA strand breakage was dose-dependent. They added that at low dose the intercalation prevented strands separation and inhibited DNA polymerase activity.

Graves and Krugh , (1983) found that doxorubicin was bound to DNA in a cooperative manner according to the ionic concentration, and that this binding enhanced its biological activity. This type of intercalation happened only with DNA of B-form because of the steric factors, B-DNA has a large radius compared with the other forms (A-DNA, Z-DNA). B-DNA is predominant in active genes, in contrast to the others which are found in the inactive regions (Chen et al., 1983).

Finally intercalation in vivo involves two further types of interaction : i) frame shift misincorporation (mutation) and ii) the production of single base substitution (Shearman and Loeb, 1983 ; Shearman et al., 1983). The first one occurs as addition and deletion of nucleotides during DNA replication caused by the intercalator.

In an important human clinical study Unverferth et al., (1983) observed that four hours of doxorubicin pretreatment induced contraction, segregation, and ring formation in the nucleoli of human endomyocardial cells. Clumping of chromatin was also seen in case of continued doxorubicin therapy as were morphologic signs of decreased RNA and Protein synthesis.

1.2.3.2. Membrane Binding :

Doxorubicin has a high affinity of binding to cell membranes causing significant changes in their structure and functions (Murphree et al., 1976). This high affinity led Burke and Tritton (1985) to suggest that the cell membrane acts as a prime target site for the cytotoxic action of the drug, and the high affinity of doxorubicin for phospholipids suggests location in the lipid domains of biomembrane. This supported the idea of Manfait and Theophanides (1983) that cell death due to plasma membrane damage could be due to doxorubicin interaction with the cellular membrane protein and / or with the phospholipid domain. This binding plays an important role in one form of anticancer activity and also in the cardiotoxicity of this drug (Tokes et al., 1982 ; Tritton and Yee, 1982). The phospholipids cardiolipin and phosphatidyl serine provide the best membrane sites for doxorubicin binding (Gianni et al., 1983). These two phospholipids differ from others by bearing a negative charge. Phosphatidyl serine has a single negatively charged carboxylic acid group, while cardiolipin has two negatively charged phosphate groups; doxorubicin is capable of binding to these negatively charged phospholipids via its protonated amino sugar group (Goormaghtigh et al., 1980 a&b).

Henry et al., (1985) suggested that in addition to the above mechanism there is binding of the embedded dihydro-anthraquinone moiety of the drug in the phospholipid bilayer of the membrane. Karczmar and Tritton, (1979) concluded that hydrophobic interaction dominates the interaction of uncharged membrane phospholipid, phosphatidyl choline, with doxorubicin. Therefore according to the above explanation, cardiolipin has greater affinity for doxorubicin than Phosphatidyl serine and that has special importance in the development of cardiotoxicity because cardiolipin (the name is related to its cardiac localization) is the major lipid component of inner mitochondrial membranes (20% of the lipid component) which are mainly localized in the heart tissues. (Tritton et al., 1978). This phospholipid is important for mitochondrial function because most respiratory chain enzymes require

it for their full activities. The interaction of doxorubicin with that phospholipid could therefore be one of multisite effects on that chain (Goormaghtigh and Ruysschaert, 1984). Moreover cardiolipin is normally a minor cell membrane component, but it is increased in some tumours upon malignant transformation which could explain the susceptibility of such tumour cells to doxorubicin (Gianni et al., 1983 ; Goormaghtigh et al., 1980 a), and could be the common site for both the antitumour activity and cardiotoxicity of doxorubicin. Schlager and Ohanian, (1979 a&b) produced evidence of the effect of doxorubicin on cardiolipin synthesis. They found that cardiolipin was lost from the cell membrane in doxorubicin pretreated cells and they were unable to resynthesize it. The loss leads to the impairment of membrane function. This was supported by the finding of Okano et al., (1984) that doxorubicin produced about 50% inhibition in the phospholipid synthesis of cardiac cells in tissue culture. They found that Phosphatidyl choline was the most sensitive to inhibition followed by cardiolipin and this could alter membrane fluidity and function. In addition Jain , (1984) found that, as a result of lipid peroxidation, the organization of the phospholipids phosphatidyl choline and phosphatidyl ethanolamine in the membrane changed, leading to altered membrane fluidity and function and possible cell damage.

Two, groups, Tritton and Yee, (1982) ; Tritton et al., (1983) and Tokes et al., (1982) concluded that doxorubicin could produce a cytotoxic effect at the level of the tumour cell membrane without the need to penetrate the cell. This work is supported by Wingard et al., (1985) who used immobilized doxorubicin on cross-linked polyvinyl alcohol and found that cytotoxicity occurred under conditions of no detectable intracellular doxorubicin. Moreover they found that the immobilized doxorubicin was more lethal than free doxorubicin and they related that to the spacer arm between the immobilized drug and the support, in addition the linkage between doxorubicin and membrane allowing enough time for the drug to interact with the cell surface, producing changes in the membrane fluidity.

As already explained, doxorubicin membrane binding is due to ionic binding of the positively charged amino group of daunosamine sugar moiety and the negatively charged phospholipid. While movement of doxorubicin into the cells occurs passively as a simple diffusion transport of the unionized form through the membrane lipid (Dalmark and Storm, 1981 ; Dalmark and Hoffmann, 1983). It is clear that doxorubicin binds to the cell membranes when protonated, the pKa of sugar amino group moiety of doxorubicin is within the physiological range (Dalmark, 1981 ; Dalmark and Storm, 1981). In addition the pH of tumour cells is somewhat low, about 6.3 compared with that of normal cells 7.2 – 7.4, and at low pH the unionized doxorubicin that passes into the cell can be easily protonated and reactivated (Vaupel et al., 1981). While the passage of doxorubicin across the normal cell membrane in both directions is governed by four factors: the pH of the cells ; the binding of the charged portion of doxorubicin to the cellular constituents; the amount of dimerization and complex compound formation ; and lastly the environmental temperature (Siegfried et al., 1985). Moreover in the resistant tumour cells the efflux of doxorubicin is also regulated by an energy-dependent active transport mechanism (Supino et al., 1988).

1.2.3.3. Metal Chelation :

Hydroxyquinone compounds such as doxorubicin are able to form stable metal complexes with a variety of metal ions (Dabrowiak, 1980). Two complexes of doxorubicin are of special importance, these formed with copper and with iron. Mailer and Petering , (1976) ; Mikelens and Levinson, (1978) ; Phillips and Carlyle, (1981) state that copper can function in vivo as a cofactor for the binding of doxorubicin to DNA ; this requires the assumption that copper binds to the DNA phosphate on one side and to doxorubicin on the other.

The doxorubicin-Fe^{3+} complex is more stable than other metal complexes because of the three charges on the ferric ion and can undergo redox cycling when physiological reducing agents (such as reduced glutathione) are present, forming superoxide, hydrogen peroxide and hydroxyl radical (Myers et al., 1982). In spite of the high stability of the doxorubicin-iron complex, the drug can intercalate between the DNA base pairs by releasing the iron. The free drug produces high antitumour activity by DNA destruction (Beraldo et al., 1985). Lipid peroxidation by doxorubicin is catalysed in the presence of ferrous ions, that facilitate free radical production (Kappus et al., 1980). Other binding processes between doxorubicin and DNA are facilitated in the presence of Cu^{2+} or Fe^{2+} and become more stable than any intercalative mode of binding (Mikelens and Levinson, 1978). The iron - chelation site on doxorubicin is between the hydroxyl at C11 and the carboxyl at C12 (Muindi et al., 1984 ; 1985), so the intact C11 hydroxyl of doxorubicin and other anthracyclines is essential for iron binding and DNA damage.

According to the conclusion of Hibbs et al., (1984) the iron requirement of rapidly dividing cells is very high. Iron depletion due to doxorubicin interaction will affect tumour growth and contribute to cytotoxicity. Although doxorubicin produces mitochodrial lipid peroxidation in the presence of NADH as a reducing agent and under the iron requirement (Mimnaugh et al., 1985), doxorubicin-iron complex can initiate lipid peroxidation directly without the need of exogenous reducing agents (Gutteridge, 1984).

1.2.3.4. Redox Behaviour and Free Radical Formation :

A second biochemical activity of doxorubicin, involved in both its antitumour activity and cardiotoxicity, is its redox behaviour leading to free radical production. Doxorubicin can easily undergo one electron reduction forming a semiquinone, and

two electron reduction forming a dihydroquinone (Figure 7).

One electron reduction of doxorubicin to the semiquinone can occur either spontaneously at physiological pH (Pietronegro et al., 1974), or is catalyzed by flavin centered oxidoreductases such as cytochrome P-450 reductase or xanthine oxidase (equation 1) (Bachur et al., 1977 ; Pan and Bachur, 1980 ; Pan et al., 1981).

$$NADPH + 2Q \xrightarrow{\text{Quinone reductase}} NADP^+ + 2Q^- + H^+ \quad (1)$$

The semiquinone is aerobically reoxidized forming the parent molecule and the superoxide anion (equation 2) which is not reactive by itself, but can undergo several reactions forming hydrogen peroxide (equation 3), and hydroxyl radical by the reaction of hydrogen peroxide with the doxorubicin semiquinone (equation 4) (Sawyer and Valentine, 1981 ; Rowley and Halliwell, 1982 ; Bates and Winterbourn, 1982 ; Kalyanaraman et al., 1984).

$$Q^- + O_2 \longrightarrow Q + {}^{\cdot}O_2^- \quad (2)$$

$$2\,{}^{\cdot}O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \quad (3)$$

$$Q^- + H_2O_2 \longrightarrow Q + HO^- + {}^{\cdot}OH \quad (4)$$

The two last reactions which involve the production of hydrogen peroxide and hydroxyl radical can occur spontanously and are accelerated by superoxide dismutase. The hydroxyl radical can also be produced from hydrogen peroxide via catalysis of the iron salts, (equations 5, 6) (Muindi et al., 1985 ; Thornalley and Dodd, 1985).

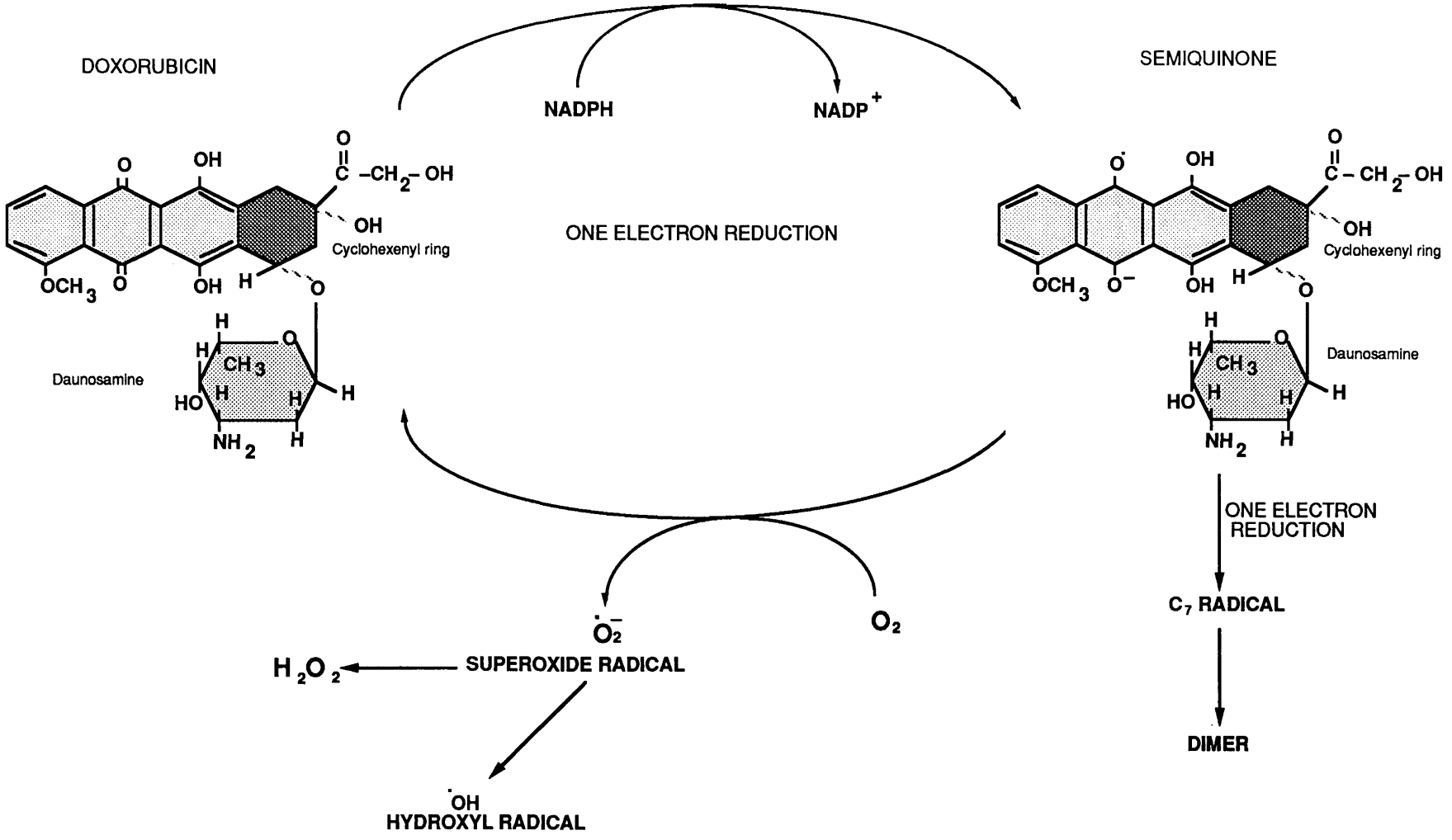


Figure 7 : One and two electron reduction of doxorubicin.

$$Fe^{3+}\text{–X} + Q^{-} \longrightarrow Fe^{2+}\text{–X} + Q \qquad (5)$$

$$Fe^{2+}\text{–X} + H_2O_2 \longrightarrow Fe^{3+}\text{–X} + HO^{-} + {}^{\cdot}OH \qquad (6)$$

Gianni et al., (1985) found that the doxorubicin - iron complex was not stable because of electron transfer from the drug to the iron forming a doxorubicin-free radical and ferrous ions (Fe^{2+}). This iron reacts with oxygen forming hydrogen peroxide or reacts with hydrogen peroxide forming hydroxyl radical. Thus, doxorubicin induced cellular injury can be due to either hydrogen peroxide production or hydrogen peroxide and superoxide mediated hydroxyl radical production under the catalytic activity of iron. Cellular injury can be established by the superoxide radical itself and also after secondary radical production (Figure 8) (Bulkley, 1987).

Normally there is a low free iron concentration in the cell and all of it seems to be chelated by doxorubicin itself (Jones et al., 1980). However Myers et al., (1982) produced evidence that the doxorubicin-iron complex can catalyze hydroxyl radical production. The oxidizing agent hydrogen peroxide and the hydroxyl radical cause DNA strand cleavage and membrane lipid peroxidation (Lown et al. , 1977 ; Fridovich , 1978 ; Mason , 1979 ; Yamauchi et al., 1989). Sugioka et al. , (1981) concluded that lipid peroxidation is iron-drug complex dependent ; in the absence of iron, the radicals produced do not cause lipid peroxidation. This is because of the low rate of hydroxyl radical production in the absence of the catalyst.

Hydroxyl radical has a short half life in the biological medium, and is active only near its formation site, thus localization of production of this radical near to the DNA is necessary for damage. This fact plays a critical role in doxorubicin cardiotoxicity (see 4.2) (Bachur et al., 1977 ; Ogura et al., 1979 ; Thayer, 1977).

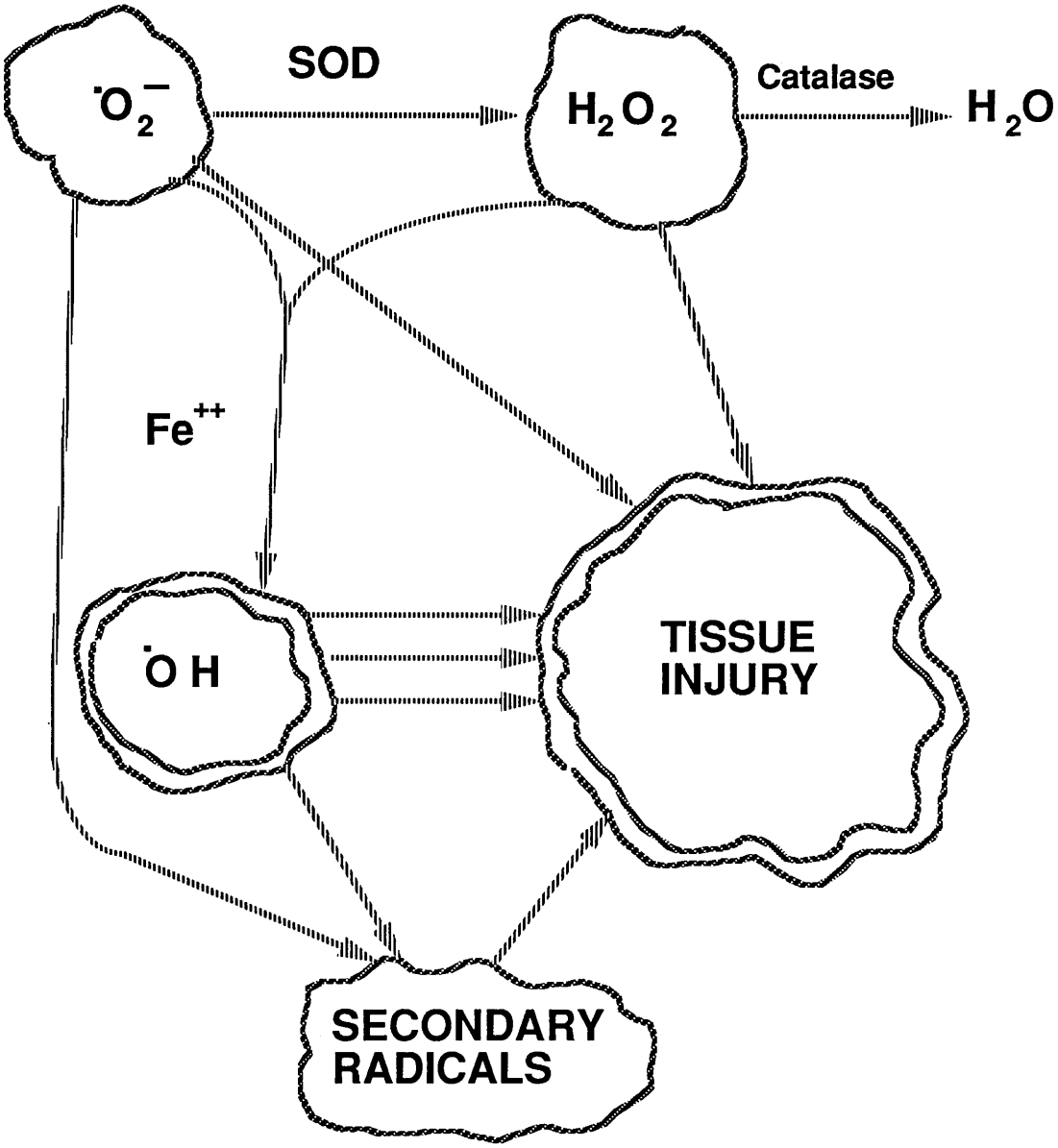


Figure 8 : Mechanism of cellular injury induced by superoxide radical.

1.2.3.5. Bioalkylation :

One-electron reduction of doxorubicin anaerobically produces the alkylating radical 7– deoxyaglycone and daunosamine (Bachur et al., 1977). This finding was supported by the work of Sinha et al., (1984) that isolated rat liver nuclei in the presence of NADPH and under anaerobic condition produced doxorubicin-semiquinone free radicals which can alkylate nuclear DNA. Doxorubicin semiquinone can rearrange, the unpaired electron moving to carbon number 7 forming C7-aglycone radical and daunosamine (Figure 9). These radical species derived from doxorubicin can easily alkylate cellular macromolecules such as DNA and protein or interact with themselves forming an aglycone dimer (Sinha and Sik, 1980 ; Sinha and Gregory, 1981). Two electron reduction of doxorubicin produces C7-quinone methide which act also as an alkylating species (Moore, 1977 ; Moore and Czerniak , 1981). Doxorubicin reduction and production of the C7-radical or quinone methide, with alkylation of DNA or protein, causes alterations in the stereochemistry of the sugar moiety and cyclohexenyl ring (Malatesta et al., 1984).

1.2.4. Doxorubicin Antitumour Activity :

In summary, the mechanisms of action of doxorubicin leading to inhibition of proliferation or cell death and its antitumour action are : i) effects related to DNA interaction; ii) effects related to membrane binding; iii) free radical formation (Gianni et al., 1983 ; Siegfried et al., 1983).

In vitro the anticancer effect depends on the dose and time of exposure to the

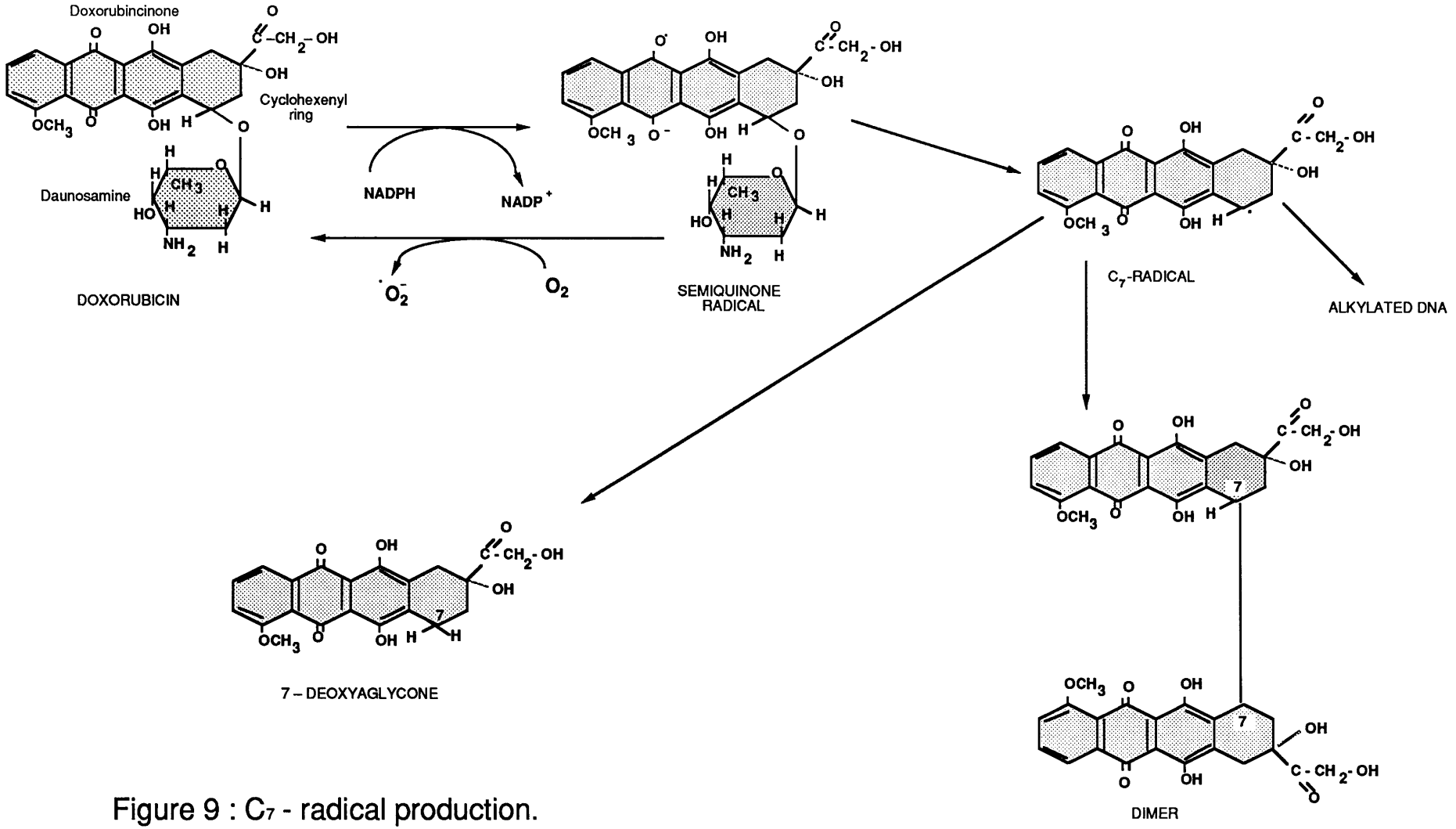


Figure 9 : C_7 - radical production.

agent, while in vivo the situation is different due to processes of drug activation or inactivation and its half life, tumour extracellular environment, and drug diffusion between tumour cells, which depends on the tissue vascularization (Tannock, 1968 ; Yesair et al., 1980). Cancers are not homogeneous because of the different metabolic characteristics of different tissues of origin and biochemical heterogeneity (Fidler and Hart, 1982), however tumours differ in their ability to effect DNA repair (Erickson et al., 1980) and in their superoxide dismutase, catalase, and glutathione peroxidase contents (Marklund et al., 1982).

Intercalation and / or binding of doxorubicin to DNA does not cause cell death by inhibiting DNA synthesis alone. Other cytotoxic effects of doxorubicin may be responsible in part, e.g. DNA damage, inhibition of polymerase and RNA synthesis (Momparler et al., 1976 ; Schwartz and Kanter, 1981). Doxorubicin kills both dividing and non dividing cells, but has more activity against dividing ones (Barranco, 1975 ; Theiss et al., 1977). Dividing cells are killed by the effects on DNA and RNA synthesis and non dividing cells as by inhibition of RNA synthesis alone (Momparler et al., 1976 ; Bachur et al., 1978 ; Barranco, 1986). Killing of nondividing cells has special importance in tumour chemotherapy, because large fractions of solid tumour in vivo are composed of non dividing cells, however these cells accumulate less doxorubicin than dividing ones, and are less sensitive (Durand and Olive, 1981 ; Bhuyan et al., 1981).

There is evidence that tumour cells are more sensitive to doxorubicin when they are treated in an aerobic state than in the hypoxic condition (Smith et al., 1980 ; Tannock and Guttman, 1981 ; Born and Eichholtz-Wirth, 1981), and that could be related to oxygen radical production. This may be another cause of lessened doxorubicin sensitivity in nondividing tumour cells, as the majority of cells in solid tumours in vivo are in a hypoxic state (Dethlefsen, 1980). The greater killing effect on tumour cells in the presence of oxygen seems to be related to ATP production, since doxorubicin sensitivity has been shown to be increased as the intracellular ATP level increases (Colofiore et al.,1982). However Teicher et al., (1981) ; Kennedy

et al., (1983) reported the opposite, that tumour cells are more sensitive to anthracyclines in hypoxic conditions in vivo. This contradiction seems to be due to the difference in tumour cell origin and possibly membrane integrity and emphasises the observed differences in response to doxorubicin of different tumours in vivo.

It has been observed that doxorubicin can kill tumour cells at a concentration which is less than that needed for inhibition of DNA synthesis (Gianni et al.,1983). There are several possible mechanisms e.g. glutathione utilization or lipid peroxidation (see below).

1.2.5. Pharmacokinetics of Doxorubicin :

Knowledge of doxorubicin pharmacokinetics in man has established a basis for its distribution and localization in both healthy and malignant tissues in order to design a chemical control program against cancer in vivo. Doxorubicin is metabolised and cleared from the body through the redox pathway under the catalytic activity of the abundant cytoplasmic NADPH-dependent enzyme, aldo-keto reductase. This enzyme is widely distributed among different mammalian tissues (Bachur et al., 1976).

1.2.5.1. Administration :

Doxorubicin cannot be administered orally because the acid pH of the stomach splits the glycosidic bond, resulting in an inactive aglycone (Bachur et al., 1976). The intravenous route is the preferred route for therapy in spite of its long terminal

elimination half-life which is due to wide distribution into tissues such as heart (Calabresi and Parks, 1985). The incidence of cardiotoxicity has been shown to be lower in patients given the drug weekly in divided doses when there are lower peak plasma concentrations compared with an intravenous bolus every three weeks (Dorr and Alberts, 1982). There are restrictions, due to local toxicity, when using different routes of drug administration, especially in the human. Intraperitoneal doxorubicin administration seems to be more effective in the treatment of certain types of neoplasm such as ovarian cancer (Tobias and Griffiths, 1976). This may be due to delayed drug clearance, because of the large fluid volume using this route (Dedrick et al., 1978).

1.2.5.2 Distribution :

The vast array of tissue binding sites for doxorubicin (1.2.3.) causes prolonged antitumour activity after a single intravenous dose and minimises its plasma concentration (Benjamin et al., 1973 ; 1977). Doxorubicin pentrates rapidly into the heart, kidneys, lung, liver and spleen, but seems not to cross the blood-brain barrier (Calabresi and Parks, 1985). The therapeutic efficacy of doxorubicin when used systemically is decided by its penetration into sites of action, such as nuclei. The variation in the drug distribution between different organs and tissues in the same organism is related the density of nuclei per weight of tissue (Terasaki et al., 1984). There are also differences in doxorubicin concentration in different tumour tissues after treatment (Ozols et al., 1979), which are dependent on the route of drug administration. Cummings et al., (1986) claimed that the long retention time of doxorubicin in different organs was due to the slow release of nuclear-bound drug from the tissues.

1.2.5.3. Metabolism :

Benjamin et al., (1973 ; 1977) observed that doxorubicin is normally metabolised and cleared from the plasma after administration, with a mean elimination half-life of about 30 hours in the human (rather long compared with other cytotoxic agents). Doxorubicin and its active metabolite doxorubicinol (DOX–ol) are split by the activity of widely distributed microsomal glycosidases to inactive aglycones. These aglycones undergo demethylation and conjugation to sulphate or glucuronide esters and are excreted in the bile (Bachur et al., 1976 ; Cummings et al., 1986). Weenen et al., (1984) observed that there was a clear individual and species discrepancy in the case of doxorubicin metabolism. They related this to the specificity of the enzymes and the production of doxorubicin metabolites, especially alcohols, which are therapeutically active.

Six doxorubicin metabolites were separated from human plasma using thin layer chromatography (Benjamin et al., 1977); three aglycones and three other polar metabolites (Figure 10). Doxorubicinol is the most active and both this metabolite and the parent drug could be reduced to inactive deoxyaglycone. Brenner et al., (1985) were able to detect 7-deoxyaglycone in human plasma after doxorubicin administration, using thin layer and high performance liquid chromatography.

1.2.5.4. Excretion :

About 50% of doxorubicin and its metabolites are cleared in the urine, bile and feces within five days after intravenous administration. The remaining 50% seems to be retained by the body tissues (Riggs et al., 1977 ; Benjamin et al., 1977). The polar metabolites appear in the urine in significant concentrations, in contrast to the small urinary concentration of aglycones, while significant amount of parent drug is excreted unchanged (Calabresi and Parks, 1985).

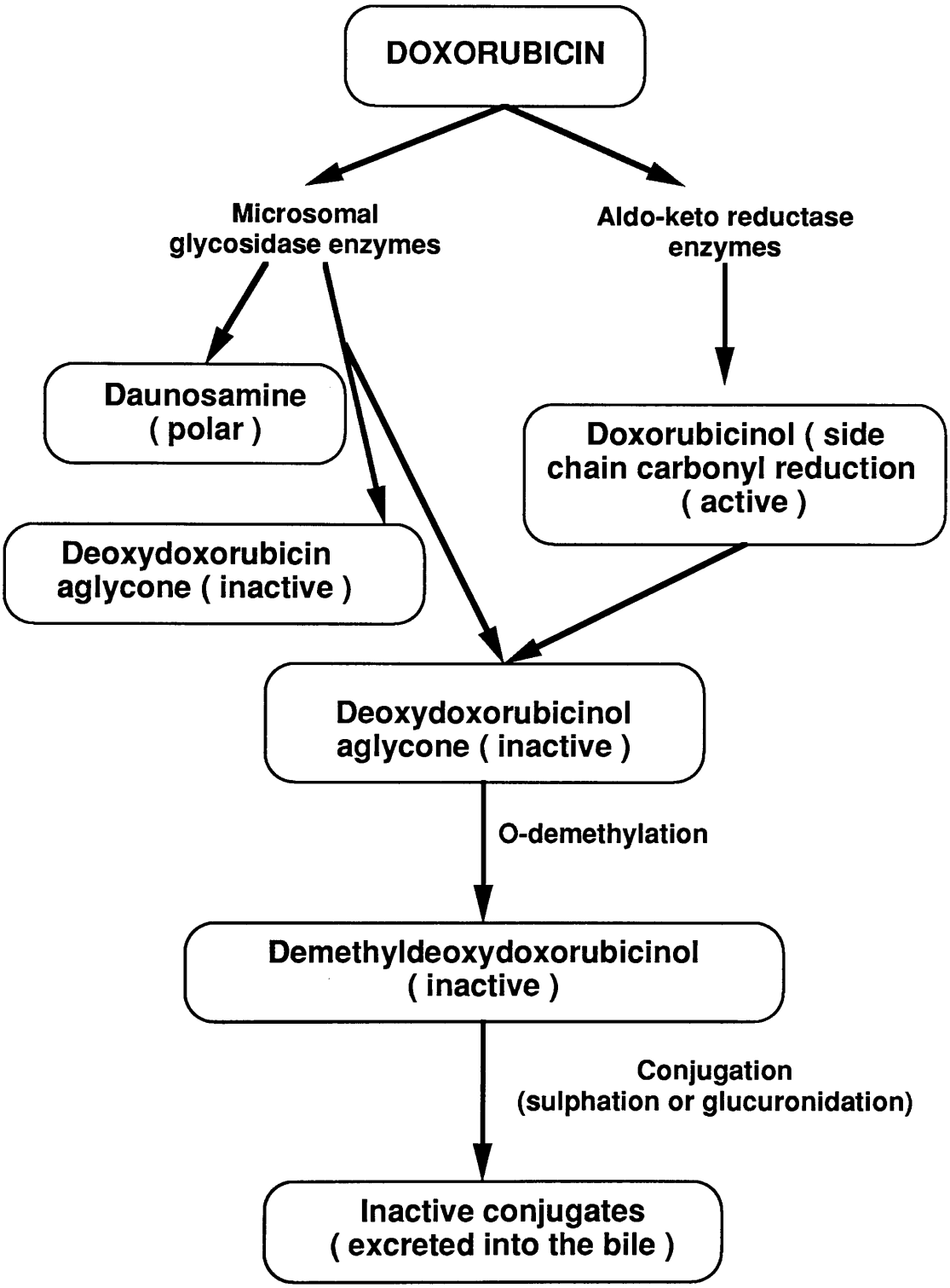


Figure 10 : Doxorubicin metabolism

1.2.6. Doxorubicin Side Effects :

One of the most important problems in doxorubicin therapy is the development of side effects i.e. toxicity to normal tissues. Doxorubicin is toxic against normal proliferating tissues such as bone marrow, gastrointestinal epithelium (presenting as stomatitis, nausea, vomiting and diarrhoea), hair follicles (alopecia), skin (necrosis) and renal epithelium (nephrotoxicity). These effects are classified as acute doxorubicin toxicity. Doxorubicin also induces nephrotic syndrome, and dose dependent acute and chronic glomerular lesions (Hall et al., 1986), however the principle treatment-limiting side effect is cardiotoxicity (Sikic et al., 1985).

1.2.6.1. General Toxicity :

Doxorubicin toxicity involves impairment of several organ functions including heart and bone marrow. This happens shortly after treatment (Bristow et al., 1978). Doxorubicin induces suppression of bone marrow proliferative elements leading to leukopaenia (Harris et al., 1975).

Skin necrosis is one of the acute side effects that develops due to intradermal doxorubicin injection or its leakage subcutaneously in the perivascular space following intravenous injection. It begins as a small ulcer due to killing of some skin cells at the site of injection. The drug is then removed from the dead cells and taken up by those nearby and the lesion extends in width and depth and may reach to the bones or joints causing extravasation of the skin (Gianni et al., 1983 ; Averbuch et al., 1986).

1.2.6.2. Cardiac Toxicity :

Doxorubicin accumulates rapidly in heart muscule and causes cardiotoxicity which may be acute or chronic according to the dose and duration of action. Lampidis et al., (1981) observed nucleolar fragmentation which indicates a severe toxic effect. The full mechanism of the cardiotoxicity is not yet understood but is thought to be related to changes in membrane fluidity (Goormaghtigh, et al., 1980 a & b).

1.2.6.2.1. Acute Cardiac Toxicity :

Symptoms depend on dose, the main symptoms are mild depression of myocardial function. Atrial or ventricular arrhythmias are seen within a few hours of doxorubicin treatment, which may develop due to a rise in plasma histamine or catecholamine concentration (Unverferth et al., 1982 ; Decorti et al., 1989). Pericarditis and myocarditis have also been reported as symptoms of acute cardiomyopathy. These symptoms mainly develop in elderly patients and disrupt normal cardiac function (Bristow et al., 1978).

1.2.6.2.2. Chronic Cardiac Toxicity :

This type of toxicity is irreversible and dependent on the total doxorubicin dose, since the effects of the drug are cumulative (Minow et al., 1975). The main clinical signs of doxorubicin cardiomyopathy are biventricular failure, tachycardia, shortness of breath, distention of neck veins, hepatomegaly, cardiomegaly and pleural effusion (Von Hoff et al., 1979). The development of congestive heart failure is dependent on cumulative dose. The dose rate of doxorubicin administration in which there is

risk of the development of congestive heart failure is about 500 to 550 mg doxorubicin / m^2 body surface area (Belli and Piro, 1977 ; Minow et al., 1977 ; Sallan and Clavell, 1984). The latency period and risk of developing heart failure varies depending on the general condition of the patient, including the age ; young adults are more tolerant to cumulative doses than the aged and small children (Von Hoff et al., 1979 ; Brockmeier et al., 1984 ; Sallan and Clavell, 1984). Another factor which facilitates development of congestive heart failure in doxorubicin treatment is previous mediastinal irradiation (Belli and Piro, 1977 ; Billingham et al., 1977 ; Praga et al., 1979 ; Von Hoff et al., 1979).

Doxorubicin cardiomyopathy begins before the development of congestive heart failure and is most severe in the left ventricle and intraventricular septum and less so in the right ventricle and both atria (Van Vleet et al., 1980).

Microscopically, human myocardial tissues that develop chronic doxorubicin cardiomyopathy are characterised by vacuolar degeneration of the cardiac cells. This is due to distention and swelling of the sarcoplasmic reticulum with interstitial edema and myofibrillar lysis. The mitochondria remain intact and degenerate after the death of myocytes (Suzuki et al., 1979). Rahman et al., (1982) described ultrastructural changes in the cardiac tissues of mice after doxorubicin administration, which included loss of myofibre elements, mitochondrial damage, swelling of sarcoplasmic reticulum, increased myeloid body accumulation and some nuclear abnormalities. Mitochondrial damage is due either to membrane lipid peroxidation, irreversible depletion of its proteins because of polymerase inhibition, or inhibition of coenzyme Q_{10} (a key enzyme of oxidative phosphorylation) (Ferrero et al., 1975 ; Folkers et al., 1977).

The mechanisms by which doxorubicin produces cardiomyopathy can not be separated from its biological activity as an antineoplastic agent. Cardiac mitochondria and sarcosomes form superoxide anion in the presence of doxorubicin (Doroshow and Reeves , 1981). These are the most prominent sites for doxorubicin injury , and are also the intracellular organelles regulating Ca^{2+} which is utilized by the contractile

protein. Disrupted calcium regulation is related to the development of cardiomyopathy (Bachmann and Zbinden , 1979 ; Gianni et al., 1983 ; Revis and Marusic , 1979). Jensen , (1986) suggested that in chronic doxorubicin cardiomyopathy the ability of sarcoplasmic reticulum to sequester Ca^{2+} is impaired, causing intracellular buildup of this ion and cell death. Ca^{2+} deficiency occurs only in the cells that are still functional. There is impairment of Ca^{2+} release from sarcoplasmic reticulum at the beginning of systole due to restriction on its gain during related diastole (Jensen , 1986).

Cardiac tissue contains little superoxide dismutase compared with others and this makes the heart more susceptible to superoxide radical injury (Doroshow et al., 1979) ; cardiac tissues also have lower catalase activity than others (Revis and Marusic , 1978). These enzymes and glutathione peroxidase are capable of disposing of hydrogen peroxide which is a product of superoxide dismutase activity. It was also noticed that within 24 hours of doxorubicin treatment glutathione peroxidase reached its nadir level in cardiac tissue, and took about five days to recover (Revis and Marusic , 1978 ; Doroshow et al., 1979).

Olson et al., (1980) studying cardiac cells, noticed that doxorubicin caused an acute reduction in the reduced form of glutathione (GSH) and concluded that GSH may play an important role in the protection of the heart against doxorubicin cardiotoxicity. Fabregat et al., (1984) suggested that doxorubicin cardiotoxicity is due to its interaction with the SH groups of certain enzymes. They also concluded that heart tissues contain low GSH level in comparison with others, nevertheless it is still the main SH-protecting compound in the cardiocytes. Heart tissues are therefore highly susceptible to radical injury by doxorubicin.

Finally, there is evidence that doxorubicin reaching the heart is metabolically reduced to doxorubicinol in cardiac tissues by the activity of reductase enzyme (Von Wartburg and Wermuth, 1980). This metabolite was found to be associated with the cardiotoxicity of doxorubicin (Tacca et al., 1985).

1.2.6.3. Lipid Peroxidation :

Doxorubicin induces lipid peroxidation, an oxygen-dependent deterioration of unsaturated fatty acids under the influence of free radicals such as superoxide and hydroxyl radicals. This is accompanied either by physiological processes such as changes in prostaglandin synthesis in aging, or pathological processes such as liver injury due to certain chemical agents (Sevanian and Hochstein, 1985). Biologically three events must be considered at the level of the cell membrane as a result of lipid peroxidation. First is the impairment of membrane enzymes and loss of function. Second is inactivation of cell organelles due to attack of highly active free radicals produced. Third is the production of certain cytotoxic compounds such as aldehyde through membrane lipid peroxidation processes (Esterbauer, 1982).

The first step in lipid peroxidation chain of unsaturated fatty acid in biological systems is the initiation of lipid radicals in the presence of free radicals (such as $^{\cdot}O^{-}_{2}$ and $^{\cdot}OH$) (equation 1).

$$RH + \text{Free radical} \longrightarrow R^{\cdot} \qquad (1)$$

The second step involves the combination of lipid radical with molecular oxygen forming lipid peroxy radical (equation 2).

$$R^{\cdot} + O_2 \longrightarrow {}^{\cdot}RO_2 \qquad (2)$$

This lipid peroxy radical can attack another unsaturated lipid molecule forming lipid hydroperoxide and a lipid radical which can initiate another reaction (equation 3).

$$^{\cdot}RO_2 + RH \longrightarrow ROOH + {}^{\cdot}R \qquad (3)$$

Lipid hydroperoxides are unstable and can be easily decomposed under the catalytic activity of metal ions such as iron, producing new lipid peroxy and alkoxy radicals which also can initiate another peroxidation chain (equations 4 and 5).

$$Fe^{2+} + ROOH \longrightarrow {}^{\cdot}RO + OH^{-} + Fe^{3+} \quad (4)$$

$$Fe^{3+} + ROOH \longrightarrow {}^{\cdot}RO_2 + H^{+} + Fe^{2+} \quad (5)$$

On the other hand ferrous ions can interact directly with the molecular oxygen forming hydroxyl radical or other reactive radicals (equations 6 , 7 and 8).

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + {}^{\cdot}O^{-}_2 \quad (6)$$

$$2{}^{\cdot}O^{-}_2 + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (7)$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^{-} + Fe^{3+} \quad (8)$$

These radicals can easily initiate lipid peroxidation in biological and non biological systems by abstraction of hydrogen atoms from unsaturated fatty acids (equations 3 , 9 , and 10).

$$^{\cdot}RO + RH \longrightarrow ROH + {}^{\cdot}R \quad (9)$$

$$^{\cdot}OH + RH \longrightarrow H_2O + {}^{\cdot}R \quad (10)$$

(Fridovich and Porter , 1981 ; Esterbauer, 1982 ; Gutteridge , 1984 ; Sevanian and Hochstein , 1985).

In these instances semiquinone radicals of doxorubicin can initiate lipid

peroxidation by the radical species derived from semiquinone autoxidation such as superoxide, hydrogen peroxide and more likely hydroxyl radicals. All of these activated oxygen radicals are involved in membrane lipid peroxidation by doxorubicin, especially those lipids in the nuclear envelope. This occurs through the oxidation-reduction cycle mediated by membrane-bound NADPH: cytochrome P-450 reductase which is abundantly available in the nuclear, microsomal and mitochondrial membranes (Figure 11) (Mimnaugh et al., 1985).

Most peroxidation of lung microsomes seems to proceed non enzymatically while in liver the mechanisms are mainly enzymatic by alteration of protective agents against lipid peroxidation (Sevanian et al., 1982). Dietary vitamin E plays an important role in controlling lipid peroxidation of lung and liver microsomes ; lipid peroxidation in lung microsomes is less than that of the liver because vitamin E concentrations are higher in the lung (Sevanian et al., 1982). The endogenous antioxidant GSH inhibits radical activity (Llesuy et al., 1985 ; Mimnaugh et al., 1985). But lipid peroxidation can also be inhibited by using exogenous agents which chelate metal cations. These results suggest that iron is intrinsically involved in the peroxidation of lipid (equations 4 – 8) (Kornbrust and Mavis , 1980 ; Aust and Svingen , 1982).

Another finding is the impairment of Ca^{2+} transport in doxorubicin treated mice (Llesuy et al., 1985), and that was due to myocardial membrane lipid peroxidation which is occurs in the early development of cardiomyopathy.

In vitro and in vivo doxorubicin induces hepatic microsomal lipid peroxidation by stimulating NADPH dependent oxygen species. At the same time and as a result of that, doxorubicin produces impairment of the hepatic drug metabolism or hepatic drug monooxygenation due to compromise of both membranous and cytosolic enzymes such as cytochrome P-450 and glucose-6-phosphate (Mimnaugh et al., 1981). Therefore more studies of doxorubicin and lipid peroxidation are required in order to improve the therapeutic efficacy and prevent or reduce related cardiotoxicity (Myers et al., 1977 ; Llesuy et al., 1985).

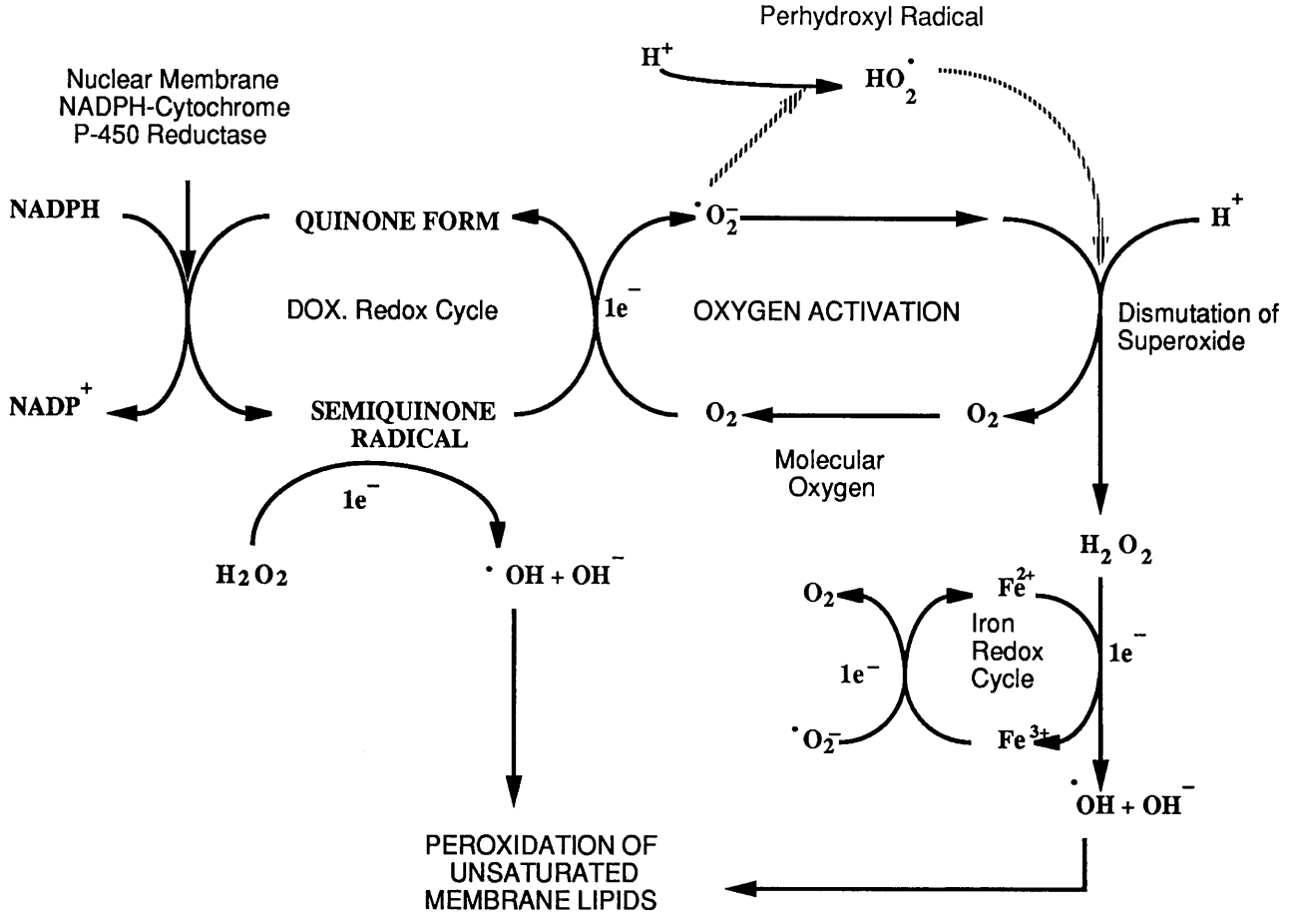


Figure 11 : Lipid peroxidation induced by hydroxyl radical.

1.2.6.3.1. Measurement of Lipid Peroxidation :

Methods for measurement of lipid peroxidation depend on determination of the decomposition products. The compound that gives most reproducible results for lipid peroxidation is malondialdehyde (MDA) which represents a common product of polyunsaturated fatty acid decomposition; it arises from fatty acid hydroperoxides during the processes of peroxidation.

Different techniques have been described for determination of MDA in tissues and biological fluids. Yagi et al., (1968) were the first to produce an acceptable method which is still used nowadays. These methods are based on the reaction of MDA with thiobarbituric acid (TBA) forming a MDA-TBA adduct, which is measured spectrophotometrically or fluorometrically after its extraction into butanol (Satoh, 1978 ; Ledwozyw et al., 1986).

HPLC methods for separation and fluorescence detection after MDA-TBA adduct formation have been described (Bird et al., 1983 ; Yu et al., 1986 ; Therasse and Lemonnier , 1987) using reversed-phase. Wong et al., (1987) adapted an HPLC method after acid hydrolysis for more sensitivity and reproducibility, but most methods of lipid peroxidation measurement are ultimately based on the Yagi method.

1.2.7. Minimizing of Doxorubicin Side Effects :

Several attempts have been made in the last few years to solve the problem of doxorubicin side effects and the interference with its antineoplastic activities. Legha et al., (1982) reported that slow continuous intravenous infusion of doxorubicin will

reduce its peak plasma levels thus reducing the amount of drug taken up by the cardiocytes but not by tumour cells. The end result is reduced acute cardiotoxicity and severity of nausea and vomiting. This result supported the idea of Bristow , (1982) that most of the acute cardiotoxicity is due to histamine , catecholamine and prostaglandin release such as occured following rapid drug administration. Raijmakers et al., (1987) supported previous results and also proved that human bone marrow clonogenic cells when exposed to either low doxorubicin doses for a long time or large doses for a short time behaved as in vivo studies i.e. increased mortality in the second case.

Carnitine, a naturally occurring compound with high levels located in the heart tissues, plays a role in the long chain fatty acid transfer into heart mitochondrial matrix. There is evidence that this compound has a protective role against doxorubicin cardiomyopathy in mice and rats (Alberts et al., 1978 ; McFalls et al., 1986).

Entrapment of doxorubicin in positively charged lipoprotein as shown by Rahman et al., (1980 ; 1982) reduced acute and chronic cardiac damage in mice. This was due to reduction of the in vivo uptake of drug by cardiac tissues compared with the net doxorubicin and doxorubicin entrapped in negatively charged liposomes. This happened without any loss in the antitumour activity (Rahman et al., 1980 ; 1982). While Hynds (1986) reached the conclusion that entrapment of doxorubicin with the low density lipoprotein could increase therapeutic efficacy of this drug as a cytotoxic agent by increasing its pentration into the tumour since the tumour cell receptors were induced for this complex, and recently Jones et al., (1989) found that incorporation of doxorubicin into controlled ion exchange microspheres can achieve significant concentration of the drug in the tumour tissue rather than the normal.

Okamoto and Ogura, (1985) proved experimentally that doxorubicin-lipid peroxidation could be blocked using tocopherol, coenzyme Q and riboflavin analogues. This was supported by the finding of Hino et al., (1985) that doxorubicin reduced FAD levels and glutathione reductase activity, but this recovered following the administration of riboflavin-butyrate. Coenzyme Q_{10} increases time

survival of mice treated by doxorubicin (Shinozawa et al., 1984).

The most important deleterious effect of doxorubicin on the heart is the destruction of free radical scavengers which can repair the DNA damage produced in vivo (Potmesil et al., 1984). In vitro and in vivo alpha-tocopherol (vitamin E) has been investigated by several authors, and can delay or lessen cardiac toxicity in animal models by prevention of destructive peroxidation (Mimnaugh et al., 1979 ; Wang et al., 1980). Another radical scavenger N-acetylcysteine (NAC), a sulfhydryl compound, was found to minimize lethality in mice when administered pre doxorubicin (Doroshow et al., 1981). This compound does not interfere with the chemotherapeutic effect of doxorubicin, but reduces or prevents lipid peroxidation and acts as a hydroxyl radical scavenger (Doroshow et al., 1981). Although NAC protects the heart against doxorubicin and lessens cardiotoxicity in dogs (Herman et al., 1985), NAC did not alter the acute nuclear effects on cardiocytes (1.2.6.2.1) when given to doxorubicin treated patients (Unverferth et al., 1983).

1.2.8. Resistance of Tumours to Doxorubicin :

One of the major obstacles in current cancer chemotherapy is the ability of tumour cells to develop resistance against anticancer chemotherapeutic agents (Griswold et al., 1981; Kaye and Merry, 1985). Such resistance may be either natural or acquired and tumour bulk may involve different clones of cells which have different drug sensitivities (Heppner et al., 1978 ; Shapiro et al., 1981). This heterogeneity of drug response may be due to :

(1) Changes in rates of transport of drug into and out of the cell.

(2) Changes in the ability to activate or deactivate the drug.

(3) Gene amplification and high molecular weight membrane glycoprotein production.

(4) Variability in DNA repair mechanism induced by the drug.

(5) The presence of alternative biochemical pathways for drug-inhibited metabolic steps.

(Lepage et al., 1964 ; Barranco et al., 1972 ; Barranco et al., 1975 ; Biedler et al., 1975 ; Schimke et al., 1978 ; Riordan et al., 1985 ; Croop et al., 1988).

In the case of doxorubicin treatment, resistance occurs due to:

(1) Reduced doxorubicin uptake by the tumour cells.

(2) Intracellular biochemical modifications leading to reduced drug-induced DNA damage.

(3) Increased drug degradation at a site other than tumour e.g liver.

(4) High drug efflux, with low intracellular accumulation and binding.

(Capranico et al., 1986 ; Vrignaud et al., 1986 ; Bellamy et al., 1988 b ; Supino et al., 1988 ; Gigli et al., 1989).

Continuous exposure of doxorubicin-sensitive tumours to increasing drug concentrations can cause them to develop a doxorubicin resistant phenotype. Due to development of resistance, modifications of doxorubicin action and changes in its intracellular accumulation and distribution compared with the wild strain have been observed (Supino et al., 1986 ; Twentyman et al., 1987 ; Supino et al., 1988).

There is evidence that some solid tumours have more than one clone of tumour cells and that the clones have a different DNA content (Barlogie et al., 1978 ; Barranco et al., 1982). This was confirmed by the finding of Schumann et al., (1978) that some of these clones are killed or inhibited by the chemotherapy , while others within the same tumour are not affected. The non-affected clones increase during the same interval producing resistant cells which predominate causing the death of the patient.

Several studies have proved that plasma membranes of tumour cells resistant to doxorubicin contain a phosphorylated glycoprotein P-180. This glycoprotein is not detected in doxorubicin-sensitive cells (Garman and Center, 1982 ; Garman et al., 1983 ; Center, 1983 ; 1985). The same authors claim that doxorubicin resistance depends on P-180 availability, and that the biological activity of this glycoprotein is highly regulated by phosphorylation. They noticed that P-180 becomes inactive when it is superphosphorylated using N-ethylmaleimide or the calmodulin inhibitor trifluoperazine ; cells revert to drug-sensitive phenotype and retain the drug. Capranico et al., (1986) clamed that membrane alteration is the only mode of doxorubicin resistance in tumours. Marsh and Center (1985) said that three glycoproteins of 20, 180 and 220 kilodaltons (P-20 ; P-180 and P-220) were phosphorylated in doxorubicin-resistant plasma membrane and are either absent or present in very low amounts in the membranes of sensitive cells. Hamada et al., (1987) involved membrane glycoproteins P-170 – P-180 in the overall activity of cellular resistance. These glycoproteins were highly phosphorylated in membranes of cells which had reverted to drug sensitivity.

Inaba et al., (1979) suggested that doxorubicin efflux mechanisms in resistant tumour cells are energy dependent. This agrees with results showing that treatment of revertant or inherently doxorubicin-resistant tumours with metabolic inhibitors such as sodium azide (an oxidative phosphorylation inhibitor) or verapamil (a calcium channel blocker) or cyclosporin A (an immunosuppressive agent) have a potential clinical role in overcoming drug resistance in human and animal tumours by inhibiting doxorubicin efflux or by altering the biophysical properties of the plasma membrane (Tsuruo et al., 1983 ; Rogan et al., 1984 ; Kessel and Wilberding, 1985 ; Merry et al., 1986 ; Twentyman et al., 1987 ; Bellamy et al., 1988 a ; Supino et al., 1988 ; Cairo et al., 1989 ; Huber et al., 1989). In addition Merry et al. (1987) showed that verapamil inhibits doxorubicin efflux by increasing intracellular binding and this was confirmed by the finding of Hindenburg et al. (1987) that verapamil displaces

doxorubicin from hydrophobic into the hydrophilic cellular components, reducing doxorubicin release.

Capranico et al., (1986) and Supino et al., (1986 ; 1988) stated that even though doxorubicin resistance appears to be related to low drug uptake and retention, there is only a weak relationship between intracellular doxorubicin concentration and cytotoxicity ; they found a difference in intracellular doxorubicin distribution shortly after treatment and noticed that the nuclear / cytoplasmic ratio seemed higher in sensitive than resistant cells while the number of DNA breaks was fewer in resistant tumour cells. Finally Chauffert et al., (1986 ; 1987) found that the antiarrhythmic drug amiodarone and its main metabolite desethylamiodarone were more efficient and less toxic than verapamil when used at their maximal tolerated levels in reversing inherent doxorubicin resistance in rat colon cancer cells. The mechanism by which amiodarone reverses doxorubicin resistance seems to be similar to that of verapamil as a calcium channel blocker, but this needs more study, especially in tumours from human sources.

The other problems which need to be resolved in cancer chemotherapy are the absence of methods for measurement in vitro of tumour susceptibility to chemotherapy, and the effect of drugs on the intermediary metabolism of tumour cells.

The standard method for assessing resistance and sensitivity is the measurement of viability following treatment in vitro. Some of the available methods are a) measuring isotopic precursor incorporated into protein of the viable cells e.g [^{3}H]-leucine and [^{35}S]-methionine (Merry et al, 1984 ; 1987) b) Determination of clonogenicity in soft agarose after doxorubicin treatment (Shoemaker et al., 1983 ; Louie et al., 1986) c) Tetrazolium based chemosensitivity assay (Ware, 1985).

The rapid measurement of markers of cell resistance and changes in these markers when tumour cells are treated by drugs could form the basis for tumour classification. The most important markers in case of anthracycline resistance are intracellular glutathione ; glutathione-S-transferase ; membrane fluidity and membrane

glycoprotein. At present there is no clear idea of the exact mechanism of development of natural and acquired resistance against doxorubicin chemotherapy and the advantages - disadvantages of each of these markers.

The glutathione-S-transferases (GST) are a complex group of isoenzymees, widely distributed in man and different animal species. They are involved in several biological functions, with a central role in the biotransformation and elimination of xenobiotics and toxic metabolites, forming GSH conjugates (Stockman et al., 1985 ; Beckett and Hayes, 1987). In man three major types have been identified (basic, neutral and acidic) subdivided on the bases of their isoelectric point (Mannervik, 1985 ; Beckett and Hayes, 1987).

Increased levels of GST have been used as an indicator of primary tumour development (Shea et al., 1988). Acidic GST has been shown to be increased in some resistant tumours (Fairchild et al., 1987 ; McQuaid et al., 1989), although this is not an invariable finding (Meijer et al., 1987). GST is however regarded as a readily measurable marker of tumour resistance or sensitivity (Smith et al., 1989). There is positive relationship between GSH and GST activity in the resistant tumour cells (Evans et al., 1987). Thus evaluation of GST and GSH could be of use as preliminary markers, both for cancer development and as an indicator of resistance against some chemotherapeutic agents.

1.3. Glutathione (GSH) :

Glutathione is one of the most prevalent non protein thiol compounds which is present in all cells, both prokaryotic and eukaryotic at concentrations up to 10 mM. It is a tripeptide containing the important biological and biochemical active thiol group (–SH). The main building blocks of GSH are the three amino acids glutamate, cysteine, and glycine (γ–glu–cys–gly). Two peptide bonds, two carboxyl groups and one each of amino and thiol groups are the main characteristic features of GSH molecule (Figure 12) (Kosower, 1976 ; Arrick and Nathan, 1984). This low molecular weight (307.3 daltons) molecule contains many hydrophilic groups and has high aqueous solubility.

1.3.1. Tissue Localization of GSH and Its Clinical Importance :

GSH is present at variable concentrations in all mammalian tissues, most plant tissues and bacteria. It is the most abundant thiol reducing agent in mammalian tissues. Generally eukaryotic cells contain two pools of GSH , a large cytoplasmic pool and a smaller mitochodrial one which is obligatory for cell survival (Gaetjens et al., 1984). Among body tissues, liver contains the highest GSH concentration, kidney contains about one third of the liver GSH while cardiac muscle contains only about one tenth of that in liver. In all aging body tissues GSH concentration decreases due to enhanced oxidation or decreased synthesis and increased utilization in the removal of peroxides and detoxification of foreign compounds (Hazelton and Lang , 1980). Depletion of the cytoplasmic pool in itself would not be expected to cause cell

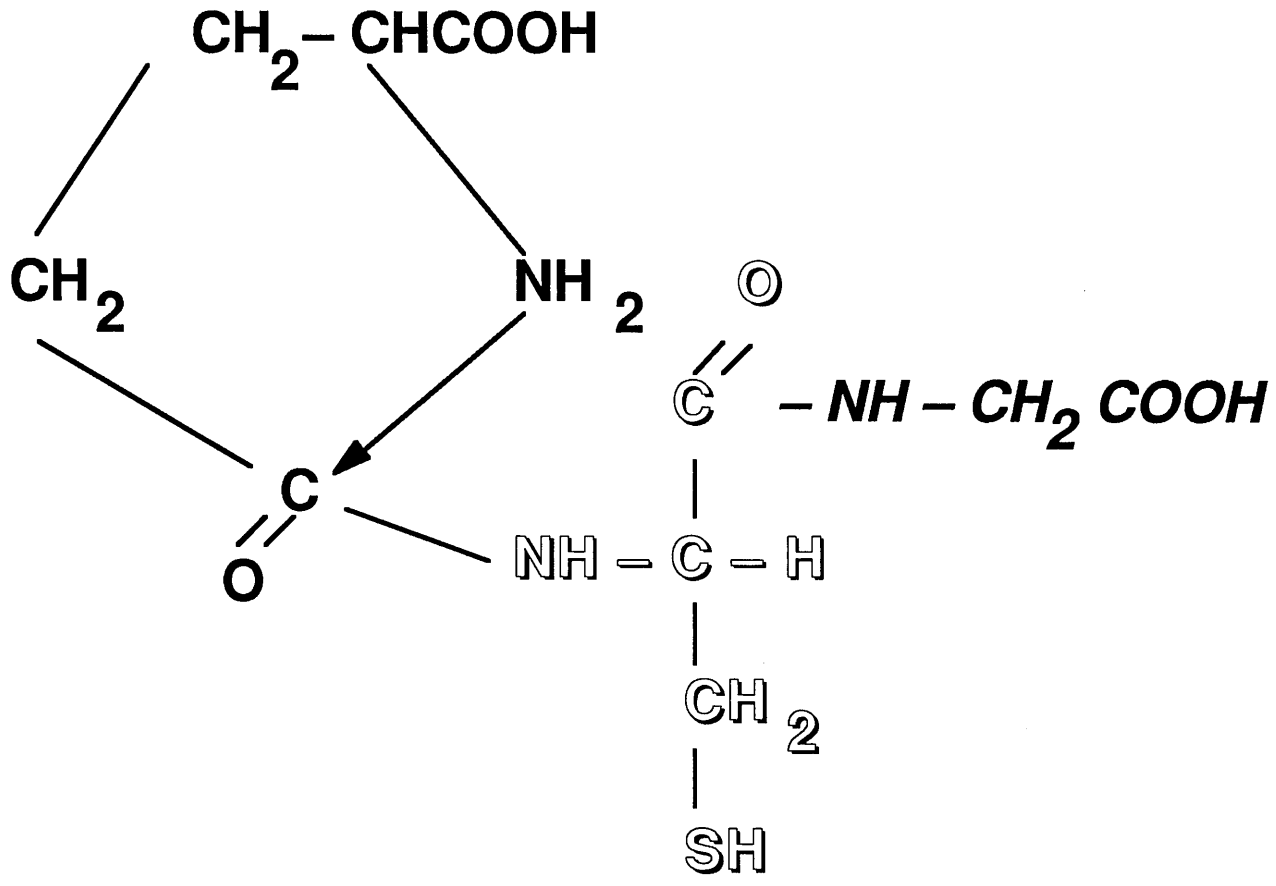


Figure 12 : Molecular structure of glutathione.

death except when there is subsequent drain of mitochodrial GSH which is derived from the cytoplasmic pool (Meister, 1984). Tumour tissues contain different GSH levels, which have been reported as low, moderate and high (Murray et al., 1987). These levels and the activity of glutathione-S-transferase enzyme in different tumour tissues play a crucial role in moderating the toxicity of certain compounds (Gianni et al., 1983 ; Evans et al., 1987). Increased GSH levels may protect cells against the free radical products induced by doxorubicin (Doroshow et al., 1979).

1.3.2. GSH Metabolism and Functions :

Understanding of GSH function comes through demonstrating its intracellular metabolism which is catalysed by different enzymatic reactions (Figure 13). During these reactions the amino acids required for GSH synthesis are transported and the molecule is synthesized and degraded and extracellular shifting take place. The most important functions of GSH are :

(1) Action as a cofactor for reduction of protein and other disulfide linkages.

(2) Reduction of ribonucleotide to deoxyribonucleotide, the precursor of DNA.

(3) Protection of cells against the effects of free radicals and reactive oxygen intermediates by which GSH is converted to GSSG in a reaction mediated by glutathione peroxidase.

$$2GSH + 2Rd^{\cdot} \longrightarrow GSSG + 2Rd^{-} + 2H^{+}$$

$Rd^{\cdot}$ = Free radical.

In addition GSH interferes with the inter-organ transport of certain amino acids and also has the ability to inactivate a number of compound such as certain drugs

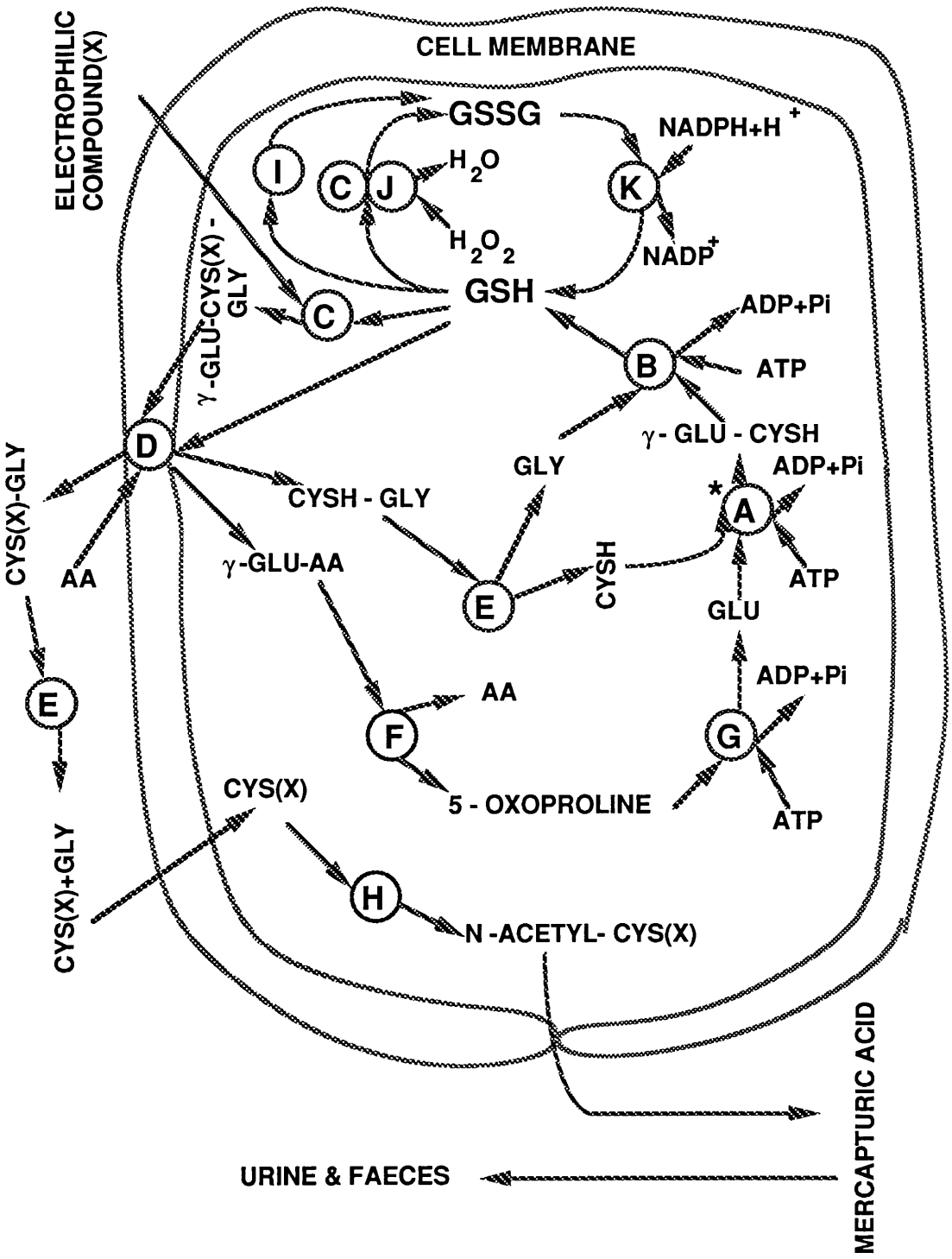


Figure 13 : Glutathione Metabolism and Functions.

A. γ-Glutamyl cysteine synthetase
B. Glutathione synthetase
C. Glutathione-S-transferase
D. γ-Glutamyl transpeptidase
E. Dipeptidase
F. γ-Glutamyl cyclotransferase
G. 5-Oxoprolinase
H. Acetylase
I. GSH transhydrogenase
J. GSH peroxidase
K. GSH reductase
* Feedback inhibition by glutathione

forming GSH conjugates. Moreover in some enzymatic reactions GSH acts as a coenzyme for their activities (Meister , 1983 ; 1984).

GSH synthesis occurs intracellularly under the catalytic activity of two enzymes: γ-glutamyl cysteine synthetase catalyses the reaction between the amino acids glutamate and cysteine forming the dipeptide γ-glutamyl-cysteine. This dipeptide reacts with the aminoacid glycine under the catalytic activity of glutathione synthetase producing GSH. These two steps of GSH synthesis need expenditure of energy which comes from the cleavage of two molecules of adenosine triphosphate (ATP) forming adenosine diphosphate (ADP) (Snoke and Bloch,1954 ; Meister , 1974).

Breakdown of GSH involves several different steps. The first step is catalysed by the membrane bound enzyme γ-glutamyl transpeptidase. This is a glycoprotein that interacts with GSH and other similar compounds forming γ-glutamyl moiety and cysteinylglycine. The γ-glutamyl moiety interacts with amino acids forming γ-glutamyl amino acids in which cysteine and some neutral amino acids such as glycine and methionine are the more active acceptors. The dipeptide cysteinylglycine is enzymatically split to cysteine and glycine (Allison and Meister, 1981 ; Thompson and Meister, 1975). γ-glutamyl transpeptidase also catalyses the hydrolysis of GSH to glutamate and cysteinylglycine and the latter is broken down by the activity of dipeptidase to its corresponding amino acids (Meister, 1981). The free amino acids and γ-glutamyl amino acids can be freely transported into the cells.

γ-glutamyl cyclotransferase converts γ-glutamyl amino acids to 5-oxoproline by cyclizing the glutamyl moiety of that compound leaving the corresponding free amino acids. 5-oxoproline is converted to glutamate under the catalytic activity of an intracellular enzyme 5-oxoprolinase in an ATP dependent reaction (Seddon et al., 1984 ; Van Der Werf et al., 1971).

The enzyme glutathione-S-transferase catalyses the reaction between GSH and electrophilic exogenous and endogenous compounds forming related GSH conjugates. These compounds are removed from the cells, being used as a substrates for γ-glutamyl transpeptidase which converts them to S-substituted cysteinyl-glycines

(CYS [X]-GLY). These substances are then converted to glycine and S-substituted cysteine (CYS [X]) by the catalytic activity of dipeptidase which can accept the γ-glutamyl moiety of GSH forming γ-glutamyl-cysteinyl(X) (Griffith et al., 1981). Intracellular acetylation of CYS(X) leads to mercapturic acid (N-acetyl-Cys [X]) which is excreted in the urine and faeces.

GSH must be supplied from the cells continuously because there is no evidence that it is synthesized exogenously, so it must be transported out of the cells. This has a role in protection of cell membranes against oxidative damage by maintaining essential –SH groups (Larsson et al., 1983). GSH is converted to GSSG intracellularly either by the action of selenium-containing glutathione peroxidase or glutathione-S-transferase or transhydrogenase.These enzymes catalyze the reduction of oxygenated intermediates as H_2O_2 and superoxide radicals (Fridovich, 1978). Through this pathway GSH can destroy the free radicals produced from high doses of irradiation and administration of oxydizing agents.

GSH is the main intracellular thiol compound involved in metabolic reactions which include protein synthesis and degradation, deoxyribose synthesis the precursor of DNA and cysteine reduction (Griffith and Tate, 1980). The maintenance of a high intracellular ratio of GSH to GSSG is due to the activity of widely distributed flavoprotein glutathione reductase with NADPH as cofactor (Meister and Anderson, 1983).

Clinically, deficiency of certain enzymes that are responsible for GSH metabolism is associated with some human diseases. Examples are 5-oxoprolinuria, pyroglutamic aciduria, hemolytic anemia, myopathy and neuropathy all due to GSH synthetase deficiency (Meister and Anderson, 1983). These diseases are associated with massive 5-oxoproline excretion in the urine and elevation in both blood and cerebrospinal fluid levels causing severe metabolic acidosis, hemolysis and mental retardation (Meister and Anderson, 1983 ; Meister, 1984). Because GSH synthesis is regulated by feedback inhibition, in cases of deficiency, γ-glutamyl cysteine

synthetase is released from inhibition, leading to accumulation of γ-glutamyl cysteine in the body. This dipeptide is converted to 5-oxoproline and cysteine under the catalytic activity of γ-glutamyl cyclotransferase causing some of above symptoms (Richman and Meister, 1975). Some patients also develop glutathionuria and excrete GSH and γ-glutamyl cysteine in their urine due to γ-glutamyl transpeptidase deficiency (Meister and Anderson , 1983 ; Meister , 1984). Deficiencies in other enzymes of GSH metabolism do not lead to severe disorders.

1.3.3. Stimulation of GSH Synthesis :

The role of GSH in the protection of cells against free radicals, reactive oxygen intermediates and toxic compounds suggests that it might be beneficial to increase its intracellular levels. Although the feedback inhibition of γ-glutamyl cysteine synthetase enzyme by GSH itself regulates its level in the cells, its intracellular levels also depend on the availability of its amino acids constituents. Therefore GSH synthesis can be improved in some cases by increasing the supply of substrates to γ-glutamyl cysteine synthetase and glutathione synthetase. Cysteine plays an important role in controlling GSH synthesis and is derived mainly from dietary protein. This is in agreement with the finding of Issels and Nagele (1989) that the main cysteine moiety of newly synthesized GSH is derived from extacellular pool of cystine. Consequently increasing cysteine supply may also increase GSH synthesis, however this is not the ideal therapy because cysteine is rapidly metabolized (Meister, 1983 ; 1984).

Thiazolidine is an intracellular cysteine delivery agent which is well transported and may help to improve GSH levels through enzymatic conversion to cysteine (Williamson et al., 1982). Administration of γ-glutamyl cysteine or its disulfide γ-

glutamyl cystine significantly increases GSH levels in kidney (Anderson and Meister, 1983). But the best way of increasing GSH levels seems to be by using derivatives which are well transported into the cells. The most effective derivative for this purpose is glutathione monoethyl or methyl ester which is effectively transported after administration producing a significant increase in hepatic and renal GSH levels in mice (Puri and Meister, 1983). It has also been reported that glutathione ester can protect human cultured cells against the lethal effects of irradiation by increasing intracellular GSH levels (Wellner et al.,1984). Certain chemical compounds and drugs can increase GSH levels, for instance phenobarbital increases hepatic GSH by about 30% after 48 hours of administration to rats (Kaplowitz et al., 1980). Cyclophosphamide at low doses increases mouse bone marrow GSH following an initial depletion (Carmichael et al., 1986). Diaminodiphenylsulfone and methylene blue increase GSH levels in rabbits and human red blood cells due to stimulation of its synthesis through activation of glutathione synthetase (Paniker and Beutler, 1972). N-acetyl cysteine spares GSH levels in mice (Williamson et al., 1982) and in Hela cells in tissue culture (Al-Kabban et al., 1988).

One of the important nutritional compounds is selenium which is a cofactor for most of cardiac glutathione peroxidase, acting as a free radical detoxification agent (Bachur et al., 1979). This was confirmed by the results of Van Vleet and Ferrans, (1980) who used a mixture of selenium and vitamin E for controlling doxorubicin cardiomyopathy in rabbits.

In contrast to the effect of GSH synthesis enhancers there are some chemical compounds which inhibit its production. Prothionine sulfoximine (Griffith et al., 1979) and buthionine sulfoximine (Griffith and Meister, 1979 ; Arrick et al., 1982 ; Gaetjens et al., 1984 ; Somfai-Relle et al., 1984 ; Crook et al., 1986 ; Russo et al., 1986 ; Jordan et al., 1987 ; Lee et al., 1987 ; Dusre et al., 1989 ; Kable et al., 1989) are selective inhibitors of the enzyme γ-glutamyl cysteine synthetase. Buthionine sulfoximine is considered as superior to all other depleting agents because of its specificity and lack of side effects. Another compound α–ethylmethionine

sulfoximine (Griffith and Meister, 1978) minimizes GSH synthesis due to its effect as a glutathione synthetase inhibitor, while 1-chloro-2,4-dinitrobenzene (CDNB) (Arrick et al., 1982) binds to GSH under the catalytic activity of endogenous enzyme glutathione-S-transferase leading to its depletion. Salicylates deplete hepatic GSH in rats by causing its leakage from hepatocytes into the blood (Kaplowitz et al., 1980).

1.3.4. The Protective Role of GSH in Radio and Chemotherapy :

The biological and clinical importance of GSH is based on its protective role against chemical and radiation injury. Both radiation and redox active drugs have special relevance in cancer management. The antitumour activity of certain quinones such as doxorubicin is thought to be due to the oxidative effects of their redox cycle which end in hydroxyl radical production (Bachur et al., 1978 ; Thor et al., 1982). The discovery that many tumour cells contain high GSH concentrations (Biaglow et al., 1983 ; Russo et al., 1986) has prompted investigation into its role in cytotoxicity and resistance against antineoplastic agents such as doxorubicin (Hamilton et al., 1985). GSH may protect cells against oxidative effects in different ways, including direct reaction with the parent quinones and their semiquinone radicals or by acting as a substrate of glutathione peroxidase to detoxify H_2O_2 or hydroperoxide formed as a result of lipid peroxidation (Nickerson et al., 1963).

An interesting approach is the experimental reduction of cellular levels of GSH prior to using cytotoxic drugs in cancer chemotherapy using these new compounds which selectively regulate intracellular GSH levels.

GSH provides substantial protection against radiation damage only at low oxygen

tensions. Accordingly, and in order to increase the curative effects of radiotherapy, GSH concentration in the tumours requires to be reduced (Mitchell and Russo, 1987). The GSH content of some human tumour cells can be high compared with normal cells and tumours from other sources (Russo et al., 1986). In these cases the GSH content of tumour tissues could determine the clinical response to antitumour agents (Jordan et al., 1987). On the other hand in some tumours one of the important signs in cells resistant to the chemotherapy is the elevation of their GSH levels (Suzukake et al., 1982 ; Somfai-Relle et al., 1984). Therefore sensitization of tumour cells to chemotherapeutic agents using GSH-depleting compounds could have a major role in cytotoxicity and the DNA damaging effects of chemotherapy (Arrick et al., 1982 ; Crook et al., 1986).

1.3.5. Measurement of GSH :

Due to the physiological activity of GSH and its ubiquity, it is desirable to be able to measure it in tissues with acceptable standards of specificity, accuracy and sensitivity. The design of such methods has been hampered by the chemical characteristics of GSH, in particular its auto-oxidable nature ; the amounts present in certain tissues require that assays should detect concentrations of the order of nmoles /l in the presence of higher concentrations of related compounds. A number of reliable methods are available for assaying GSH in tissues with relatively high concentrations. Initially chemical methods based on the nitroprusside reaction (Hopkins, 1921), were developed for colorimetric measurement (Owens and Belcher, 1965). But these methods, like the Ellman reaction used nowadays were

not specific, and in some of them oxidation of GSH to GSSG occurred during sample preparation producing large errors in the measurement of both. Proof that the need for a good method to measure GSH has not been fully satisfied is the existence of a wide varety of techniques based on different principles to assay both the reduced and oxidized forms.

Spectrophotometry using Ellman's reagent (5,5-dithio-bis [2-nitrobenzoic acid]) (DTNB) conjugation (Ellman, 1959) ; fluorometry based on the conversion of thiol compounds to fluorescent derivatives using (O-phthalaldehyde) (Cohn and Lyle, 1966 ; Hissin and Hilf, 1976) and enzymatic methods based on the catalytic activities of glutathione-S-transferase or glutathione reductase enzymes (Tietze, 1969 ; Wendell, 1970 ; Koivusalo and Uotila, 1974 ; Griffith, 1980 ; Davies et al., 1984) have all been used. The enzymatic methods gave GSH and GSSG values which were consistently lower than those measured by Ellman method.

Thin layer chromatography using silica gel has been used for GSH separation (States and Segal, 1969) ; gel electrophoresis was used by (Klein and Robbins, 1970) during different cycles of cell division. But the advent of more modern and sophisticated techniques for proteins, peptide and amino acid analysis has been provided with new tools like high performance liquid chromatography (HPLC) coupled to colorimetric detection by means of which it has been feasible to separate and quantitate GSH from cysteine and γ-glutamyl cysteine. There are now several HPLC techniques which have been developed for GSH determination in small biological samples. Some of these involve precolumn derivatization of the thiol compounds with a fluorescent reagent such as monobromobimanes and monobromo-trimethyl-ammoniobimane and separation by using HPLC (Fahey et al., 1981 ; Newton et al., 1981 ; Burton and Aherne, 1986).

Modification of the Ellman method coupled to derivatization of thiol compounds, using reversed phase HPLC has been used by Reeve and Kuhlenkamp, (1980) for GSH separation. But the most sensitive and specific method for measuring GSH and GSSG independently is the recycling post-column reaction on an anion exchange

HPLC technique. By this method GSSG is separately determined after alkylation of the GSH using N-ethylmaleimide (Alpert and Gilbert, 1985). HPLC with electrochemical detection is a sensitive procedure for detection of GSH , GSSG and other thiol compounds using dual gold mercury electrodes (Lunte and Kissinger, 1984 ; Stein et al., 1986).

The main problem in all of the above methods is the time required for sample preparation and analysis, and the need for special equipments which are not widely available . In addition GSH measurement needs a simple, fast and accurate method to reduce the errors. In the present work a direct sensitive and rapid method for GSH assay designed by the author was used. It involves cationic ion-pair chromatography (HPLC) followed by ultraviolet detection and is explained in detail in the materials and methods section. Better methods would allow changes to be detected in the intact tumour cells, and one method of studying intracellular components in intact cells is nuclear magnetic resonance (NMR) spectroscopy.

1.4. ^{1}H Spin Echo Nuclear Magnetic Resonance (NMR) :

^{1}H spin echo NMR spectoscopy is a non-invasive probe which is now an acceptable method for the study of cellular metabolism in intact erythrocytes (Reglinski and Smith, 1986). Although NMR is less sensitive than other methods, it has other major advantages in that it can identify selective metabolic processes directly in the intact viable cell (Brown and Campbell, 1980 ; McKay et al., 1986 ; Rabenstein, et al., 1985).

^{1}H NMR has been shown to be very useful in monitoring low molecular weight metabolites in tissue fluids such as blood, plasma and urine. This includes measuring natural endogenous compounds and drugs. Using the same technique, metabolic abnormalities such as hyperglycaemia in cases of diabetes can be detected (Lindon, 1986), although this is obviously not the method of choice. Another important use of the NMR is to study membrane transport by indicating changes in signal strength of cellular components (Reglinski and Smith, 1986). More recently spin echo NMR has been used in Hela cells (Reglinski, et al., 1987) to study intracellular glutathione and in leukaemic J-111 cells to detect lactate and study glycolysis, to follow the changes which occur on the addition of doxorubicin (Reglinski, et al., 1988b) and to confirm the effect of doxorubicin on the cellular biochemistry of Hela cells (Al-Kabban, et al., 1988).

As an analytical technique it can be quantitative (Rabenstein, et al., 1985), but is more effective in conjunction with HPLC where the selectivity of NMR and it's sensitivity to molecular conformational changes in the intact and viable cells can be supported by the quantitative in vitro HPLC method.

1.5. Hypothesis :

(1) The development of a simple method (HPLC following direct injection of cell lysates) for estimation of intracellular GSH in human tumour cells will allow investigation of the differences in basal levels in sensitive and resistant cell lines and effects of doxorubicin treatment on this.

(2) Examination of GSH levels in intact tumour cells using NMR in a manner which excludes the effect of disruption on that compound should demonstrate similar differences between resistant and sensitive tumour cell lines and a similar effect of doxorubicin.

(3) Sensitive cell lines can be protected from the toxic effects of anthracycline by protecting their GSH content using NAC or cysteine.

(4) Reduction of GSH in the heterogenic resistant tumour cells using buthionine sulfoximine will increase their sensitivity to doxorubicin.

(5) Treatment of heterogenic resistant tumour cells with amiodarone could increase intracellular retention of doxorubicin, and reduce the protective effect of high intracellular GSH.

II. MATERIALS AND METHODS

2.1. Materials :

Suppliers of materials used in the following experimental section are detailed below. All other reagents and solvents were of AnalaR grade, BDH, Poole, Dorset, England. Doxorubicin was a generous gift from Farmitalia Carlo Erba Italy. Daunorubicin hydrochloride was from May and Baker Ltd., Dagenham England. Amiodarone hydrochloride and fluphenazine were from Labaz: Sanofi U.K. Ltd., Floats Road, Wythenshawe, Manchester, M23 9NF.

Materials	Suppliers
Buthionine-S,R-Sulfoximine (BSO)	Sigma Chemical Co.Ltd., Poole, England.
1-Chloro-2,4-dinitrobenzene (CDNB)	Sigma
Cysteine chloride	Aldrich Chemical Co. Englabd.
Deuterium oxide D_2O (gold label)	" "
EMIT free drug level filters	Syva (U.K.) Ltd., Maidenhead, Berks, England.
Foetal bovine serum (FBS)	Gibco (U.K.) Ltd., Scotland and Northumbria Biological Ltd., Cramlington, Northumberland, England.

Materials	Suppliers
Glutathione GSH (reduced form)	Sigma
Glutathione-S-Transferase (GST) from human placenta	"
HPLC Apex Octadecyl 5µ, 25 cm column	Jones Chromatography (U.K.) Ltd. Midglamorgan, CF8 8AU (Wales).
L-Glutamine solution (100X)	Gibco
Methanol (HPLC grade)	Rathburn Chemicals Ltd., Walkerburn, Scotland.
Micro well plates 96 U	Nunclon, Denmark.
Millipore filters	Millipore, S.A. Molsheins France.
MTT [3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide : Thiazolyl blue]	Sigma
N-acetylcysteine	"
ODS Hypersil 5µ	Shandon Southern Products (U.K.) Ltd., England WA7 IRR.
Penicillin-Streptomycin solution	Gibco
Phosphate buffer saline (PBS)	"
Resorcinol	Sigma

Materials	Suppliers
1X RPMI 1640 medium with 20 mM hepes buffer	Flow Laboratories, Irvine Scotland.
1XRPMI 1640 (Dutch modification) medium with 20 mM hepes buffer, 1g /l Sodium bicarbonate and 6.4 g /l Sodium chloride.	" "
Sodium bicarbonate 7.5% solution	" "
Tetrabutyl ammonium dihydrogen phosphate	Sigma
40% Tetrabutyl ammonium hydroxide	"
1,1,3,3-Tetraethoxy propane (Malondialdehyde tetraethyl acetal)	"
Thiobarbituric acid	"
Tissue culture flasks	Nunclon
Tissue culture multiwell plate 96 Flat bottomed wells	Flow Laboratories
Tris buffer	Sigma
Trypsin	Flow Laboratories
Water (HPLC grade)	Rathburn

2.2. Tumour Cell Lines :

Hela tumour cells were taken from stock held in liquid nitrogen in our laboratory. **A549** an alveolar type II cell adenocarcinoma of lung (CCL 185) normally resistant to doxorubicin was from American type culture, Rockville Maryland. **J 111** human leukaemia cell line was supplied by Flow laboratories. These three cell lines were grown as monolayers in tissue culture flasks.

GLC_4 210 a small cell carcinoma of lung sensitive to doxorubicin, and **GLC_4 210** a small cell carcinoma of lung showing acquired resistance to doxorubicin originated from the Department of Medical Oncology, University of Groningen, Holland. These were grown in suspension. The three lung tumour cell lines were obtained through the department of Medical Oncology, Glasgow University.

2.3. Culturing :

2.3.1. Tissue Culture Medium and Feeding :

Hela and J 111 cell lines were grown on 1XRPMI 1640 (Dutch modification) medium with 20 mM HEPES buffer, 1g / l sodium bicarbonate and 6.4 g / l sodium chloride. A549 and GLC_4 210 cells (sensitive) were grown on 1XRPMI 1640 with 20 mM HEPES buffer. GLC_4 210 cells (resistant) were grown on the same medium with the addition of 0.9 nmol /l doxorubicin to obtain acquired resistance to doxorubicin.

2.3.2. Cell Culture :

Hela cells, A549 and J 111 were grown routinely as monolayers in F120 flasks containing enriched RPMI 1640 medium supplemented with 20 mM HEPES buffer , 1g / l sodium bicarbonate and with or without 6.4 g / l sodium chloride, 10% v/v fetal bovine serum (FBS) , 1% v/v L-glutamine (200 mM) and 1% Penicillin–Streptomycin antibiotic solution (10000 IU / ml, 10000 µg / ml respectively).

GLC_4 210, sensitive and resistant, were grown in a suspension in F120 flasks containing enriched RPMI 1640 medium as described above except that doxorubicin was added in a concentration of 0.9 nmol /l continuously to induce resistance. Cells were grown at 37°C for 3 – 4 days with replacement of the medium at the middle of growing interval. J 111 cells were grown at 37°C in a 5% CO_2 atmosphere.

2.3.3. Detachment of Cells from Culture Flasks :

Hela, A549 and J 111 cells were detached from culture flasks using 0.25% trypsin in calcium and magnesium free phosphate buffered saline (PBS). After removing the medium, the cells were soaked in trypsin solution for 30 seconds. The trypsin solution was aspirated and the flasks sealed and incubated at 37°C until the cells had detached from the surface of the flasks, this usually required 15 - 20 minutes, after which the cells were harvested using culture medium prior to counting cell density using a haemocytometer or Coulter counter. In the case of GLC_4 210 cells there was no need to use trypsin because they were grown in suspension ; centrifugation of the suspensions was adequate for harvesting.

2.4. Development of a High Performance Liquid Chromatographic (HPLC) Assay for Glutathione Measurement :

A specific new HPLC method for glutathione measurement in cell lysates was developed using simple equipement (Figure 14), which can be adapted to measure other cellular components.

2.4.1. Apparatus :

The HPLC analysis of cell lysates was carried out on a 250 x 4.6 mm (i.d.) column supplied packed with Apex Octadecyl Silica (5 µ) with a guard column of 5 x 4.6 mm (i.d.) slurry packed in our laboratory with ODS Hypersil (5 µ).

Manual injection was made via a Rheodyne 7125 injection valve (Scotlab Instrumentation Ltd., Law, by Carluke, Scotland) with a 20 µl loop, and using a single piston reciprocating pump model 302 with a manometric module model 802 from Gilson, 72 Rue Gambetta B.P. 45, 95400 Villiers. L.E. Bel, France.

UV detection was carried out using a Waters 490 multiwavelength detector (Waters Associates, England) at 200 and 210 nm, (0.02 and 0.01 AUFS respectively) in the case of Hela cells, and at 205 and 210 nm, (0.01 and 0,02 AUFS respectively) in the case of the other three cell lines (A549 and GLC_4 210 [S and R]), since this gave a better specificity. Recording was carried out using a CR6525 double pen recorder (JJ Lloyd Instruments Ltd., Southampton SO3 6HP England).

Figure 14 : Photograph of the instrument used in the HPLC assay.

2.4.2. Initial Development of the HPLC Method :

The above apparatus was used first for the detection of standard and cellular GSH at different UV wavelengths. Normal distilled water, HPLC grade methanol and water in addition to the AnalaR grade methanol were investigated as an eluting solvent. In all cases solvent polarity was decided by changing the ratio of methanol to water to obtain good resolution. Tetrabutyl ammonium hydroxide and tetrabutyl ammonium dihydrogen phosphate, as ion-pairs were added to the methanol / water, and buffers of a range of pHs were also investigated.

2.4.2.1. The Effect of Ion-Pairing :

This experiment was carried out using aqueous standards, on reverse phase using initially methanol : water only without ion-pair. To the appropriate mobile phase two different cations were added (Tetrabutyl ammonium hydroxide or Tetrabutyl ammonium dihydrogen phosphate). The first was found the best for glutathione separation, and different concentrations (0.05 – 0.2 %) were added to the HPLC buffer in order to get the best resolution.

2.4.2.2. The Effect of pH on the Resolution of Glutathione from Different Intracellular Componants :

This experiment was carried out using 10 % v/v orthophosphoric acid to control the pH of the HPLC buffer over the range of 2.5 – 8 in order to achieve on the best resolution. Hela cell lysate was used in this experiment with HPLC buffer as described above.

2.4.2.3. Final Development of the HPLC Method :

Elution was isocratic, the eluant being Methanol : Water : 40% w/w Tetrabutyl ammonium hydroxide (100 : 899 : 1) for Hela cells and (75 : 924 : 1) for the other three cell lines. These conditions resolved a component which interfered with the internal standard peak. The pH was adjusted retrospectively to 3.5 by the addition of 10% v/v Orthophosphoric acid. Elution was at ambient temperature at a flow rate of 2 ml / minute in case of Hela cells and 1.5 ml / minute in case of the other lines for the reason stated above.

2.4.3. Linearity :

The linearity of the method was studied by injecting onto the HPLC system 20 µl of a glutathione solution prepared in mobile phase containing 11.4 µmol / l resorcinol as internal standard over the concentration range 0 –1.2 µg on column (0 – 60 mg / l standard) (0 – 194.4 µmol / l).

2.4.4. Precision :

Precision of chromatography was assessed by determining glutathione dissolved in mobile phase (n = 21) at a concentration of 20 mg / l (0.4 µg on column) (65.1 µmol / l) and glutathione from Hela cells using same cell suspension for each injection (n = 10) after lysing in mobile phase as 6.25×10^9 cells / l (1.25×10^5 cells on column).

2.4.5. Sensitivity :

The sensitivity of detection of GSH was determined by finding the lowest concentration which would give rise to a peak with a height equal to 2 x base line noise level.

2.4.6. Glutathione Standard Curve :

In this experiment serial dilutions of a glutathione solution were made using HPLC buffer containing 11.4 µmol / l resorcinol as internal standard. 20 µl of glutathione (final concentration 8.1 – 194.4 µmol / l) were injected onto the HPLC system. The standard curve was plotted for glutathione : resorcinol peak height vs. glutathione concentration.

2.5. Growth Experiment :

This experiment was carried out using the four cell lines Hela, A549, and GLC_4 210 (resistant [R] and sensitive [S] to doxorubicin) to examine the baseline behavior of these cells in culture. One F25 flask of each line was harvested as described in (2.3.3.) and counted using a haemocytometer. Cells were diluted using culture medium to give 2 x 10^4 cells / ml in the case of Hela and A549 cells, and 3 x 10^4 and 4 x 10^4 cells / ml in the case of GLC_4 210 (S) and GLC_4 210 (R) respectively.

Sixteen F25 flasks were set up for each cell line with 5 ml of cell suspension in normal tissue culture medium in each. Cells were incubated at 37°C and paired flasks of each cell line were removed for counting at 24 hour intervals for eight days, at the end of which growth curves were plotted for the mean values of each pair of flasks.

2.6. Exposure of Cells to Doxorubicin :

In these experiments the four cell lines (Hela, A549 and GLC_4 210 [S and R]) were included. Different doxorubicin concentrations were used for each line. The experiments were run as described in figure 15.

2.6.1. Hela Cell Line :

Seven F25 flasks were prepared, each containing 7.3 x 10^6 cells in 5 ml of culture medium. One was used as a control and the other six were treated with doxorubicin at concentrations of 0.6 – 6.0 nmol / 10^6 cells respectively. After 12 hours the cells were harvested and centrifuged at 1500 rpm for 10 minutes, washed twice with PBS and centrifuged each time. Cells were lysed by the addition of 0.4 ml of HPLC buffer containing 11.4 µmol / l resorcinol as internal standard and using an Ultrasonic probe (MSE 150 Watt Ultasonic Disintegrator MK2 MSE Scientific Instruments, Manor Royal, England). The lysate was filtered by centrifuging at 2500 rpm through EMIT free drug level filters designed for the ultrafiltration of plasma. Twenty µl of filtrate, equivalent to approximately 3.63 x 10^5 cells were injected directly onto the HPLC

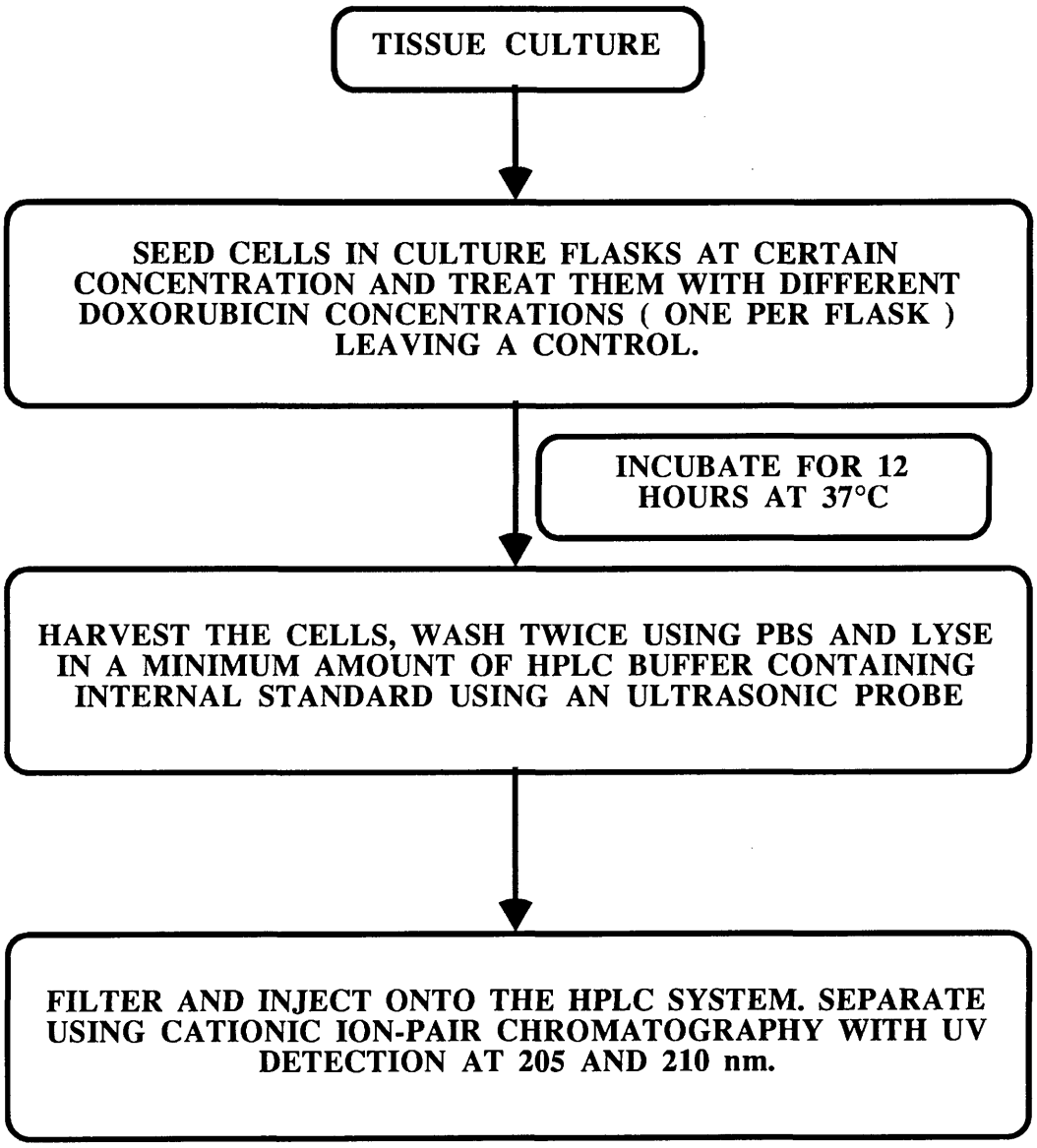


Figure 15 : Protocol for measuring the effect of doxorubicin on the glutathione content of the cells in tissue culture.

column. GSH standards, prepared in mobile phase containing resorcinol internal standard were run to calibrate the system.

2.6.2. A549 Alveolar Type II Cells (Adenocarcinoma of Lung) :

Six F25 flasks were prepared each containing 2 x 10^6 cells in 5 ml of culture medium. One was used as control and the other five were treated with doxorubicin at concentrations from 4.3 – 68.8 nmol / 10^6 cells respectively. The other steps of the experiment were completed as described in (2.6.1.) except that the cells were lysed in 0.3 ml of HPLC buffer containing internal standard resorcinol (11.4 µmol / l). The 20 µl sample of filtrate which was injected into the HPLC system was equivalent to approximately 1.33 x 10^4 cells.

2.6.3. GLC_4 210 Small Cell Carcinoma of Lung (Sensitive to Doxorubicin) :

Six universal tubes were prepared each containing 7.8 x 10^6 cells suspended in 5 ml of culture medium. They were treated as described in 2.6.2. except that the doxorubicin concentrations were from 1.1 – 17.6 nmol / 10^6 cells respectively. Cells were lysed using 0.4 ml HPLC buffer containing internal standard resorcinol (11.4 µmol / l). The 20 µl sample of filtrate was equivalent to approximately 3.9 x 10^5 cells.

2.6.4. GLC_4 210 Small Cell Carcinoma of Lung (Resistant to Doxorubicin) :

These cells were grown under the stress of doxorubicin in order to induce resistance as explained (2.3.2.). For this experiment six universal tubes were prepared as in 2.6.3., each tube containing 6.9 x 10^6 cells in 5 ml of their normal culture medium. The rest of the experiment was as described in 2.6.3., except that the doxorubicin concentrations were from 1.25 – 20 nmol / 10^6 cells respectively. The 20 µl sample of filtrate was equivalent to approximately 3.45 x 10^6 cells.

2.7. Effect of the Added Thiols on Cellular Glutathione :

2.7.1. Effect of N-acetylcysteine (NAC) on Hela Tumour Cells Treated with Doxorubicin :

In this experiment seven F25 flasks were prepared, each containing 3.5 x 10^6 cells in 5 ml of culture medium. Five flasks were treated with doxorubicin (5 nmol / 10^6 cells) four of them had been already pretreated for one hour with NAC at concentrations of 0.35 – 2.80 µmol / 10^6 cells respectively. Two flasks were used as controls, one with and one without NAC (0.35 µmol / 10^6 cells). Twelve hours later the cells were harvested and analysed as described (2.6.1.). The 20 µl sample of filtrate injected was equivalent to 1.75 x 10^5 cells.

2.7.2. Effect of Cysteine on Hela Tumour Cells Treated with Doxorubicin :

This experiment was carried out as described in 2.7.1. except that the concentration of doxorubicin was 4 nmol / 10^6 cells and cysteine (1.2 – 4.8 μmol / 10^6 cells) was used in stead of NAC.

2.8. ^{1}H Spin Echo NMR Spectroscopy :

The NMR method used in this study is well documented for erythrocyte biochemistry (Rabenstein 1978 ; Rabenstein and Nakashima, 1979 ; Brown and Campbell, 1980). A Bruker WM 250 MHz spectrmeter was used to record all spectra. Spin echo NMR spectra were obtained using a Hahn spin echo pulse sequence ($90° - t - 180° - t$) with a delay time (t) of 60 ms, the sequence creates a time delay (2t) between signal generation and accumulation. Samples were maintained at 20°C during data collection and the data from 2000 complete pulse sequences were accumulated for each Fourier transform. Cellular systems can be considered loosely to consist of two types of NMR active components; large molecules (e.g., membranes, proteins and nucleic acids) and small or mobile molecules (e.g., cytosolic metabolites and substrates). The relaxation times of these two categories differ; large molecules, by virtue of cross relaxation, have short relaxation times whereas small molecules have substantially larger values. The delay time (t = 60 ms) used (as an ideal value for GSH) is sufficient to allow the polarisation signal from the large molecules to relax back to equilibrium and thus be absent from the spectrum. The small molecules, as a direct consequence of their longer signal life, still provide a resonance line in the NMR spectrum on completion of the pulse sequence. The spectra obtained have modulated signals, which precludes the use of signal integration and hence quantitation, but the peak heights do reflect the relative ratios of metabolites. Thus the introduction of a suitable reference compound or the identification of an invariant naturally-occuring species allows the determination of the relative change in concentration of individual metabolites. Chemical changes at specific sites within molecules can still be observed. An example of this behaviour is the change in resonance intensity observed in the g_2 - β-methylene resonance in glutathione on oxidation (Brown et al., 1977). Species may

be removed from the NMR spectrum by two mechanisms. They can be metabolised (degraded) or can interact with the cell macrostructure. The latter depends on the molecule, the change in relaxation time means that the signals from the interacted or bound molecules are filtered from the spectrum (Reglinski et al., 1988 a). when the instrument is tuned to intracellular water, molecules which leak from dead or dying cells into the surrounding medium show a reduction in signal of around 20%.

All spectra were recorded by Dr J. Reglinski, Department of Pure and Applied Chemistry, Strathclyde University.

2.8.1. Hela Cells :

2.8.1.1. Sample Preparation for NMR Studies :

Cells were harvested from culture flasks as described in 2.3.3., washed twice in a minimum amount of physiological saline ^2H_2O / NaCl (0.154 M) to remove excess medium and provide a deuterium lock for the NMR spectrometer. It has been reported that excessive washing of the Hela cells with physiological saline causes cell lysis (Levine, 1960). The cells were transferred to a previously autoclaved 5 mm NMR tube with a small amount of saline (^2H_2O / NaCl 0.154 M) to produce a suspension of 80 % packed cells. The average sample size was approximately 10^9 cells in 0.4 ml saline.

2.8.1.2. Study of Glycolysis :

Study of cellular metabolism by NMR depends on the ability to maintain cell viability. For these initial experiment it was decided to suspend the cells solely in ^2H_2O saline and to estimate the viability under these conditions. This test is quite stringent for cells in which much more complex nutrient media are usually used to increase life time but it does give a conservative estimate for the time scale in which

experiments can be done with viable cells. In this instance cell viability was checked after harvesting by resuspending them in sterile physiological saline at 0.5 x 10^6 cells in 3 ml. To 200 µl of this suspension was added 200 µl of trypan blue solution. The percentage of living cells which did not stain was counted using a haemocytometer. Cells were found to remain viable in physiological saline for at least 5 hours (> 90 % viability) at room temperature.

Glucose was added to the cells while they were in the NMR tube at a concentration of 2 mg (11 µmol) / 10^9 cells. The NMR spectra for both glucose and lactate were recorded at zero time and 10 hours after the addition of glucose.

2.8.1.3. Exposure of Hela Tumour Cells to Doxorubicin :

Cells were prepared as described in (2.8.1.1.) and maintained at 20°C during the experiment. In all cases an initial reference spectrum of the culture under study was recorded prior to the addition of glucose 0.3 mg (1.66 µmoles) and doxorubicin (30 and 300 nmol / 10^9 cells). A control experiment was conducted with no doxorubicin present.

2.8.1.4. Effect of NAC on Hela Cells Treated with Doxorubicin as shown by lactate measurement :

This experiment was carried out as described in 2.8.1.3. except that NAC was added to the cells in the NMR tube at a nontoxic concentration (2 µmol /10^9 cells) prior to the addition of doxorubicin (300 nmol / 10^9 cells). The protective mechanism of NAC was shown by lactate profial and NAC reduction.

2.8.1.5. Transport of Amino Acids into the Cells :

Hela cells were used in this study. The NMR samples were prepared as described (2.8.1.1.) . Glycine 1.18 mg, cysteine chloride 1.55 mg and glutamate 2.05 mg (15.9, 9.6, and 13.5 μmoles) respectively were added as concentrated solutions (20 μl) to the cells in the NMR tube. An initial reference spectrum was recorded, and the mixture was treated with doxorubicin (30 nmol / 10^9 cells). The NMR spectra were recorded at one and two hours time intervals.

2.8.2. Leukaemia J 111 Cells :

2.8.2.1. Sample Preparation for NMR Study :

In the case of J 111 cells various culture media were used. A simple saline solution as above was used initially to profile the cells and identify the resonances arising from the cytosol rather than the culture medium. For the metabolic studies, 10 ml of the RPMI 1640 solution was freeze dried and re-dissolved in 10 ml of ^2H_2O. This solution was further diluted with physiological saline (^2H_2O / NaCl, 0.154 mol / 1) (1 to 10) to produce 10% RPMI in ^2H_2O. This procedure ensured the homogeneity and integrity of the culture medium. Doxorubicin was added at a concentration of 30 nmol / 10^9 cells in a final volume of 0.5 ml 100 % ^2H_2O RPMI 1640 medium .

2.8.2.2. Study of Glycolysis :

In this experiment cellular glycolysis was followed using human leukaemia J111 cells in the presence of doxorubicin (30 nmol / 10^9 cells) after the addition of glucose (5.5 μmoles). Cellular glycolysis was indicated by increased lactate production after administration of doxorubicin.

2.9. Glutathione-S-Transferase (GST) activity assay :

GST activity was measured in sensitive and resistant lung tumour cell lines of high, moderate and low glutathione content (A549, GLC_4 210 [S] and GLC_4 210 [R]) respectively, using a kinetic method on an Encore centrifugal analyser, (see 2.9.1.). The effect of doxorubicin pretreatment on GST activity was also measured. This assay was modified from that of Dr G. Beckett, Department of Biochemistry, Royal Infirmary, Edinburgh (personal communication). The aim of this assay is to find the relationship between glutathione content in different tumour cells and GST activity and also to estimate the GST activity in doxorubicin-resistant and sensitive tumours.

2.9.1. Reagents Preparation and Assay procedure :

The assay buffer consisted of 100 mmol / l sodium phosphate buffer pH 6.5 . Starting reagent was prepared by dissolving 13 mg of 1-chloro-2,4-dinitrobenzene

(CDNB) in 7.5 ml ethanol, made up to 60 ml using warm (37°C) assay buffer to give a final concentration of 1.1 mmol / l. Glutathione solution was prepared by dissolving 26 mg of reduced glutathione in 8 ml assay buffer to give a final concentration of 10.6 mmol / l. Standard GST from human placenta was prepared and run in the same manner.

The absorbance of GST was measured using an Encore Chemistry System (Baker corporation) connected to a CENTRIFICHEM system Pipettor 1000 (100, Cascad Drive Allentown, Pennsylvania 18001), programmed as follows :–

Temperature	37°C
Test Code	74
Test Name	GST
Wavelength	340 nm
Mode	C
Time (for blank)	20 sec
Mix time	1.6 sec
Linearity	0.01
Concentration factor	2
T_1	25 sec
T_w	5 sec
Rate time	10 sec
Abnormal absorbance limit	3.50
T_f	300 sec
Sample volume (cells lysate)	5 µl
Diluent (assay buffer)	15 µl
Starting reagent (CDNB)	240 µl
Second reagent (GSH)	50 µl

2.9.2. Sample preparation for the Assay :

Two F25 flasks were prepared for each cell line, one as a control containing cells in 10 ml of culture medium only and the other containing the same number of cells (4 x 10^6, 10 x 10^6, and 8.8 x 10^6 cells for A549, GLC_4 210 (S) and (R) respectively) in 10 ml of doxorubicin-containing medium (3.5 µmol / l). Twenty four hours later the cells were harvested and lysed as described in 2.3.3. and 2.6.1. after dissolving in 0.3 ml of assay buffer. Cell lysates were centrifuged at 2500 rpm to obtain the supernatant for the GST assay. The GST activity was corrected according to the cell number in the different cell lines used.

2.9.3. GST Standard Curve :

A GST standard curve was prepared using human placental enzyme. Stock solution was prepared with 10 enzyme units per ml of assay buffer. Four different GST concentrations (0.125, 0.25, 0.5, and1.0 units / ml) were prepared from the stock using same assay buffer. A standard curve was plotted for Δ absorbance vs. GST concentration.

2.10. Measurement of Lipid Peroxidation Due to Doxorubicin Treatment :

This assay was carried out on the two sensitive cell lines, one with a high glutathione concentration (Hela) and the other with a low glutathione concentration (GLC_4 210 [S]). Cells were grown and prepared as described (2.3.2.) and (2.3.3.). This assay was as described by Ledwozyw et al., (1986) and is based on the measurement of malondialdehyde (MDA) as a product of lipid peroxidation due to the decomposition of cell membrane unsaturated fatty acid caused by the oxygen free-radicals (1.2.6.3.) produced by doxorubicin metabolism.

2.10.1 Cell Preparation and Treatment :

MDA was measured after doxorubicin treatment. For each of the above cell lines seven x 3 ml batches of the same concentration of cells were prepared, as 10^7 cells / flask in case of Hela cells and 7 x 10^7 cells / flask in case of GLC_4 210 (S), one batch for use as a control and the other six for treatment with doxorubicin at a final concentration of 10 µmol / l. Cells were incubated at 37°C and one batch of each cell line was removed at intervals for MDA measurement. Cells were pelleted by centrifugation at 1500 rpm for 10 minutes, washed using PBS buffer, pH 7.4 and recentrifuged. The cells were lysed in 0.5 ml of buffer as described (2.6.1.).

2.10.2. Malondialdehyde Measurement :

MDA was measured using the thiobarbituric acid reaction by mixing cell lysate with 2.5 ml of 1.22 M tri-chloroacetic acid in 0.6 M HCl and allowing to stand for 15 minutes, after which 1.5 ml of thiobarbituric acid solution was added to each mixture (Thiobarbituric acid solution was prepared by dissolving 500 mg of this acid in 6 ml 1 M NaOH then adding 69 ml H_2O). The mixture was then heated for 30 minutes in a boiling water bath, and after cooling 4 ml of n-butanol was added. The mixture was shaken vigorously for 3 minutes and then centrifuged at 1500 rpm for 10 minutes. The absorbance of the organic layer was measured using a Unicam SP 1800 Ultraviolet Spectrophotometer (Pye Unicam) at 515, 532, and 555 nm for cell extracts, an Allen correction was made after subtracting the control reading.

$$A_{corr} = A332 - \frac{(A515 + A555)}{2}$$

A blank was prepared in the same manner for each experiment and standard MDA was run at the same time. Standard curves for different MDA solutions were prepared using PBS pH 7.4 at a final concentrations (0.142 – 4.54 µmol / l) and the curves were plotted for MDA concentration vs. absorbance.

2.10. 3. Effect of NAC on Hela Cells Treated with Doxorubicin :

This experiment was performed to confirm whether NAC can reduce lipid peroxidation caused by doxorubicin. In this assay cells were pretreated with NAC at 0.7 µmole / 10^6 cells (as effective non toxic concentration) (2.7.1.) leaving a control. The rest of the experiment including cell preparation is as described in 2.10.1.; the MDA was estimated in Hela cells as in 2.10.2.

2.11. Tetrazolium Based Chemosensitivity Assay :

This protocol was designed to determine cell viability using a colorimetric microtiter technique. The technique can be used to measure the activity of compounds which are cytotoxic or cytostatic to the cells (Ware, 1985). In this assay the effect of different doxorubicin concentrations on sensitive and resistant cells was examined. Cells were plated at low density and exposed to doxorubicin at different concentrations. The drug was then removed and the cells allowed to recover and pass through two to three cell doubling times. The surviving cell numbers were then determined by the ability of live but not dead cells to reduce the tetrazolium dye (MTT).

2.11.1. Determination of Optimum MTT Concentration :

The assay relies on a linear relationship between cell number and MTT formazin production during the incubation period. Four of the above cell lines described in 2.2. were used in this experiment. The adherent cells (Hela and A549) were plated out at a constant number per well i.e. 5 x 10^3 cells / ml (1000 cells per well in 200 µl of culture medium) in a 96 flat bottomed well plates. Cells were incubated at 37°C under 2 % CO_2 for 24 hours . The next day the medium was replaced by fresh and 50 µl aliquots of 10 different MTT solutions (0.5 – 5.0 mg / ml prepared in PBS) were added to the plate wells, one concentration to each row leaving the first and last rows as blank. Plates were wrapped in tinfoil and incubated at 37°C under 2 % CO_2 for four hours, after which the medium was removed from the wells and the insoluble MTT formazan and crystals were dissolved in 200 µl DMSO and 25 µl of 0.1 M, tris-buffer pH 9.5 to dissolve the non-dissolved dye. The plates were read using Bio-

Rad ELISA plate reader at an absorbance of 570 nm (Bio–Rad Laboratories Ltd. Watford Business Park, Watford, Herts. WD1 8RP, England).

The non-adherent cells (GLC_4 210 [S] and [R]) were plated out at 2 x 10^4 cells / ml (3000 cells per well in 150 µl of culture medium) in 96 round bottomed well plates. The MTT concentrations were added at the same time and treated as in the case of adherent cells except that the plates were centrifuged at 1000 rpm for 10 minutes before removing the medium in order to pellet the cells (Figure 16). Curves were plotted of MTT absorbance (mean of six) vs. MTT concentrations. The concentration which gave the maximum absorbance without being toxic to the cells was used.

2.11.2. Chemosensitivity of Adherent Cells (Hela and A549) :

Cells were plated as described in 2.11.1. and incubated at 37°C under 2 % CO_2 for three days to allow attachment and growth. Doxorubicin was added with fresh tissue culture medium after removal of the old medium. Eight different doxorubicin concentrations (8.4 nmol / l – 1075 nmol / l) were used, one concentration per three wells with three wells at each end as a control. The first and last rows contained culture medium only as blanks for the plate reader. Cells were incubated at 37°C under 2 % CO_2 for 24 hours in the presence of doxorubicin. They were fed with fresh culture medium after removing the old doxorubicin-containing medium, then again on each of the next two days which was a recovery period for the cells. On the last day of the assay the cells were fed with the usual culture medium, and 50 µl of optimised MTT solution was added to each well. The experiment then continued as in 2.11.1. and figure 17. Graphs were plotted of absorbance (mean of three) vs. doxorubicin concentration.

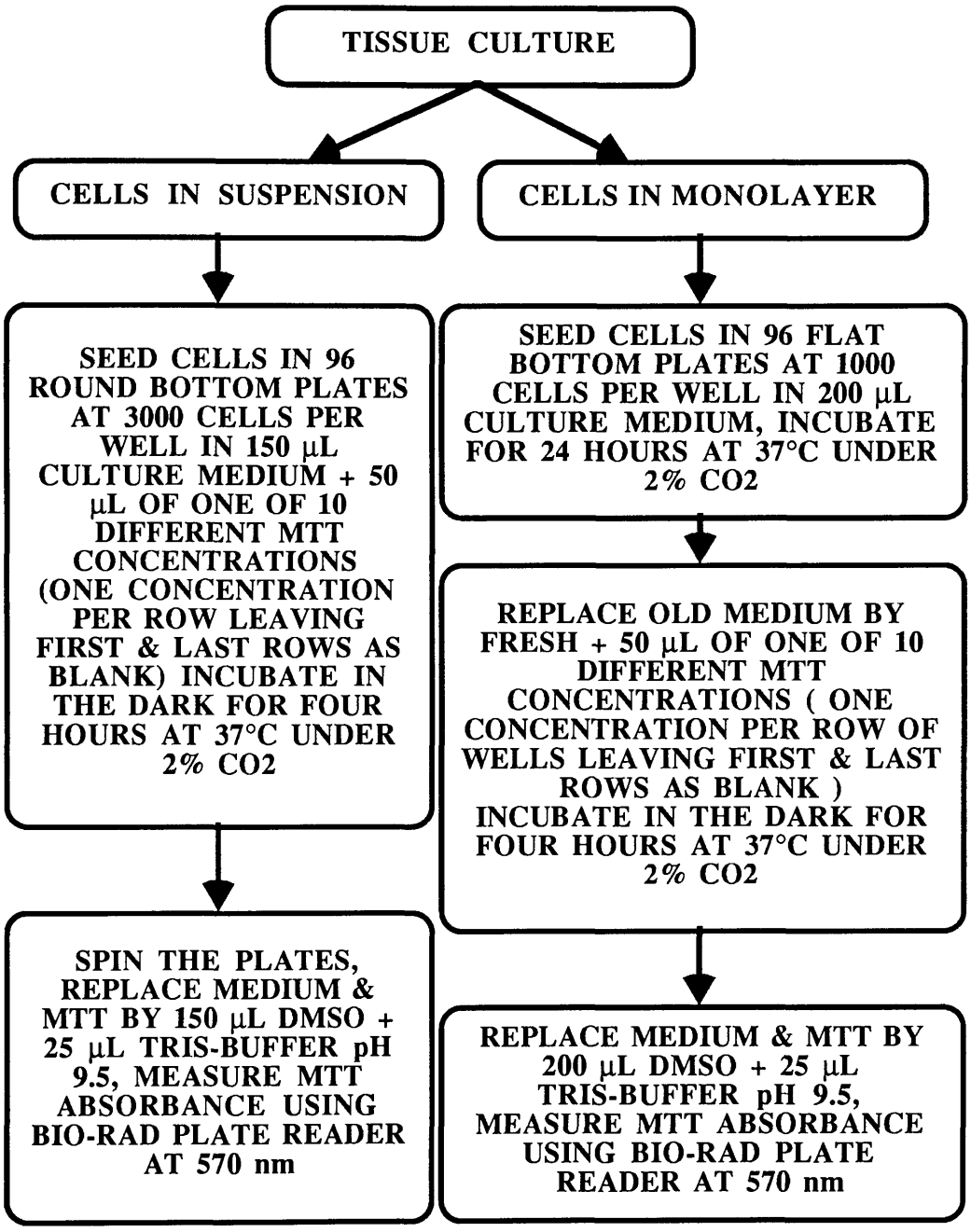


Figure 16 : Determination of the optimum MTT absorbance in different cell lines.

Chemosensitivity assay of cells grown as monolayer :

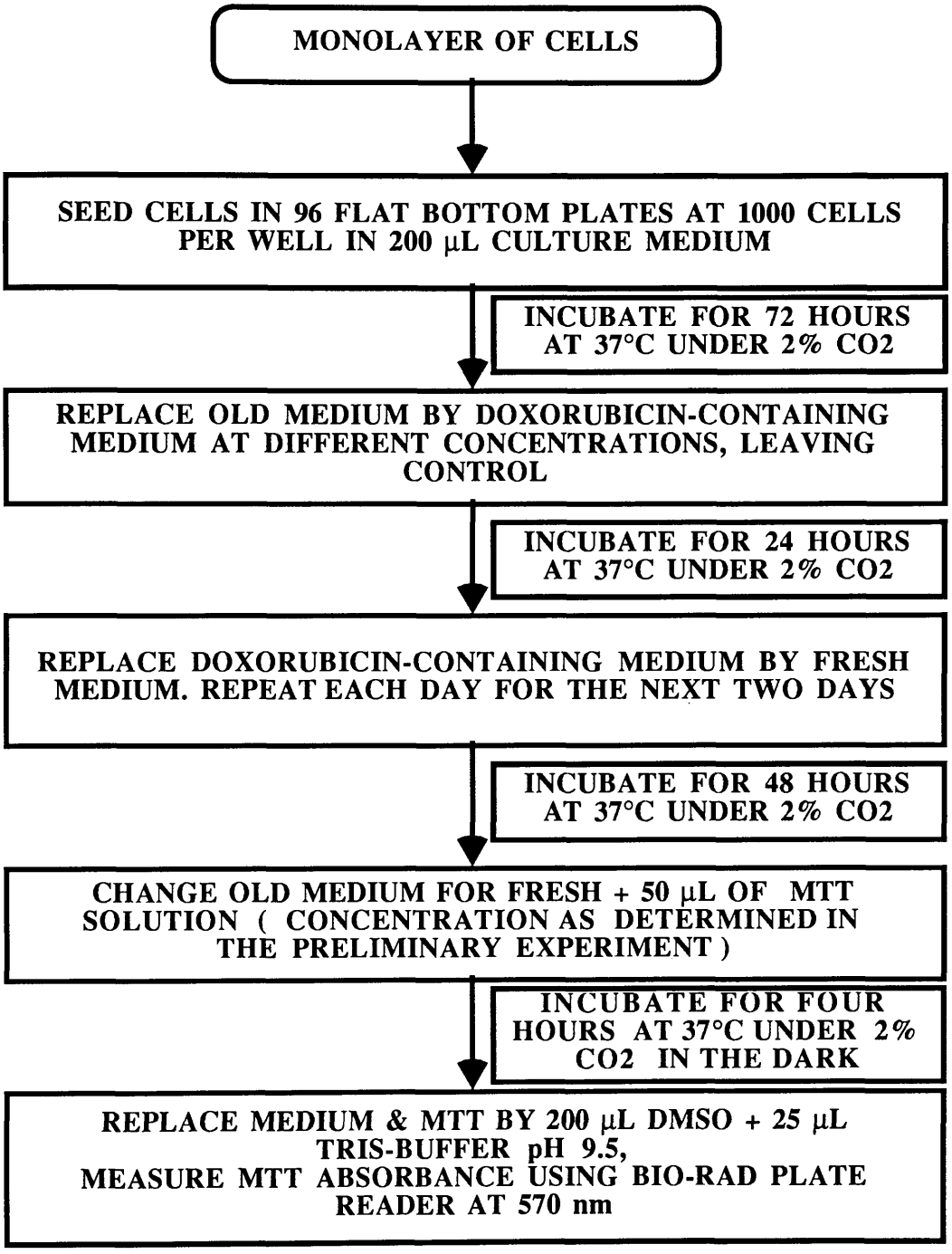


Figure 17 : Protocol for chemosensitivity assay used in the testing of drug effect against cells in monolayer.

2.11.3. Chemosensitivity Assay of Non-adherent Cells (GLC_4 210 [S] and [R]) :

Cells were subcultured three days prior to use and in the case of GLC_4 210 resistant cells grown in a doxorubicin-containing medium, the drug was removed at this stage. Cells were plated as described in 2.11.1. at 3 x 10^4 cells / ml as (3000 cells per well in 100 µl of culture medium). Doxorubicin was added at the same time in another 100 µl of medium to give the same final concentrations used in 2.11.2. in the case of the sensitive line and ten different concentrations (8.4 nmol /l – 4.3 µmol / l) in the case of the aquired resistance line, three wells for each concentration. Other conditions were as described in 2.11.2. except that the plates were centrifuged before feeding and the MTT formazine crystals were dissolved in 150 µl of DMSO (Figure 18). The graphs were plotted as in 2.11.2.

2.12. Effect of Buthionine Sulfoximine (BSO) Pretreatment on the Resistance of A549 Cells to Doxorubicin :

Following a preliminary experiment in which the plated cells were treated with different BSO concentrations to decide the best non-toxic dose by measuring MTT absorbance after six hours, two 96 flat-bottomed well plates were used in this experiment, plated as described (2.11.1.) . Three days later, and after removing the old medium, one plate was treated with BSO-containing medium at a concentration of 2 mM (as effective non-toxic concentration) in 200 µl ordinary medium. One row of wells was left as a control using BSO free medium. The other plate was fed as before using fresh medium. Plates were incubated at 37 $^{\circ}$C under 2 % CO_2 . Six hours

Chemosensitivity assay for cells grown in suspension :

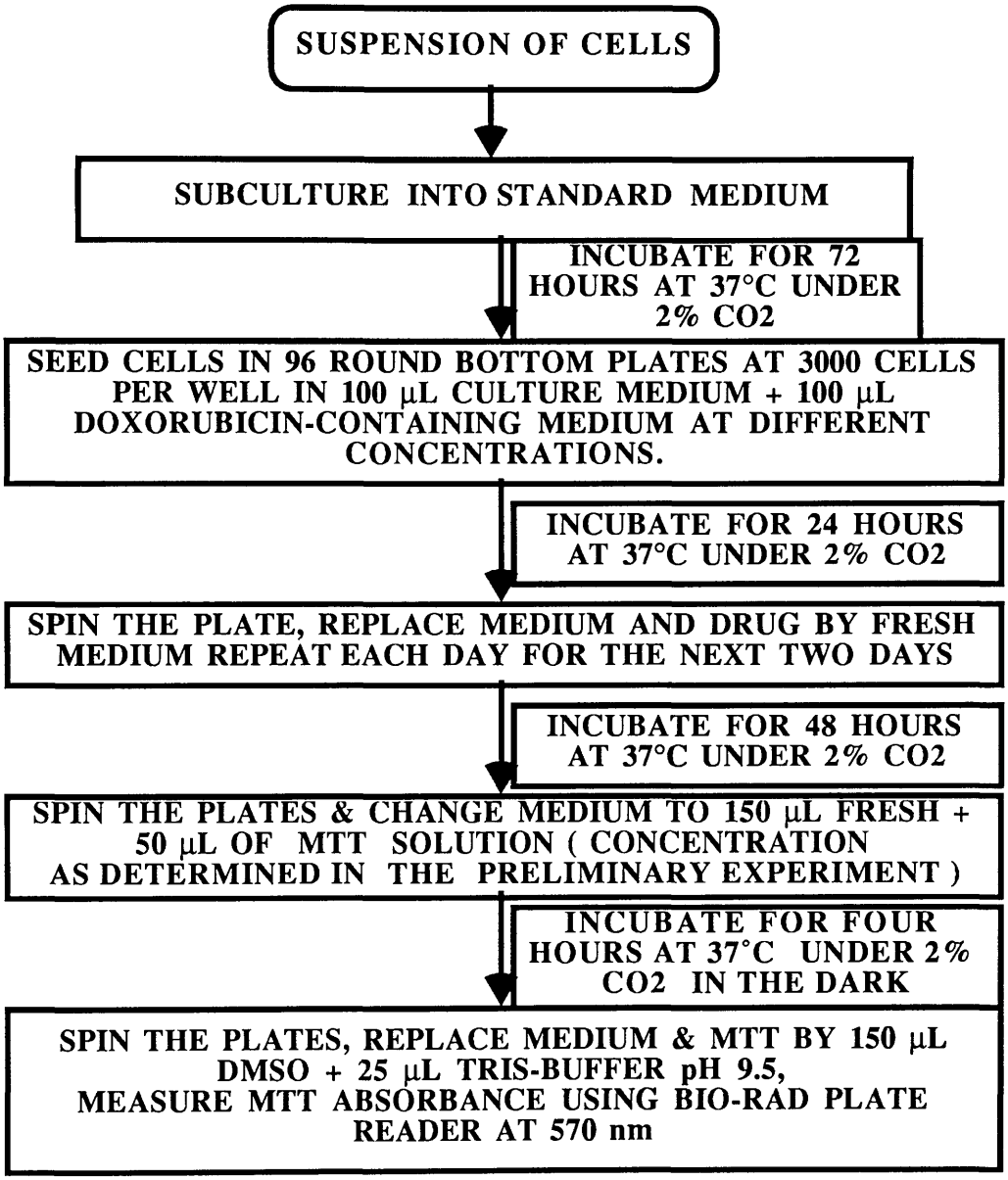


Figure 18 : Protocol for the chemosensitivity assay used in testing drug effects against cells grown in suspension.

later 50 µl of doxorubicin-containing medium was added at six different concentrations, one concentration per six wells to give a final concentrations of (1.72 – 55.2 nmol / l) leaving one row with BSO on the first plate and one with medium only on the first and second plates. Plates were incubated again for 90 minutes with the drugs. The medium was exchanged for drug-free medium three times in the next three days. On the final day the MTT absorbance was measured as described in 2.11.2. and figure 19. Graphs were plotted for doxorubicin concentrations vs. MTT absorbance.

2.13. Enhancement of Doxorubicin Cytotoxicity Using Amiodarone :

The A549 cells used in this assay were resistant to doxorubicin and had a high intracellular glutathione concentration. The effect of amiodarone in enhancing the sensitivity of these cells to doxorubicin was studied by observing the increased glutathione utilization in these cells compared with control cells both doxorubicin-treated and untreated. Amiodarone and doxorubicin uptake was also compared as was sensitivity to doxorubicin after amiodarone pretreatment.

A preliminary qualitative experiment was carried out using the same cell number (10^8) of A549 and GLC4 210 (S) strain in order to examine the penetration of doxorubicin into resistant and sensitive tumour cells by treating them with the same drug concentration in two separate test tubes. One hour later the doxorubicin-containing medium was removed by centrifugation at 1500 rpm for 10 minutes and its concentration in the two cell lines was examined by viewing the fluorescence emitted by doxorubicin under excitation at 366 nm.

Depletion of glutathione using buthionine–S–R–sulfoximine (BSO)

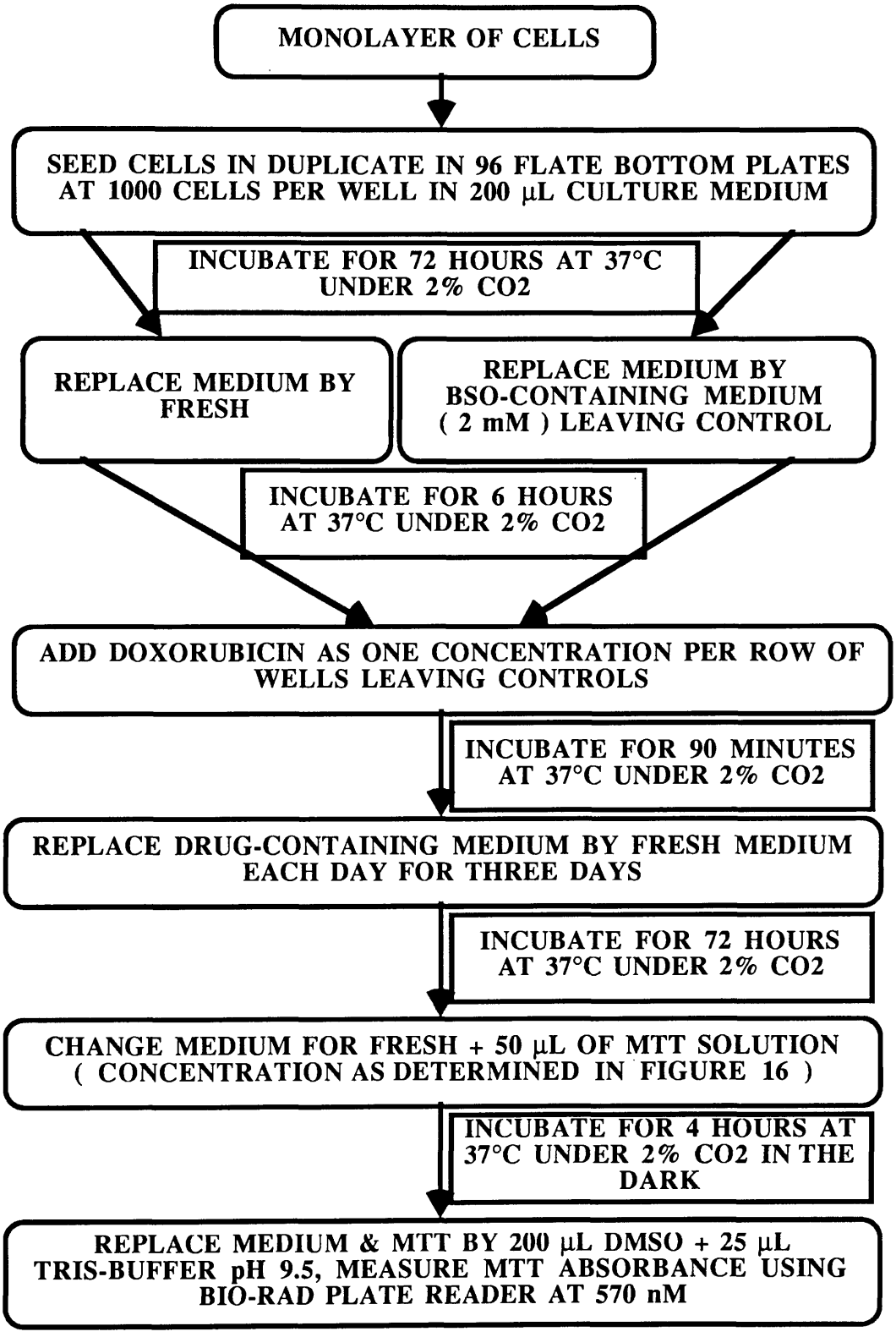


Figure 19: Method of assaying the effect of GSH depletion using BSO on doxorubicin chemosensitivity of cells in monolayer.

2.13.1. Cell Treatment and Preparation :

Sixteen F25 flasks were prepared, each containing 1 x 10^6 A549 cells in 10 ml culture medium. Four days later, the medium was removed from twelve flasks and replaced by 5 ml of fresh medium containing amiodarone; the other four flasks were used as controls, their medium being replaced by fresh medium free of amiodarone. Amiodarone was used at four different concentrations (2 – 8 µmol / l), three flasks for each concentration. One hour later the amiodarone-containing medium and the controls medium were replaced by doxorubicin-containing medium at a concentration of 5 µmol / l after first washing the cells and the flasks with warm fresh medium. One of the control flasks was left with doxorubicin-free medium as a control for glutathione measurement. Three hours later the doxorubicin-containing medium was removed and cells were washed rapidly three times using iced PBS, 10 ml for each wash , for detail see figure 20.

For measurement of doxorubicin and amiodarone uptake the cells were harvested in two different ways. First by adding 5 ml of trypsin solution (0.25 % in PBS) to each flask, and leaving the cells in contact for 15 minutes at 37°C. Second by scraping the cells in 5 ml of iced PBS using a disposable cell scraper (Costar, 205 Broadway, Cambridge, MA 02139, U.S.A.). For glutathione measurement cells were harvested and GSH was measured as described in sections 2.3.3. and 2.6.1.

2.13. 2. Doxorubicin and Amiodarone Measurement :

4.5 ml of cell suspension from each flask in 2.13.1. were used for the estimation of doxorubicin and amiodarone uptake. Cells were disrupted using an ultrasonic probe as described in 2.6.1. and the extraction of the drugs from the lysate was

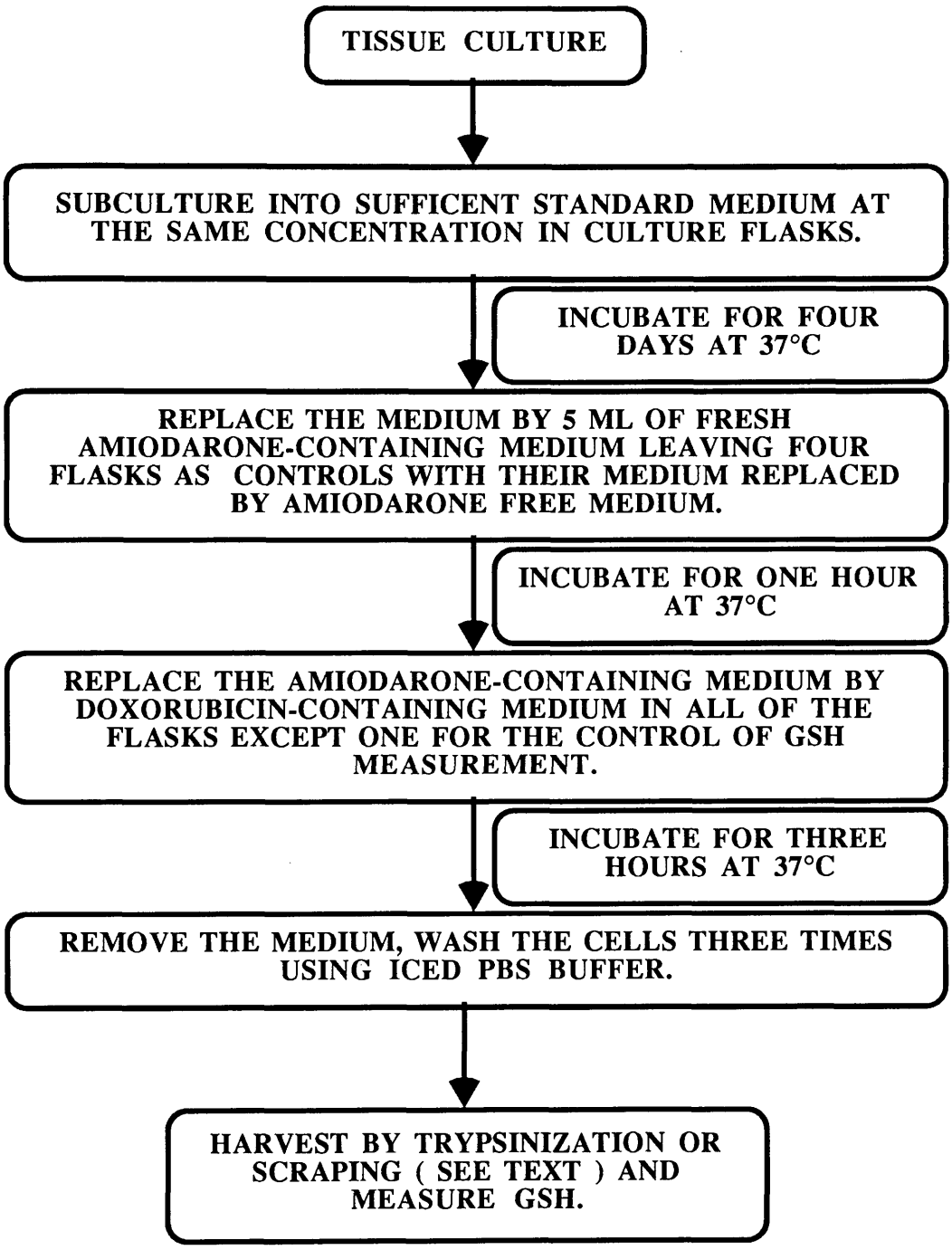


Figure 20 : Cell preparation for the assay of amiodarone induced cell sensitivity in A549 resistant cells.

carried out as described by Cummings and McArdle, (1986). Briefly, cell homogenates were treated with 0.9 ml of silver nitrate solution (33 % w/v) and mixed for 10 minutes in order to release doxorubicin from the DNA and to precipitate protein (Schwartz, 1973). 100 µl of methanol containing 17.73 µmol / l daunorubicin and 91.43 µmol / l fluphenazine as internal standards, first for doxorubicin and second for amiodarone, were added to each tube. The homogenate was extracted with 10 ml of chloroform : propan-2-ol (2 : 1) for 30 minutes, followed by centrifugation at 2000 rpm for 10 minutes at which three distinct phases separated. The lower organic layer was transferred to a clean test tube and evaporated to dryness using a vacuum evaporator (Buchler Vortex-Evaporator, U.K.). The residue was dissolved in 100 µl of methanol and 20 µl of it were injected onto the HPLC system for doxorubicin measurement and 50 µl onto the HPLC system for amiodarone measurement (Figure 21). Standard samples of doxorubicin and amiodarone were prepared in the same manner and injected onto the columns at the same time. Standard curves of doxorubicin and amiodarone were prepared by making serial dilutions of both of them in the related HPLC buffer containing 17.73 µmol /l daunorubicin and 91.43 µmol /l fluphenazine as internal standard respectively.

2.13.2.1. Doxorubicin measurement :

These were carried out as described by Watson et al., (1985) with some modifications. The system consisted of a single piston reciprocating pump (Gilson model 302) with a manometric module 802 as described (2.4.1.) . The LC - FL fluorescence detector was fitted with a 450 nm excitation interference filter and a composite emission filter formed from two sharp cut off filters with minimum absorbances at 528 and 650 nm giving a minimum absorbance at 555 nm (Pye Unicam Ltd., Cambridge, U.K.). This was used with a CR 6525 double pen chart

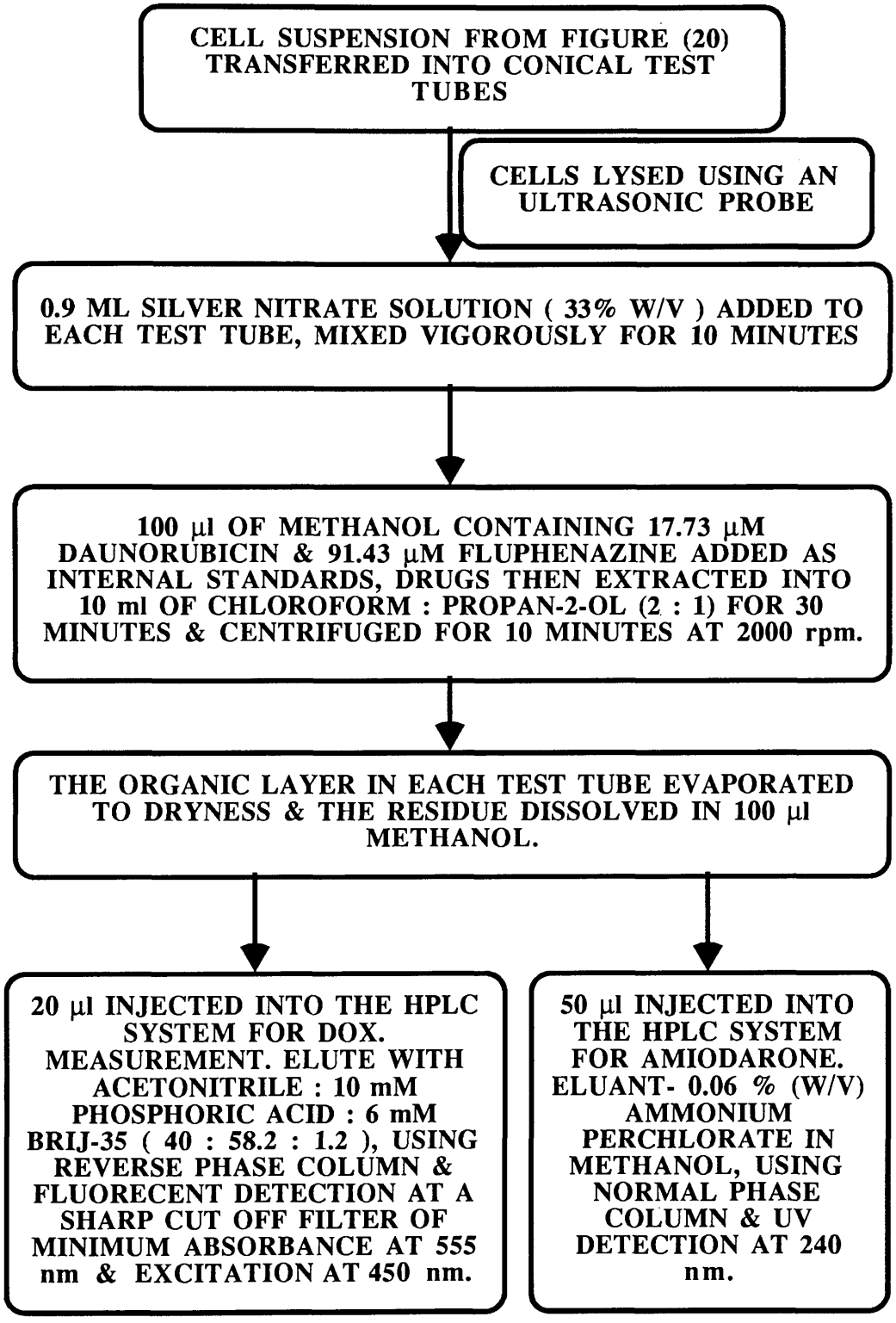


Figure 21 : Extraction and measurement of doxorubicin and amiodarone.

recorder described (2.4.1.). The column used for doxorubicin detection was 250 x 4.6 mm (i.d.) stainless steel supplied packed with Apex Octadecyl (5 μ) from Jones Chromatography.

Manual injection was made via a Rheodyne 7125 injection valve (Scotlab) with a 20 μl loop. The initial chromatographic eluant consisted of acetonitrile : 10 mM phosphoric acid : 6 mM Brij-35 (non-ionic surfactant) (40 : 58.2 : 1.2), filtered, degassed and run at a flow rate of 1.8 ml / minute.

2.13.2.2. Amiodarone Measurement :

This was carried out as described by Storey et al., (1982). Briefly the equipment was similar to that for doxorubicin measurement except that detection was by a single wavelength LC-UV detector (Pye Unicam) set at 240 nm and 0.08 AUFS, with a single-pen chart recorder (Chessel Ltd., Worthing, Sussex, England). Separation was on a 250 x 4.6 mm (i.d.) stainless steel column supplied packed with Hypersil silica (5 μ) from HPLC Technology, (Burke Electronics Ltd., 4 Park Gardens, Glasgow G3 7YE). Injection was as in the case of doxorubicin. Elution was isocratic ; the eluant was 0.06 % (w/v) ammonium perchlorate in methanol, at flow rate of 2 ml per minute.

2.14. Chemosensitivity Assay of A549 Cells Following Amiodarone pretreatment :

This assay was carried out as described in 2.11.2. and figure 17. 200 µl per well of amiodarone-containing medium were added to the wells at four different concentrations (2 – 8 µM), as one concentration per six wells. One hour later amiodarone-containing medium was replaced by doxorubicin-containing medium at a concentration of 100 nM (200 µl per well). Two controls free from amiodarone were used, one with doxorubicin-containing medium at the same concentration as above and the other with fresh medium only, six wells for each control. Three hours later the doxorubicin-containing medium was replaced by fresh medium and the experiment continued as in (2.11.2.).

III. RESULTS

3.1. Development of HPLC Method for GSH measurement :

Separation of endogenous compounds at the cellular level at different concentrations without interference is not simple. For GSH the best HPLC buffer was found to be water : methanol : tetrabutyl ammonium hydroxide. Figure 22 gives a clear idea of the best resolution using cationic ion-pair chromatography for GSH separation rather than reverse phase partition on which GSH is unretained. Figure 23 shows the resolution of different cellular components using tetrabutyl ammonium hydroxide and tetrabutyl ammonium dihydrogen phosphate as cation pairs, and the reliability of using the first of these for the best resolution and absorbance. HPLC grade water and methanol improve the baseline, by reducing noise seen in figure 24 due to the absorbance of impurities in AnalaR grade solvents. Comparison of figure 23 and figure 24 indicates the improvement in signal to noise ratio when using HPLC grade water and methanol (Figure 23) rather than AnalaR grade methanol and distilled water (Figure 24).

The optimum tetrabutyl ammonium hydroxide concentration was found to be 0.1 % which gave high resolution of the GSH from other cellular components and good separation. The best capacity ratio (k') can be reached at this concentration of tetrabutyl ammonium hydroxide when other variable are fixed (Figure 25). Adjustment of the HPLC buffer pH to 3.5 using 10% v / v orthophosphoric acid was also found to increase resolution giving a value of 3.5 with k' 7.75. At pH 8 the resolution was 2.9 and k' was 6.1 (Figures 26 and 27). Resorcinol was chosen as the internal standard as it is soluble in the HPLC buffer and does not co-elute with other cellular constituents, it also shows good absorbance in the UV range.

Ultrasonic lysis was found to be the best method for treatment of cells releasing their components in a more reproducible manner than other methods of lysis such as

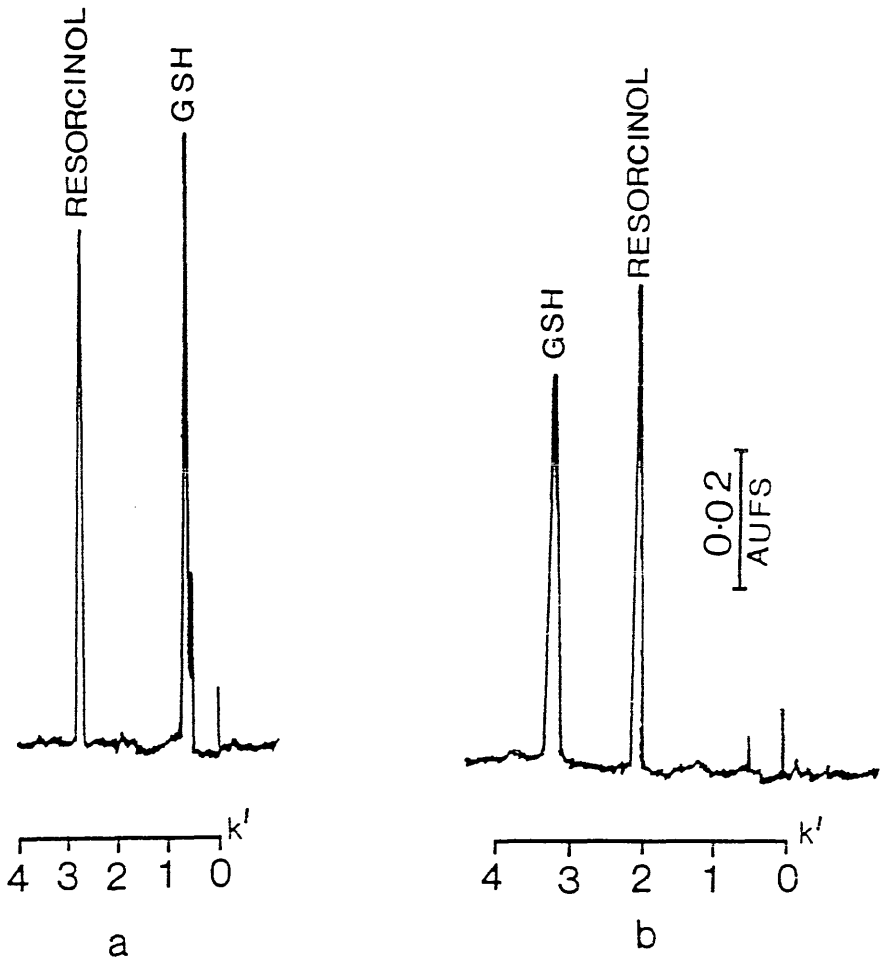


Figure 22 : HPLC chromatograms of standard GSH with the internal standard resorcinol measured at 200 nm ; a) no ion-pair used ; b) 0.1 % tetrabutyl ammonium hydroxide.

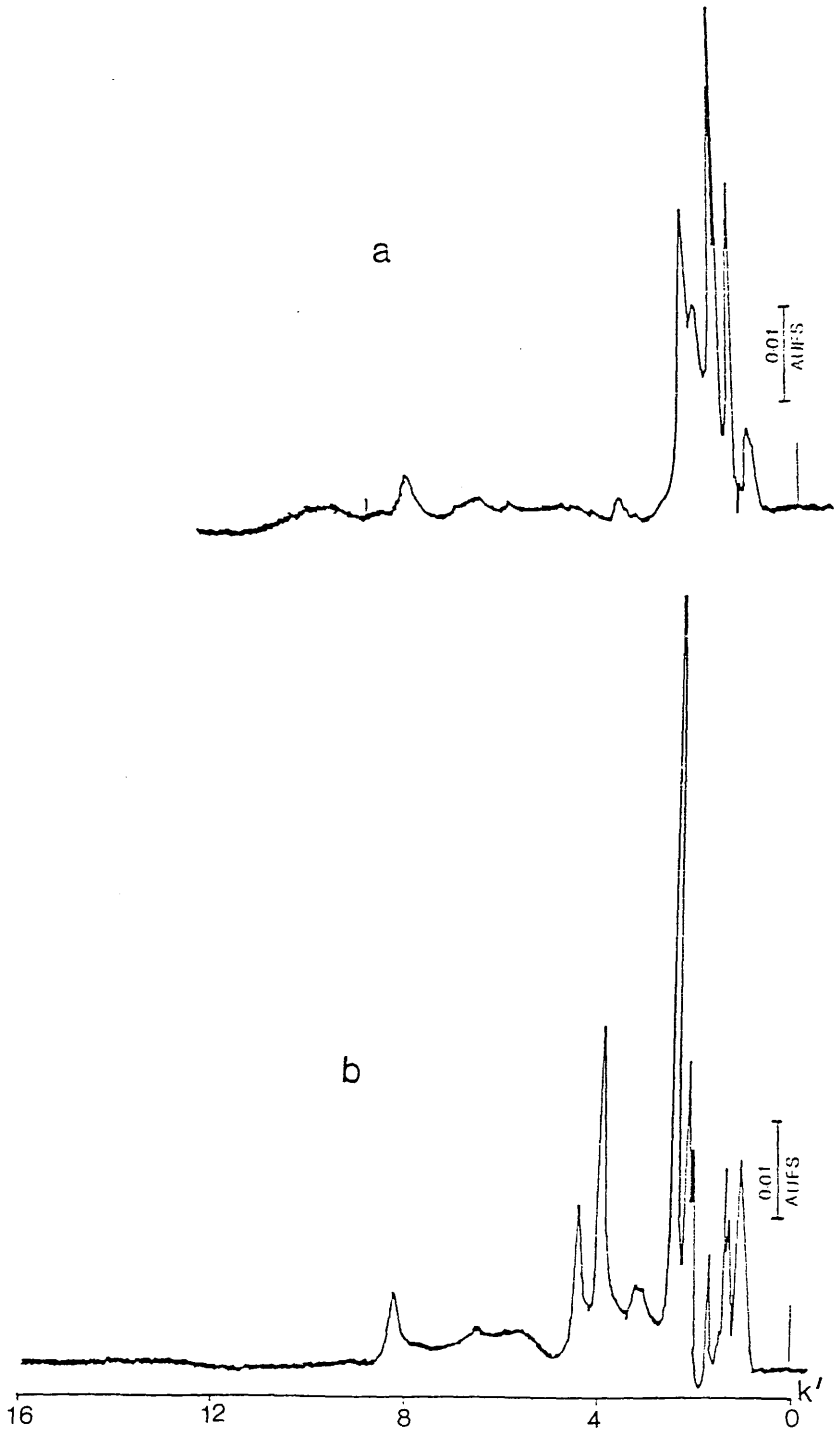


Figure 23 : Chromatograms of cell lysate obtained from Hela cells using the final HPLC method showing resolution of components with two different ion-pairs. a) Tetrabutyl ammonium dihydrogen phosphate (0.1 %) ; b) Tetrabutyl ammonium hydroxide (0.05 %).

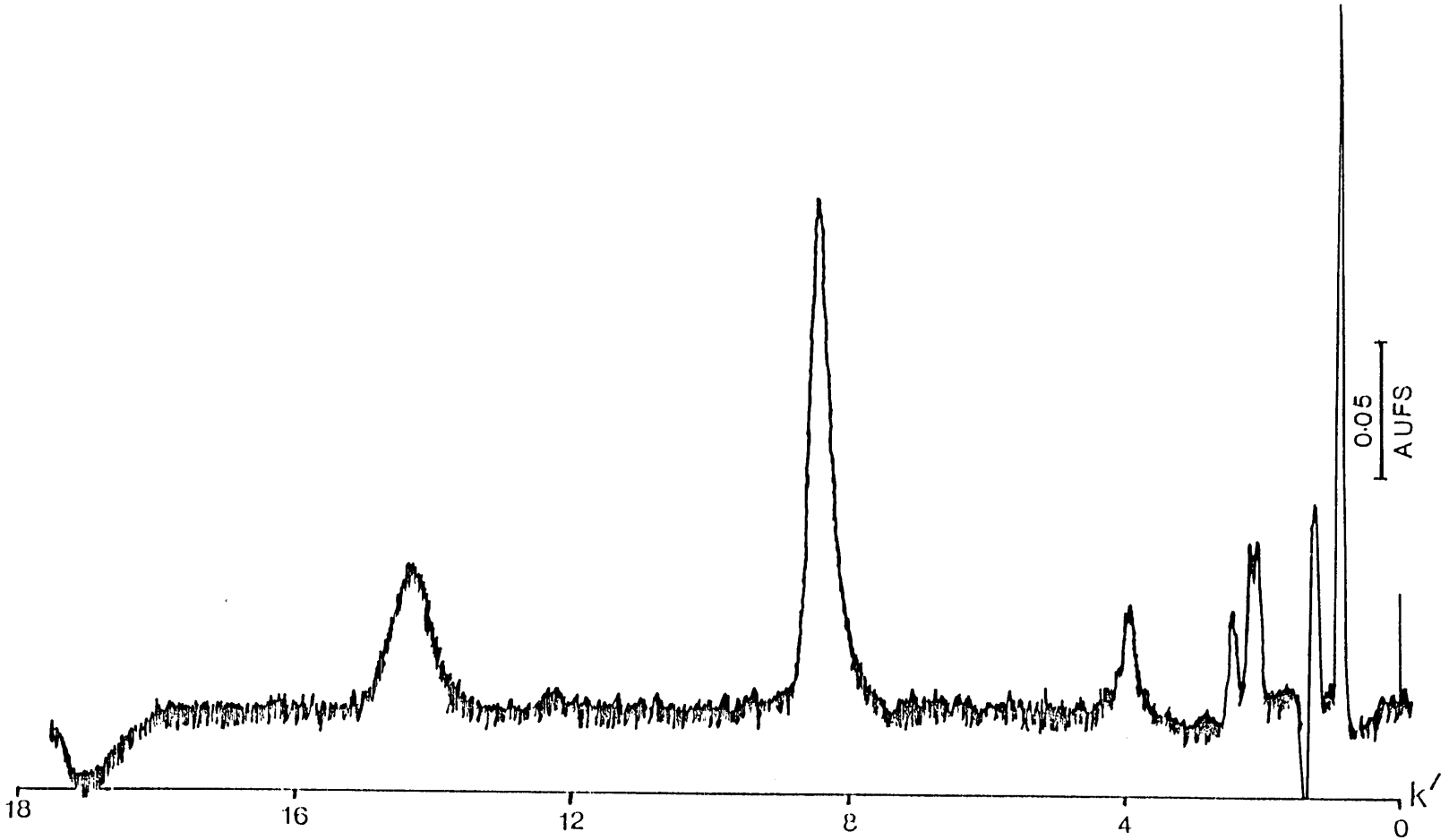


Figure 24 : HPLC chromatogram of cell lysate obtained from Hela cells using analar grade methanol and distilled water ; other variables were as described for the final HPLC development.

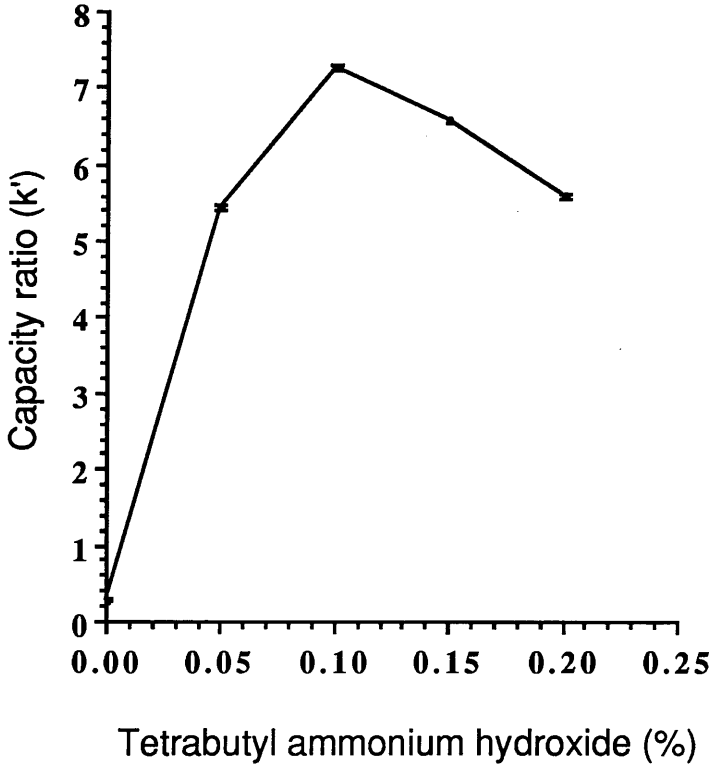


Figure 25 : The relationship between the capacity ratio (k') of GSH and the concentration (%) of tetrabutyl ammonium hydroxide in the HPLC buffer. Each point is a mean of five different readings, using standard GSH. Bars = standard error.

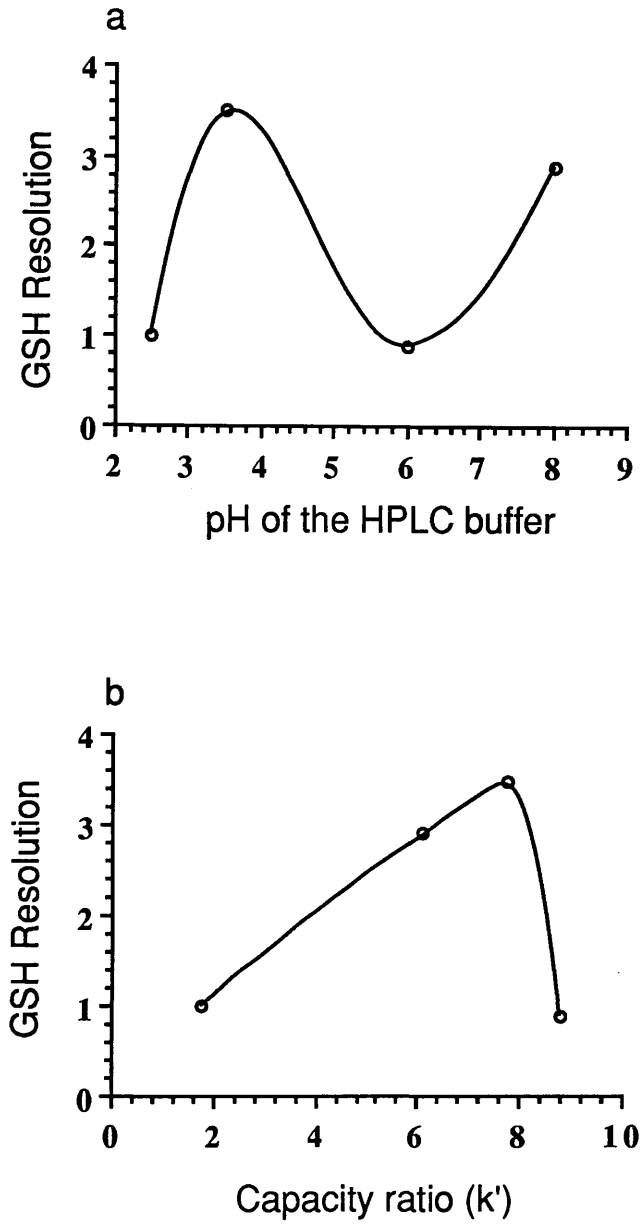


Figure 26 : The relationship between GSH resolution and a) the pH of the HPLC buffer used for separation ; b) the capacity ratio (k') of the GSH peaks at different pH values. Each point is a mean of two different GSH measurements using Hela cell lysates.

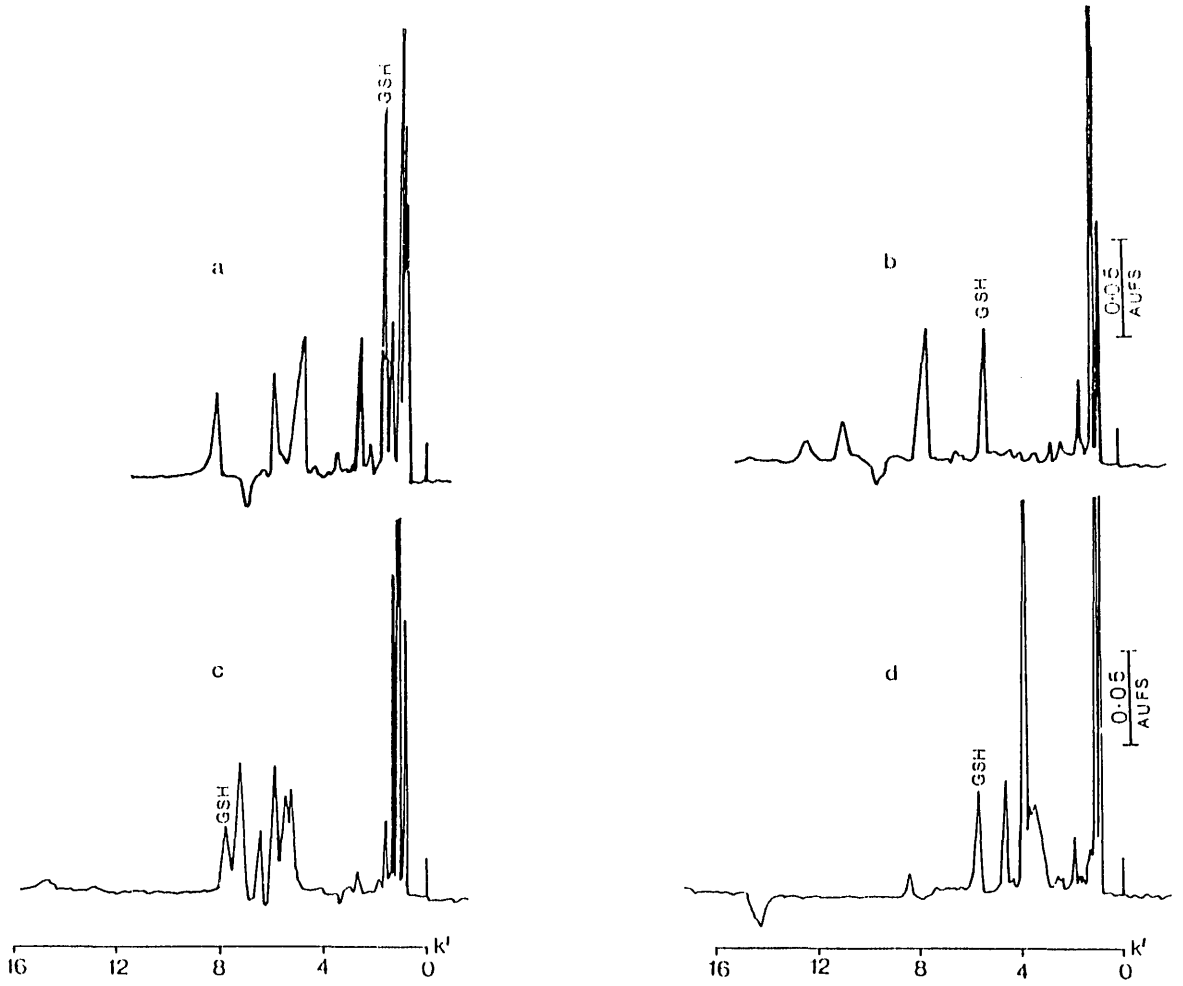


Figure 27 : HPLC chromatograms of cell lysates obtained from Hela cells at the final HPLC development with different mobile phase pH. a) pH 2.5 ; b) pH 3.5 ; c) pH 6 ; d) pH 8.

freezing and thawing. The lysate was found to be stable at – 20°C for more than 12 hours. Figures 28 – 31 show HPLC analysis of cell lysates by this method of the four cell lines used in these studies in which the differences in glutathione content of these different cells appears clear. Figure 32 shows the standard GSH separation using the same method.

3.1.1. Linearity :

The standard curve for glutathione was found to be linear over the range 0 – 1.2 μg on column sample weight (0 – 3.9 nmoles) (Figure 33).

3.1.2. Sensitivity :

The limit of sensitivity for standard glutathione measured by this method using HPLC buffer as a diluent was found to be 1 mg / l (20 ng on column ≅ 65 picomoles) (≅ 3.25 μmol / l).

3.1.3. Precision :

The precision studies using aqueous standards of glutathione or Hela tumour cells are summarised in table 3. All coefficients of variation and standard deviations are below two percent.

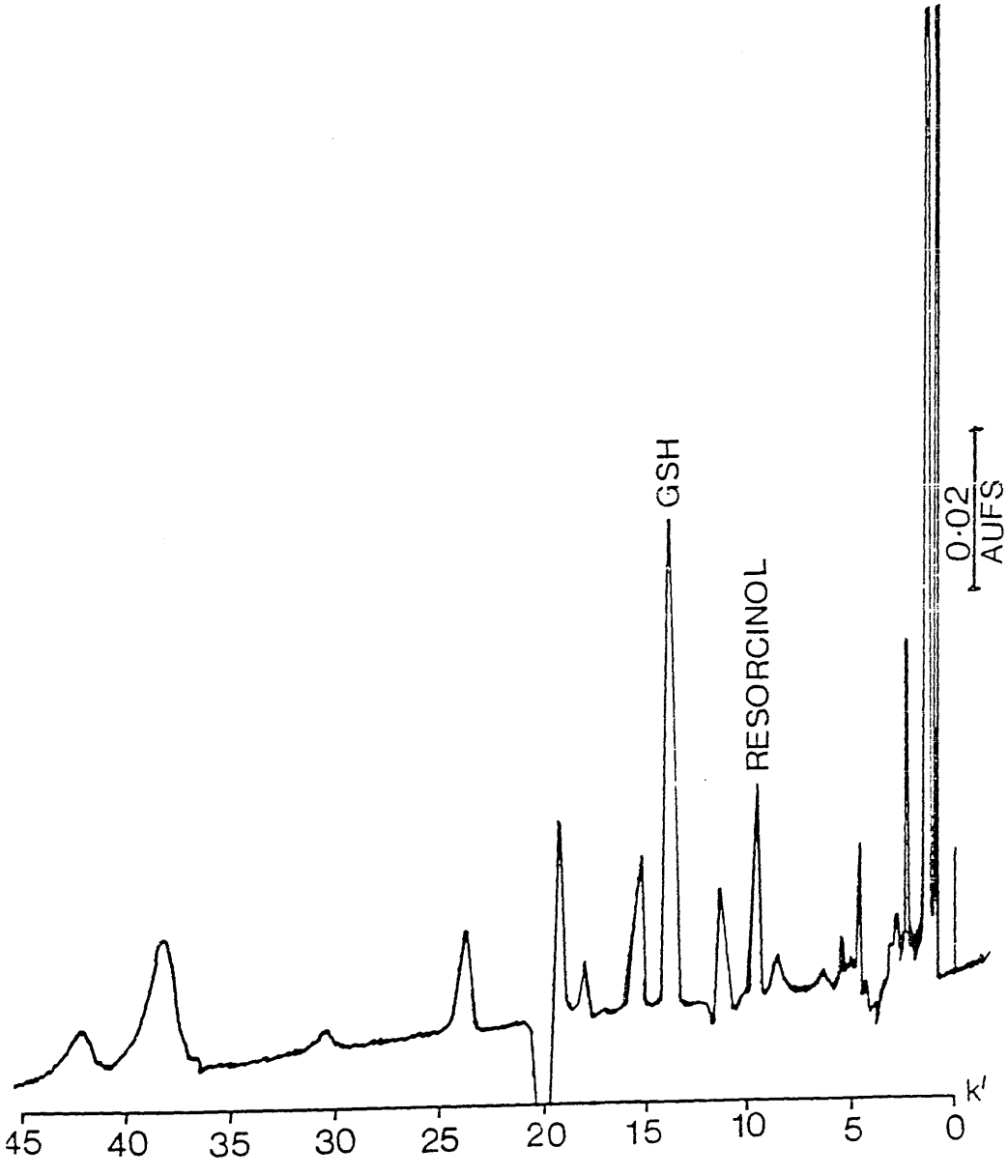


Figure 28 : HPLC chromatogram of cell lysate from Hela cells using the final HPLC method measured at 200 nm.

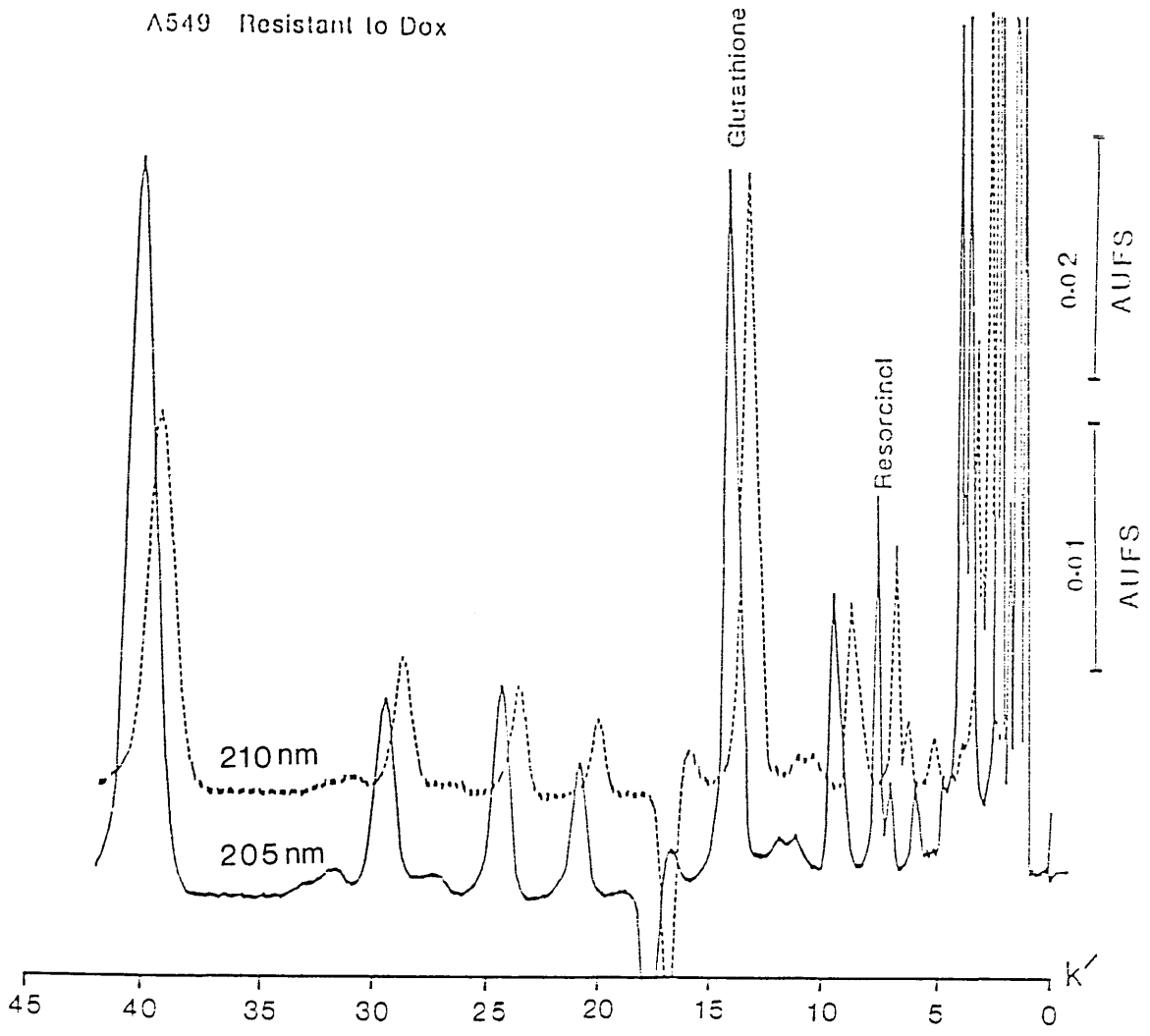


Figure 29 : HPLC chromatogram of cell lysate from A549 cells using the final HPLC method.

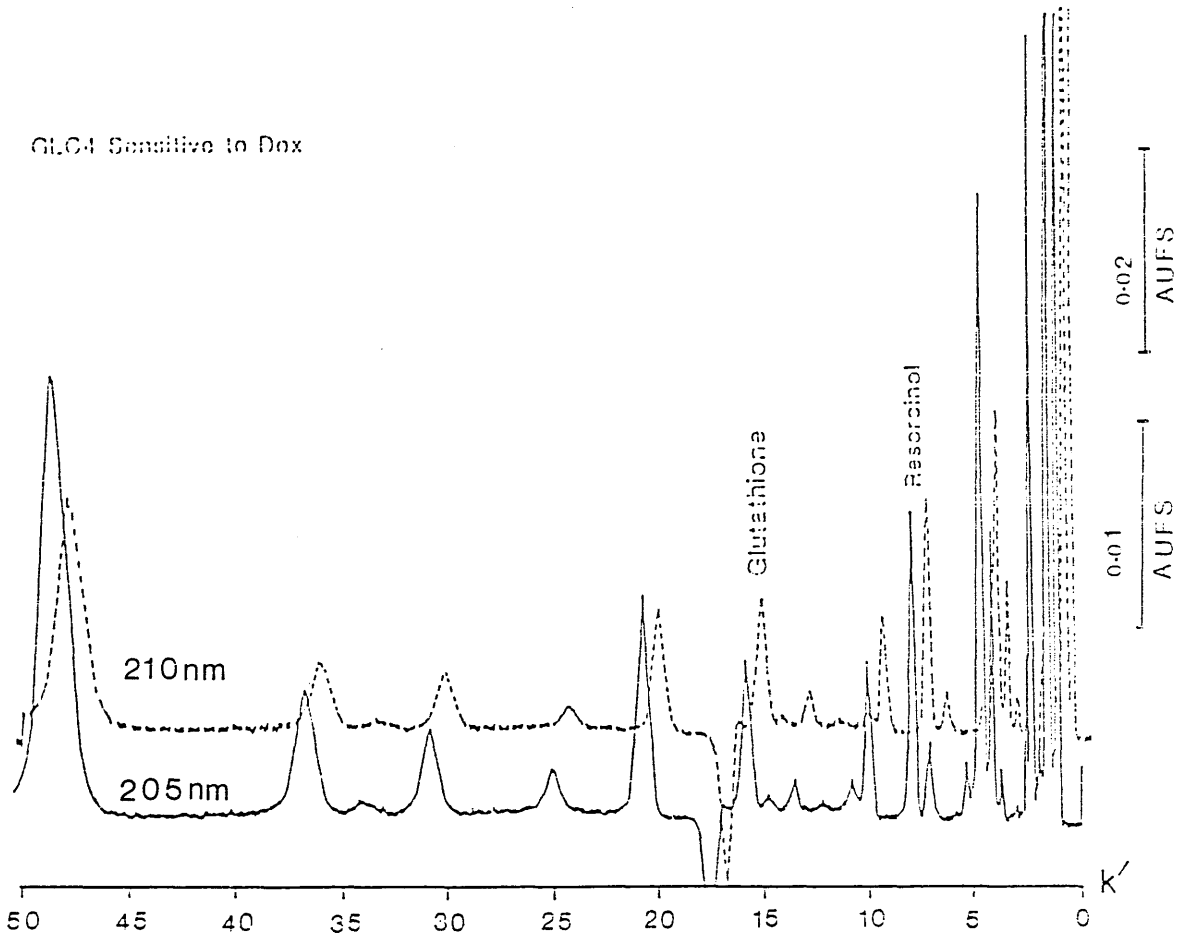


Figure 30 : HPLC chromatogram of cell lysate from GLC_4 210 (S) cells using the final HPLC method.

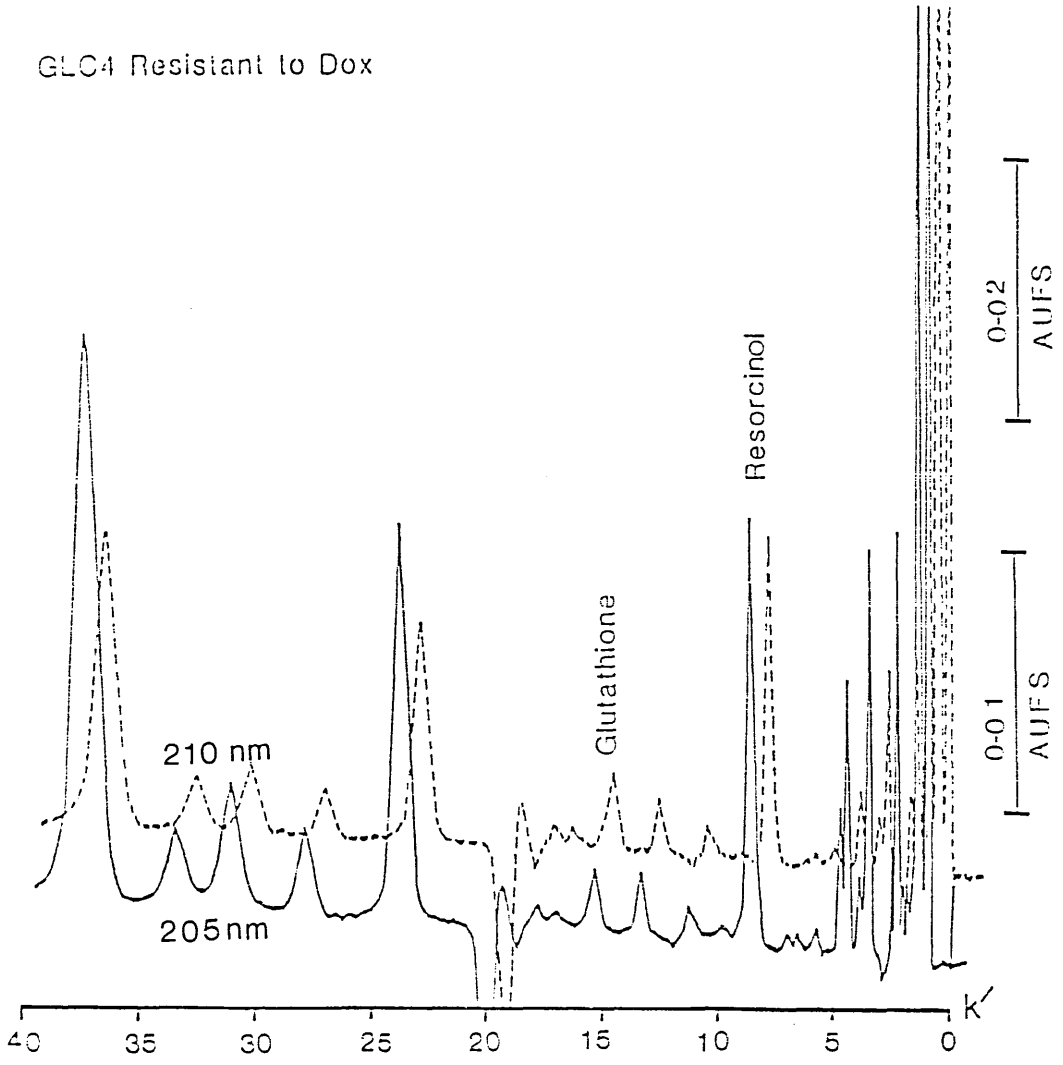


Figure 31 : HPLC chromatogram of cell lysate from GLC_4 210 (R) cells using the final HPLC method.

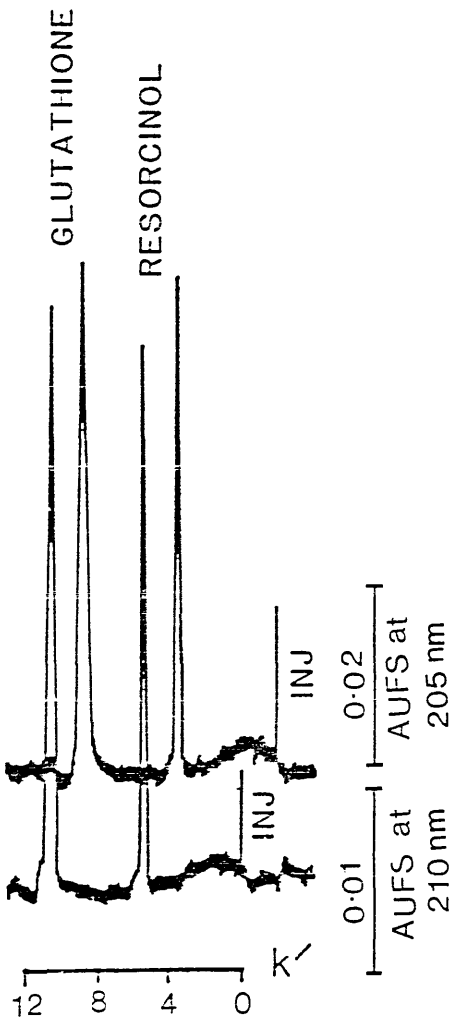


Figure 32 : HPLC chromatogram of standard GSH with the internal standard resorcinol measured at 205 and 210 nm.

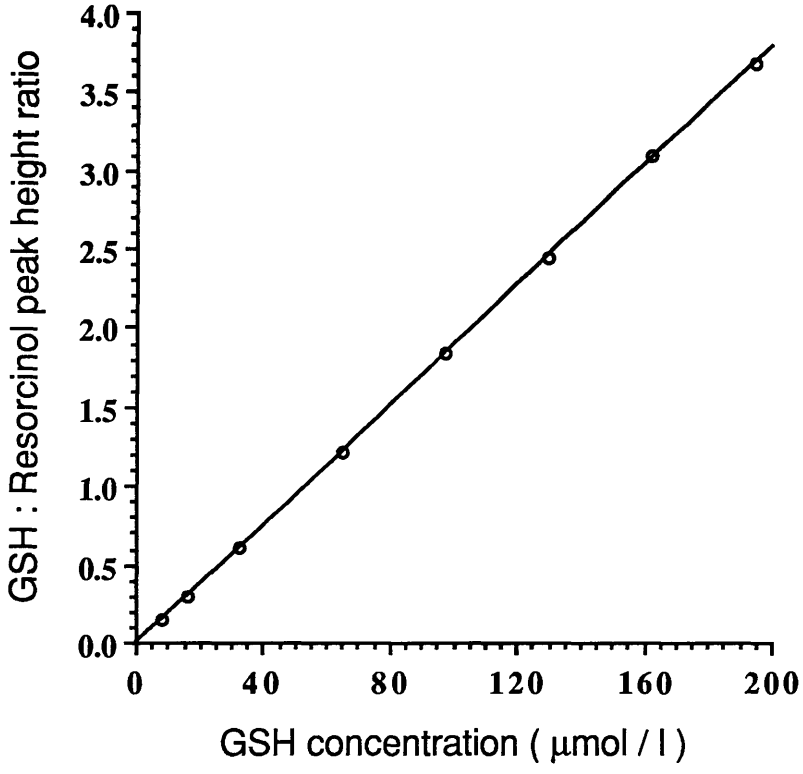


Figure 33 : Standard curve for glutathione as measured by HPLC. Each point is the mean of duplicate measurements.

Table 3 :

Precision studies on glutathione standard (n = 21) and glutathione from Hela cells (n = 10).

GSH SOURCE	MASS UNIT OR CELLS / L	MOLAR UNIT OR NUMBER OF CELLS INJECTED	PRECISION (%)
STANDARD GSH	20 mg / L	6.51×10^{-5} MOLAR	± 1.89
CELLS	6.25×10^{9} CELLS / L	1.25×10^{5} CELLS	± 0.95

3.1.4. Identification of Peaks Other than GSH in Cell Lysate :

A number of the larger peaks in the lysate chromatograms were collected, freeze dried and reconstituted in salt free loading buffer pH 2.1 (42 g citric acid, 200 ml methanol and 5 ml thiodiglycol 25% v/v in deionized water). These were then analysed using a specific amino acid HPLC method with post-column ninhydrin detection. The amino acids, glycine, glutamine, leucine, alanine, arginine and phenylalanine were identified. No consistent changes were found in these peaks after doxorubicin treatment and the separation was not optimised for these. Confirmation of identities using mass spectrometry was not successful due to the high phosphate concentrations in the freeze dried material. Since these findings were not central to the study, no further attempts were made to quantitate the amino acids.

3.2. Growth Experiment :

The growth curves for the four cell lines, Hela, A549, GLC_4 210 (S and R) are unremarkable and are shown in figure 34.

3.3. Effects of Exposure to Doxorubicin :

3.3.1. Hela Tumour Cells :

In this experiment the effect of doxorubicin exposure on Hela tumour cell lysates as measured by HPLC (Figure 28) showed a rapid dose-related depletion of the

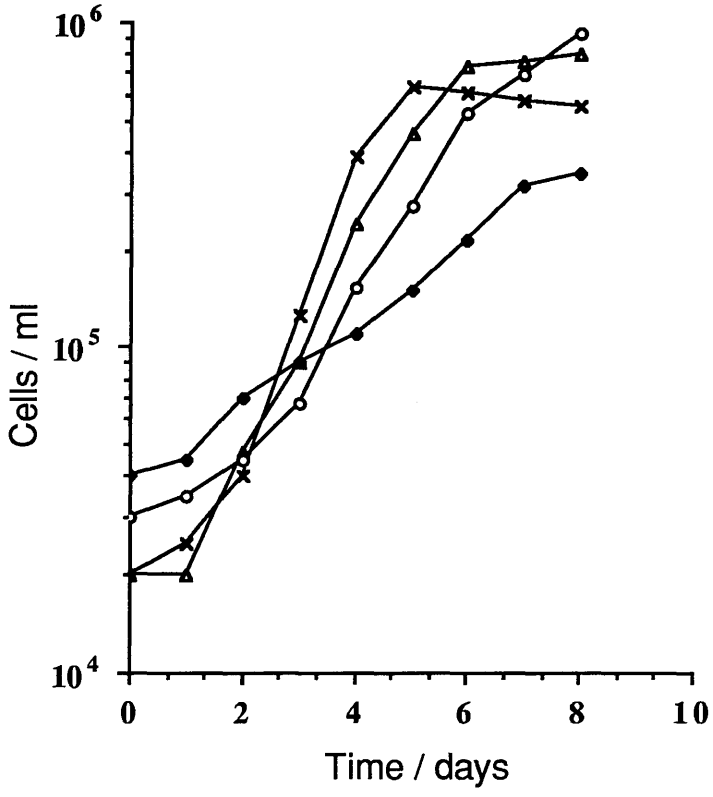


Figure 34 : Growth behaviour of Δ Hela ; x A549 ; o GLC_4 210 (S) ; ♦ GLC_4 210 (R). Cell numbers were measured using a haemocytometer. Each point is the mean of duplicate counts.

cytosolic glutathione pool. The basal glutathione concentration of Hela tumour cells was found to be 14 – 15 nmol / 10^6 cells as a result of two different experiments. The depletion of glutathione was up to 85% of the control value after 12 hours exposure using doxorubicin at 6 nmol / 10^6 cells (Figure 35). The result of this experiment indicates that Hela tumour cells have high intracellular glutathione concentrations but are highly sensitive to doxorubicin.

3.3.2. A549 (Resistant) Cells :

The result of this experiment shows that these cells also contain high glutathione levels as measured by HPLC (Figure 29). The basal glutathione concentration of A549 tumour cells was found to be 14.2 – 15 nmol / 10^6 cells which is about the same as Hela cells as measured in two different experiments. However in this case reduction of the cytosolic glutathione pool was minimal even with high doxorubicin doses reaching 68.8 nmol / 10^6 cells (Figure 36). Glutathione depletion using high doxorubicin doses in this tumour was about 50% of the control value after 12 hours exposure. The result of this experiment suggests that the high GSH content in A549 tumour cells may be related to their resistance to doxorubicin.

3.3.3. GLC_4 210 Sensitive Tumour Cells :

These cells contain little glutathione as measured by HPLC (Figure 30). The basal glutathione concentration of this type of tumour cell was found to be 1.1 – 1.3 nmol / 10^6 cells when measured in two different experiments. However these cells

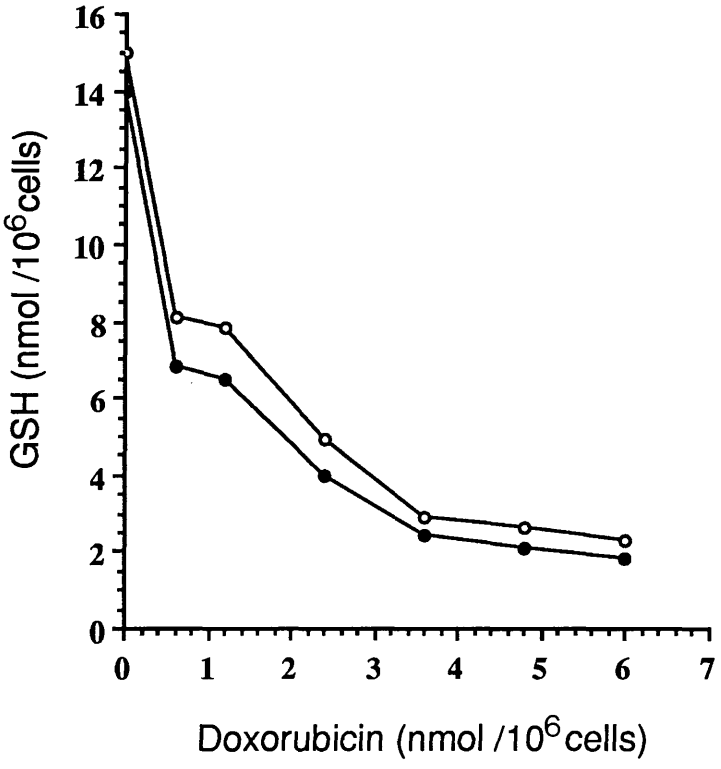


Figure 35 : Effect of doxorubicin on the GSH concentration of Hela cells as measured by HPLC after 12 h exposure. The two curves are from duplicate experiments.

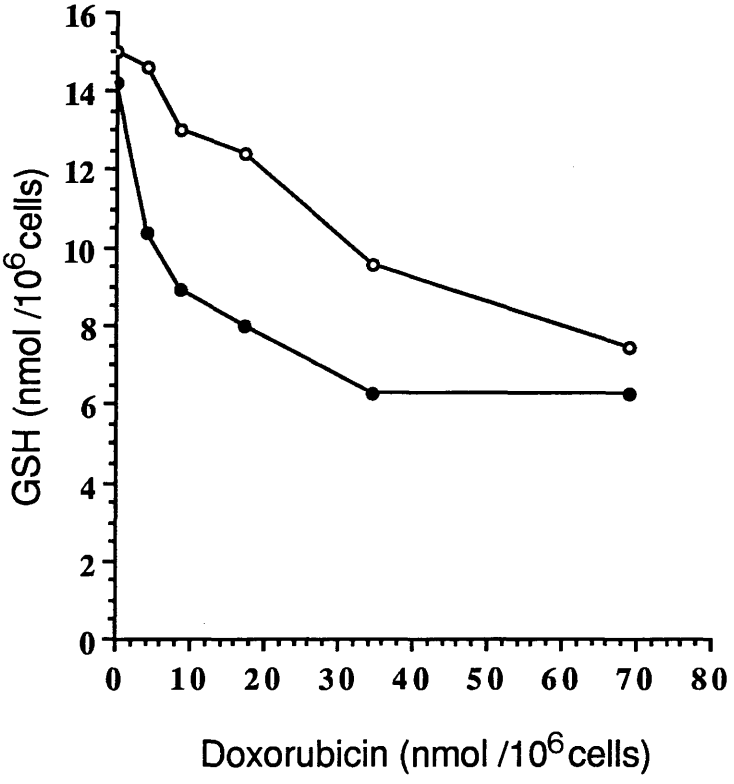


Figure 36 : Effect of doxorubicin on the GSH concentration of A549 cells as measured by HPLC after 12 h exposure. The two curves are from duplicate experiments.

are very sensitive to the doxorubicin effect and exposure to this drug resulted in a rapid depletion of the small cytosolic glutathione pool. The depletion of glutathione was up to 64% of the control value after 12 hours of exposure using doxorubicin at 17.6 nmol / 10^6 cells (Figure 37).

3.3.4. GLC_4 210 Resistant tumour Cells :

In this tumour, with acquired resistance to doxorubicin, the cells have a low level of glutathione as measured by HPLC (Figure 31). The basal glutathione concentration of these resistant tumour cells was found to be 0.7 – 0.8 nmol / 10^6 cells when measured in two different experiments. Doxorubicin exposure did not lead to measurable depletion of the cytosolic glutathione pool ; depletion of glutathione was around 5% of the control value after 12 hours of exposure using doxorubicin at 20 nmol / 10^6 cells (Figure 38).

3.4. Effect of Thiols on Cellular Glutathione :

3.4.1. Effect of N-acetylcysteine on Hela Tumour Cells Treated with Doxorubicin :

In this experiment treatment of Hela tumour cells with N-acetylcysteine one hour prior to treatment with the previously effective doxorubicin dose of 5 nmol / 10^6 cells was found to offer a protective mechanism against the drug effect as shown by measuring glutathione using HPLC. The most effective N-acetylcysteine

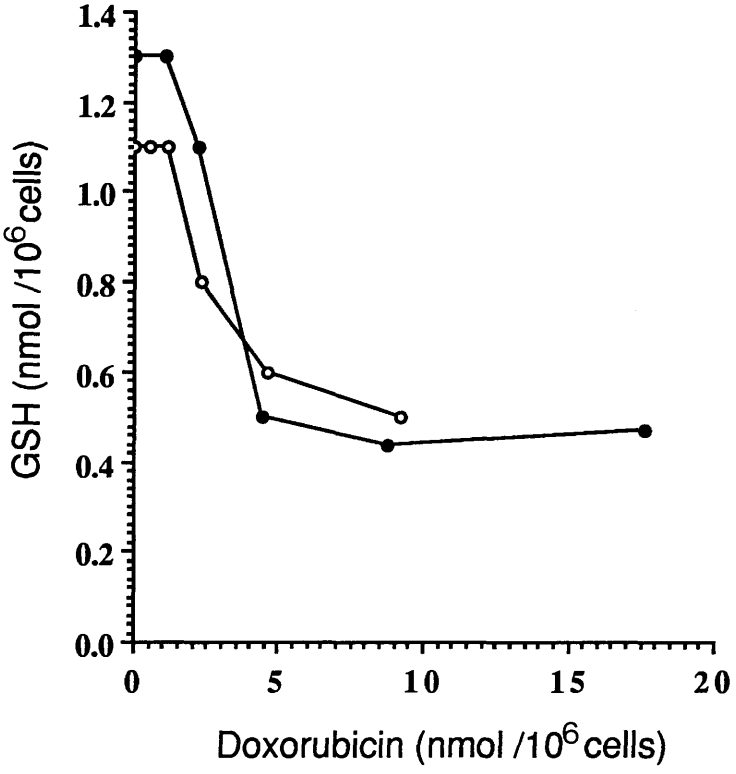


Figure 37 : Effect of doxorubicin on the GSH concentration of GLC_4 210 (S) cells as measured by HPLC after 12 h exposure. The two curves are from duplicate experiments.

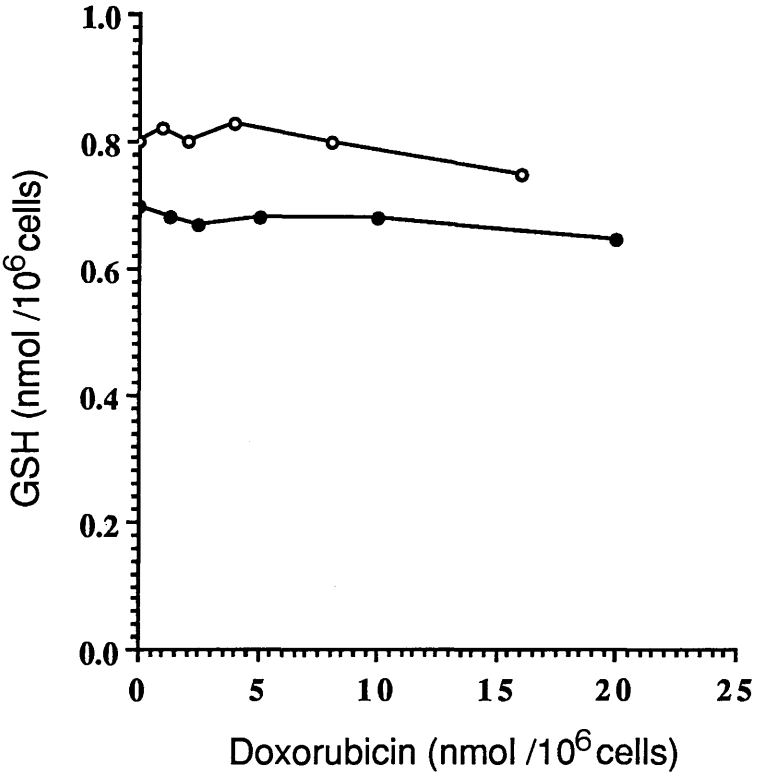


Figure 38 : Effect of doxorubicin on the GSH concentration of GLC_4 210 (R) cells as measured by HPLC after 12 h exposure. The two curves indicate duplicate experiments.

concentration was 1.4 μmol / 10^6 cells above which NAC became toxic (Figure 39). Using N-acetylcysteine at 1.4 μmol / 10^6 cells maintained the cytosolic glutathione pool at 87.7 – 89.5 % of the control value in the presence of doxorubicin, while treatment with doxorubicin alone reduced it to 53 – 62 % of the basal values. There was therefore a 27.5 – 34.7 % reduction in glutathione consumption by pretreatment with a non toxic concentration of NAC.

3.4.2. Effect of Cysteine on Hela Tumour Cells Treated with Doxorubicin :

As in (3.4.1.) pre-treatment of Hela tumour cells with cysteine under the same conditions also minimized the effect of doxorubicin on glutathione as measured by HPLC. Cysteine seems to be non-toxic to the cells and increased the cytosolic pool of glutathione by the same amount at each of the three doses used (Figure 40), with the plateau level being reached at the lowest cysteine concentration used. These cysteine concentrations maintained the cytosolic glutathione pool at 85.3 % of the control value, use of doxorubicin alone reduced the GSH pool to 75 % of the basal level. So, there was a 10.3 % improvement in intracellular glutathione using cysteine pretreatment.

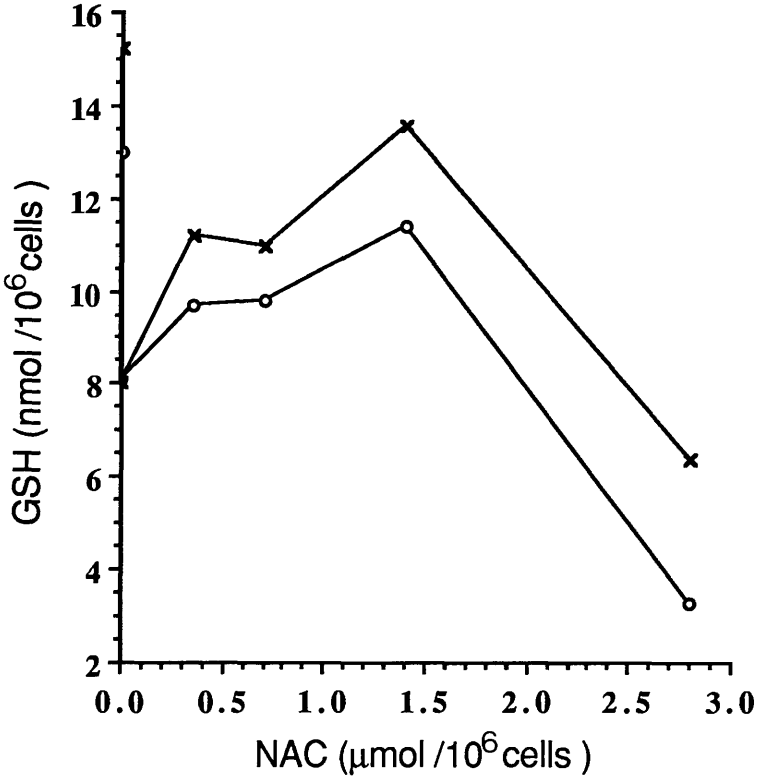


Figure 39 : Effect of 1 h pretreatment with N-acetylcysteine on the GSH content of Hela cells treated with doxorubicin (5 nmol per 10^6 cells) as measured by HPLC after 12 h exposure. The two curves are from duplicate experiments.

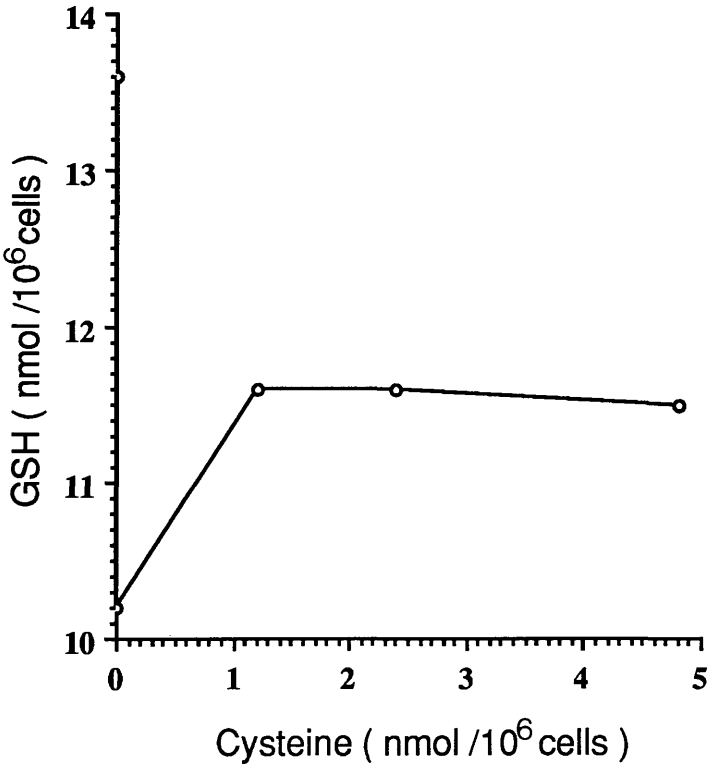


Figure 40 : Effect of 1 h pretreatment with cysteine on the GSH content of Hela cells treated with doxorubicin (4 nmol per 10^6 cells) as measured by HPLC after 12 h exposure.

3.5. ^1H Spin Echo NMR Spectroscopy :

3.5.1. Hela Cells :

The normal FT NMR spectra obtained from the Hela cells are shown in figure 41 in which trace (a) shows the normal resonance arising from large molecules such as cell membranes, proteins and other macromolecules.

Applying the spin echo pulse sequence filters the large molecules from the spectrum by virtue of their shorter relaxation times. Thus, figure 41 (b – e) represent the spin echo spectra of the small resonant cytosolic components of Hela tumour cells. Since the technique relies on relaxation times a series of spectra are shown (T_2 = 30 to 70 ms). Some of the molecules with a short delay time (T_2) are edited from the spectrum (i.e. Protein and membranes).

The resonance in figure 42 have been assigned on the basis of reported information by Klein and Robbins (1970) ; Righetti et al., (1971) and by the procedure of standard addition to the cells. According to this glycine can be easily observed at T_2 = 30 ms, glutathione and triglyceride are observed to the exclusion of other cellular components because of their intense resonances, due to the glycyl methylene of glutathione and the methyl and methylene resonances of triglyceride.

This study using a non–invasive real time method also detects phosphorylcholine, phosphorylcreatine and lactate in the cells. These compounds appeared as prominent species in the ^1H spin echo NMR. These peak assignments are made on the basis of standard addition to the whole-cell spin echo NMR and on the basis of the direct comparison with NMR studies previously reported (Evans and Kaplan, 1977 ; Mountford et al., 1982 ; Evanochko et al., 1984 ; May et al., 1986). However, certain differences were observed between cell types.

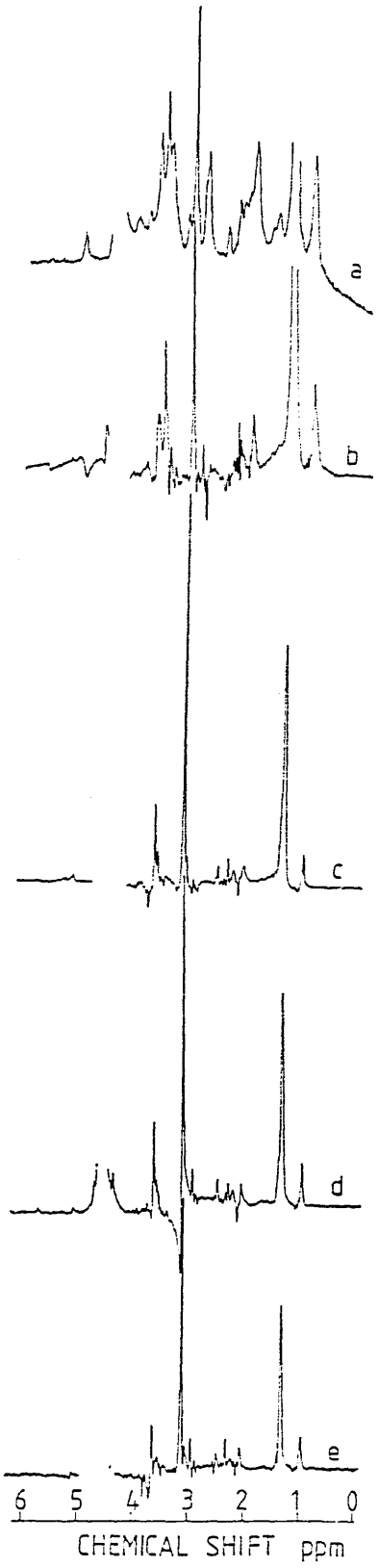


Figure 41 : Normal 250 MHz FT NMR (a) and spin-echo NMR (b – e) of the Hela cell ; (b) $T2$ = 30 ms ; (c) $T2$ = 50 ms; (d) $T2$ = 60 ms; and (e) $T2$ = 70 ms. Each spectrum consists of 1000 scans on a total sample size of 10^9 cells / 0.4 ml ^2H_2O/NaCl (0.154 M). All spectra were recorded at 20°C.

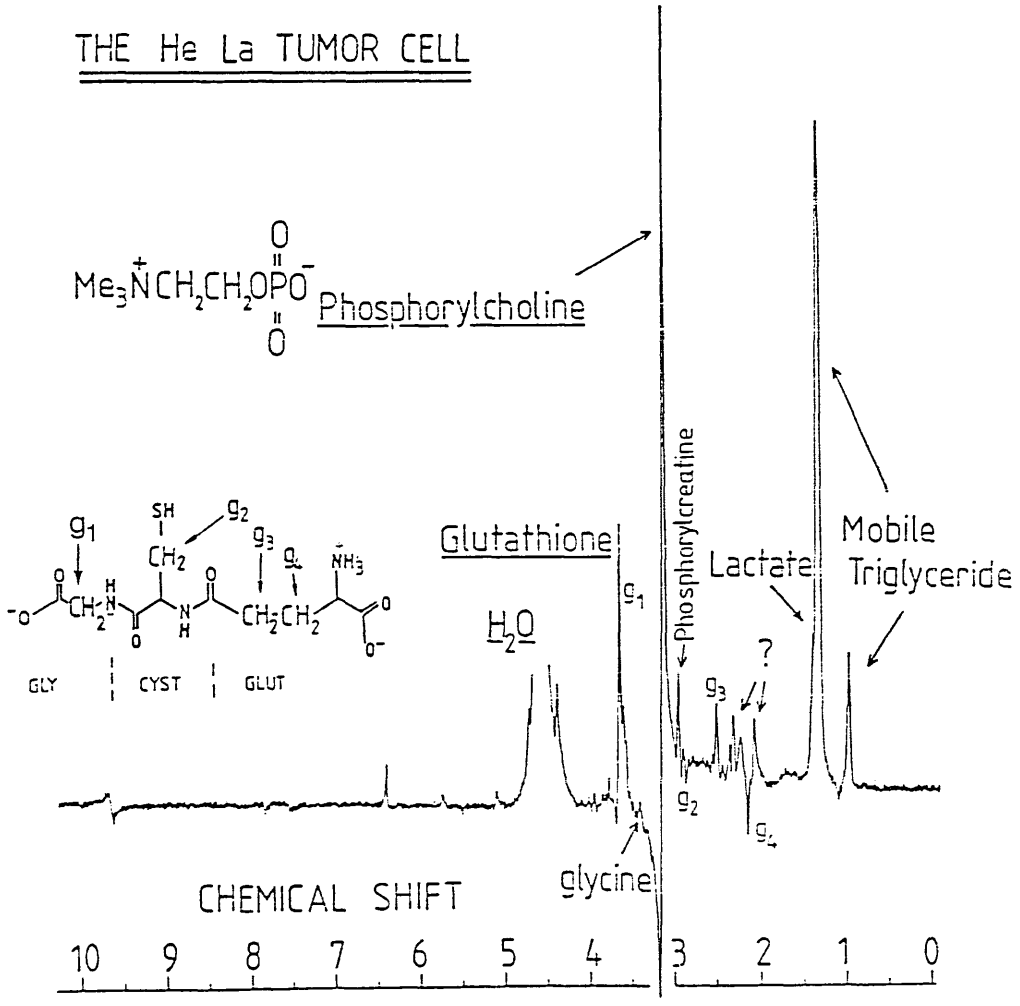


Figure 42 : ^{1}H spin echo NMR of the Hela cell, $T2$ = 60 ms. Total sample size of 10^9 cells / 0.4 ml $^{2}H_2O$ / NaCl (0.154 M). The spectra were recorded at 20°C.

3.5.1.1. Study of Glycolysis :

Study of cellular metabolism by NMR methods depends on the ability to maintain cell viability. Cells were found to be viable for > 5 hours when suspended in unsupplemented $^{2}H_2O$ saline. Because the non-invasive real time NMR method is capable of detecting intracellular lactate (Figure 42), it is possible to study anaerobic glycolysis by the cells as a measure of the energy requirements of the cells. Added glucose [2.0 mg (11 μmoles) / 0.4 ml packed cells] appears as an asymmetric doublet at δ 5.0 in the spectra (Figure 43). The doublet arises as a result of the two glucose anomers (α and β). The upfield line is assigned to the α anomer (Nicholson et al., 1984). The Hela cells have a definite preference for the α anomer, behavior previously reported by Ugurbil et al., (1978) in ^{13}C NMR experiments with micro-organisms. Using the signals from α-glucose and lactate the glycolytic process can be monitored as a function of time (Figure 44), the rate of cellular uptake of the sugar and production of lactate can be clearly seen to be linked considering the basic stoichiometry of lactate production, (equation 1).

Glucose $\longrightarrow$ 2Lactate (1)

The pulse sequence used to obtain spin echo NMR spectra modulates the intensities of the various lines. Thus signal intensities in the spectra, while still reflecting the individual species concentrations, are no longer reliable for direct comparison without prior calibration. Standard mixtures of glucose and lactate (lithium salt, monohydrate) were used. The intensities of the important lines were measured in the normal FT NMR experiment (ratio 2 : 3.04, glucose : lactate) compared with those obtained in the spin echo for the same sample (ratio 2 : 4.21 glucose : lactate).

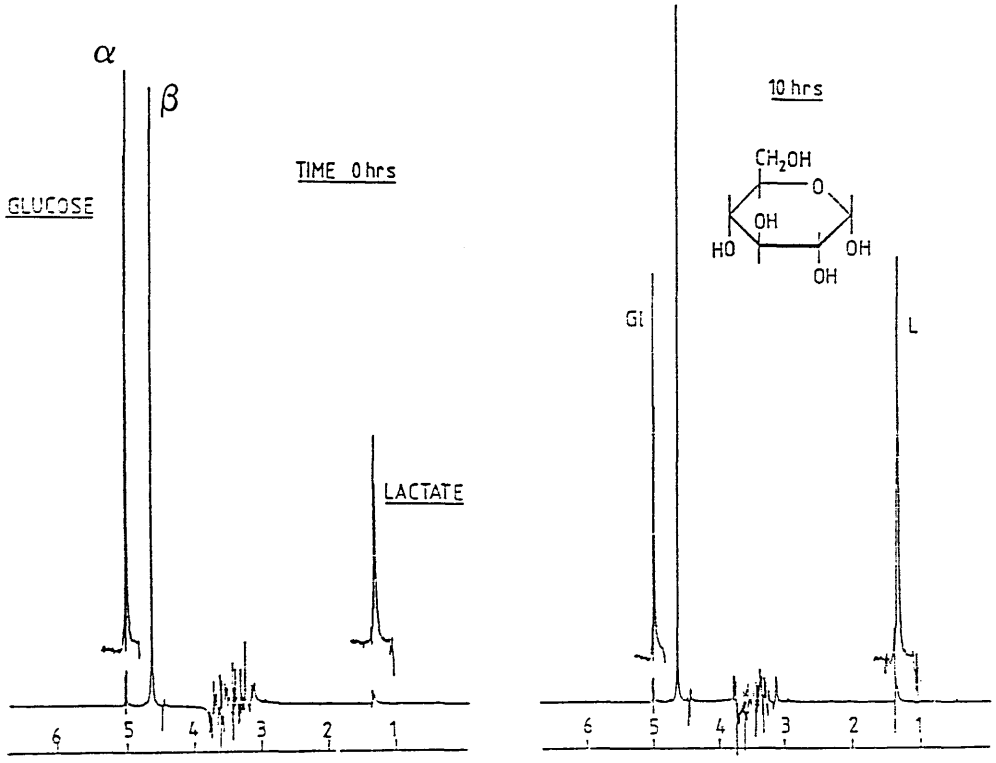


Figure 43 : ^{1}H spin echo NMR of the Hela cell ($T2$ = 60 ms) at time t= 0 h and t = 10 h, the medium being supplemented with 11 μmoles glucose. The upfield line is assigned to the α glucose anomer.

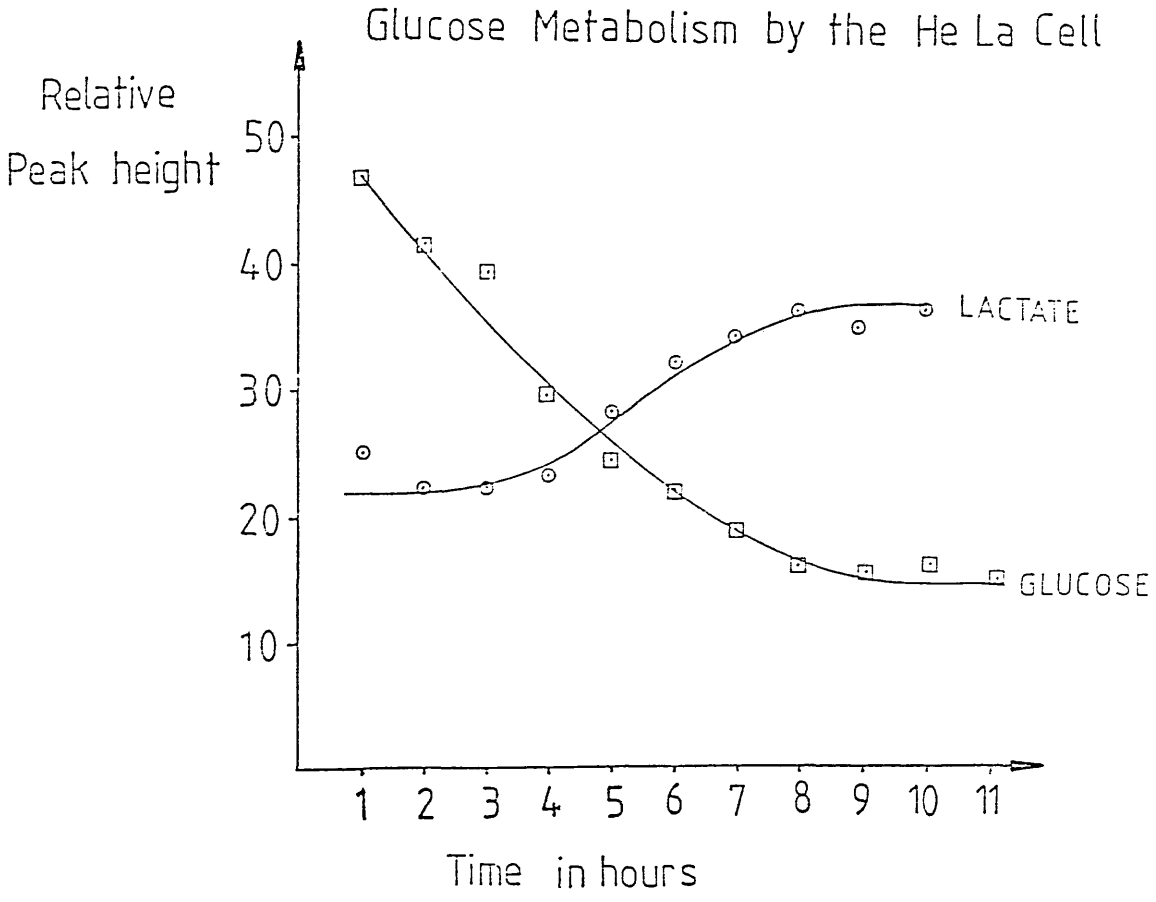


Figure 44 : The time course of glycolysis in the Hela cell as depicted by the observed changes in the concentrations of α glucose (at δ 5.2) and lactate (δ 1.5)

3.5.1.2. Exposure of Hela Tumour Cells to Doxorubicin :

The spin echo NMR detected a rapid dose-related depletion of the cytosolic glutathione pool. This NMR study shows comparable, but qualitative results (Figure 45 and 46) to the HPLC method (Figure 35), however changes can be conclusively assigned to cellular activity in the intact cell by doxorubicin. The single control used (doxorubicin absent) clearly shows no change in cytosolic glutathione. The result of this experiment indicates that the effect of doxorubicin on the glutathione pool takes two forms. At high doses the glutathione depletes rapidly ($t_{1/2}$ = 30 minutes) ; lower doxorubicin doses depleted the glutathione pool with a $t_{1/2}$ of 4 hours (Figure 46), with the added feature of a lag phase.

Treatment of Hela cells with different doxorubicin concentrations (30 and 300 nmol / 10^9 cells) gives us clear evidence of glycolysis through lactate production (Figure 47). Inspection of figures 46 and 47 indicate that the maximum rate of glycolysis as reflected in lactate production occures at 3 – 4 hours respectively at high and low doxorubicin doses at which point glutathione depletion is about 60 % and 25 % respectively.

3.5.1.3. Effect of NAC on Hela Cells Treated with Doxorubicin as Shown by Lactate Measurement :

Addition of NAC to Hela tumour cells increases the cytosolic pool of thiol offering a protective mechanism as indicated by extension of the lactate lag phase which is coincident with the time required to deplete the larger cytosolic small thiol concentration (Figure 48). Thus NAC presence offers a protective mechanism against lactate stress from high doxorubicin doses ; stress is not observed until intracellular NAC falls to insignificant levels.

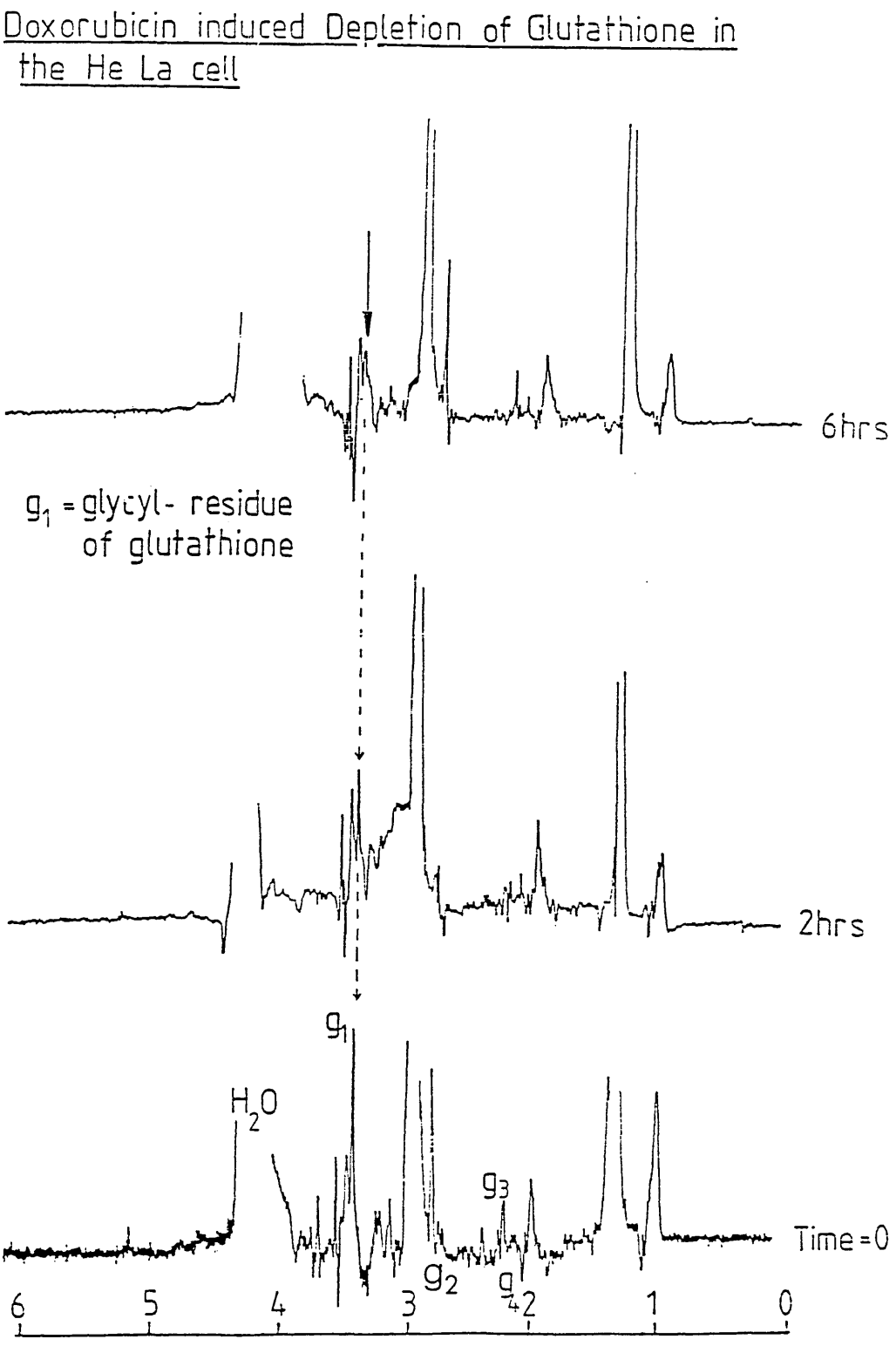


Figure 45 : Doxorubicin induced depletion of GSH in Hela cells as measured by ^1H spin-echo NMR ($T2$ = 60 ms) at times shown on the figure and at 20°C. g_1 arising from glycyl residue of GSH, g_2 cysteine residue of GSH while g_3 and g_4 are glutamyl residues of GSH.

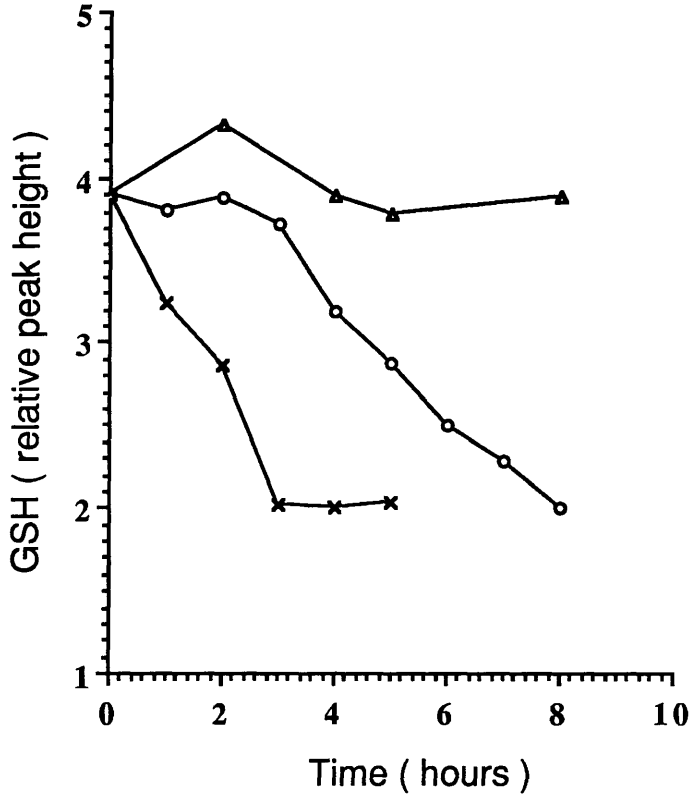


Figure 46 : Effect of different concentrations of doxorubicin on the GSH concentration in intact Hela cells as measured by ^{1}H spin-echo NMR. Δ control no doxorubicin ; o Doxorubicin 30 nmol per 10^{9} cells ; x Doxorubicin 300 nmol per 10^{9} cells.

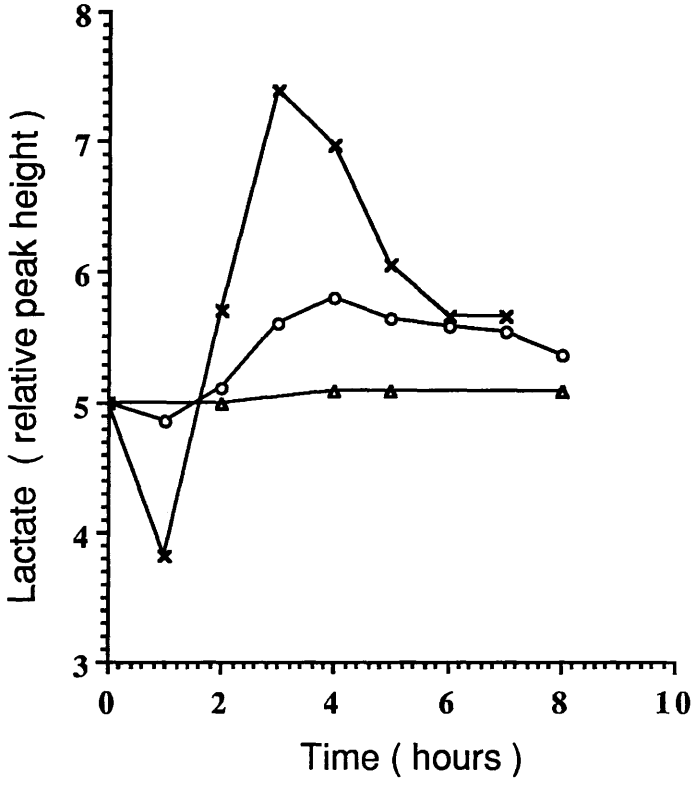


Figure 47 : Effect of different concentrations of doxorubicin on the intracellular lactate concentration of Hela cells as measured by spin-echo NMR. Δ control no doxorubicin ; o Doxorubicin 30 nmol per 10^9 cells ; x Doxorubicin 300 nmol per 10^9 cells.

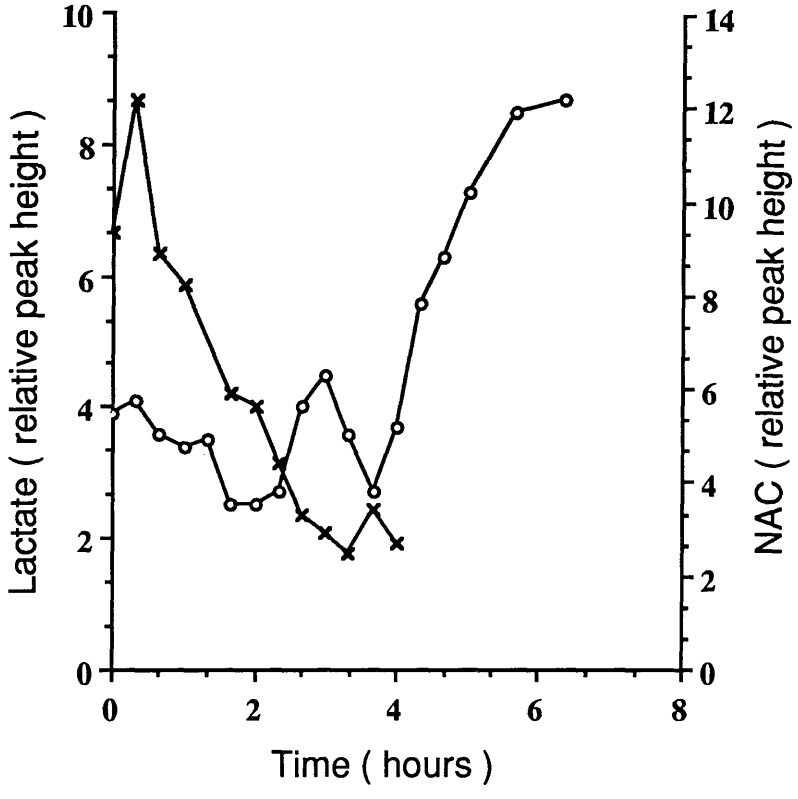


Figure 48 : Time course of N-acetylcysteine (NAC) and lactate in Hela cells pretreated with NAC and later with doxorubicin (300 nmol / 10^9 cells) as measured by spin-echo NMR ; o intracellular lactate ; x intracellular NAC.

3.5.1.4. Transport of Amino Acids into the Cells :

This experiment examined the treatment of Hela tumour cells with the individual components of glutathione (glutamate, cysteine and glycine) in the presence of a low doxorubicin dose, in order to eliminate the lack of precursor as a cause of reduced synthesis of glutathione. The nature of the instrument tuning creates an experimental framework in which the machine is more sensitive to the intracellular rather than the extracellular environment. Thus as a species crosses the membrane from a less sensitive to a more sensitive domain a small increase in the resonance intensity is to be expected (Brown and Campbell, 1980). Glycine showed the simplest characteristics, passing across the cell membrane barrier into the cytosol. Glutamate also crossed the membrane, but is a substrate for intermediary metabolism in the cell and is consumed post transport giving a reduction in its resonance intensity. Cysteine shows a late fall after transport cross the cellular membrane. The lactate profile in this experiment showed no stress when compared with unprotected cells exposed to the same concentration of doxorubicin (Figure 49).

3.5.2. Leukemia J-111 Cells :

As shown in 3.5.1. an initial profile of the cellular metabolites which can be observed by the spin echo method was required. Spectra (Figure 50) were collected using cells suspended in a simple physiological saline solution (^2H_2O / NaCl, 0.154 mol /l). The comparison with standard FT NMR spectra where the bulk of the resonances arise from the cell membrane is shown. There is considerable contribution to the spectrum in the lactate region arising from methyl- and methylene resonances of the lipids and proteins. Applying the Hahn spin echo pulse sequence simplifies the spectrum to just four lines identified as arising from phosphorylcholine,

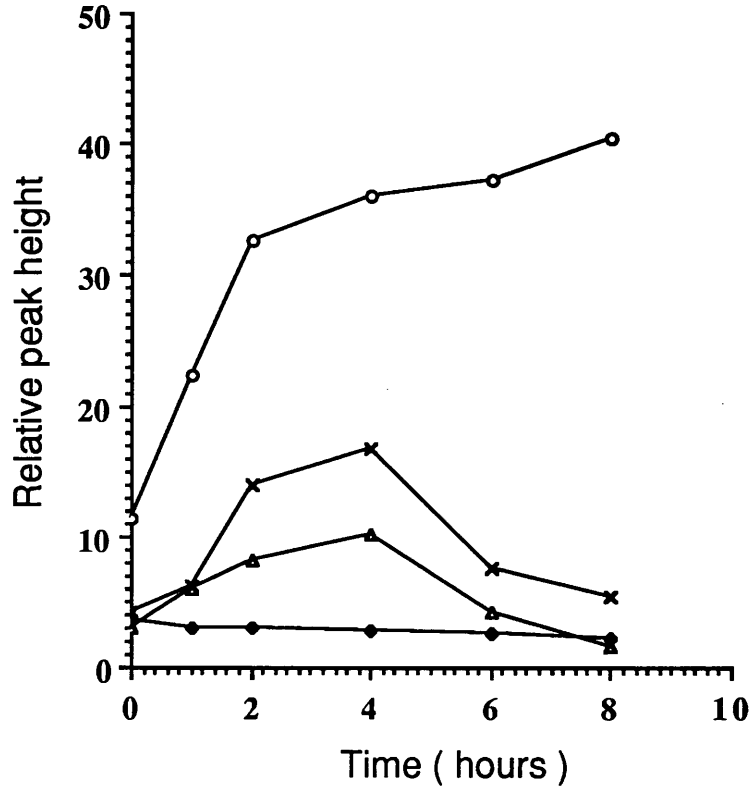


Figure 49 : Effect of doxorubicin at a concentration of 30 nmol per 10^9 cells on the intracellular concentration of amino acids added to the medium as measured by spin-echo NMR ; o glycine ; x cysteine ; Δ glutamate; ♦ lactate.

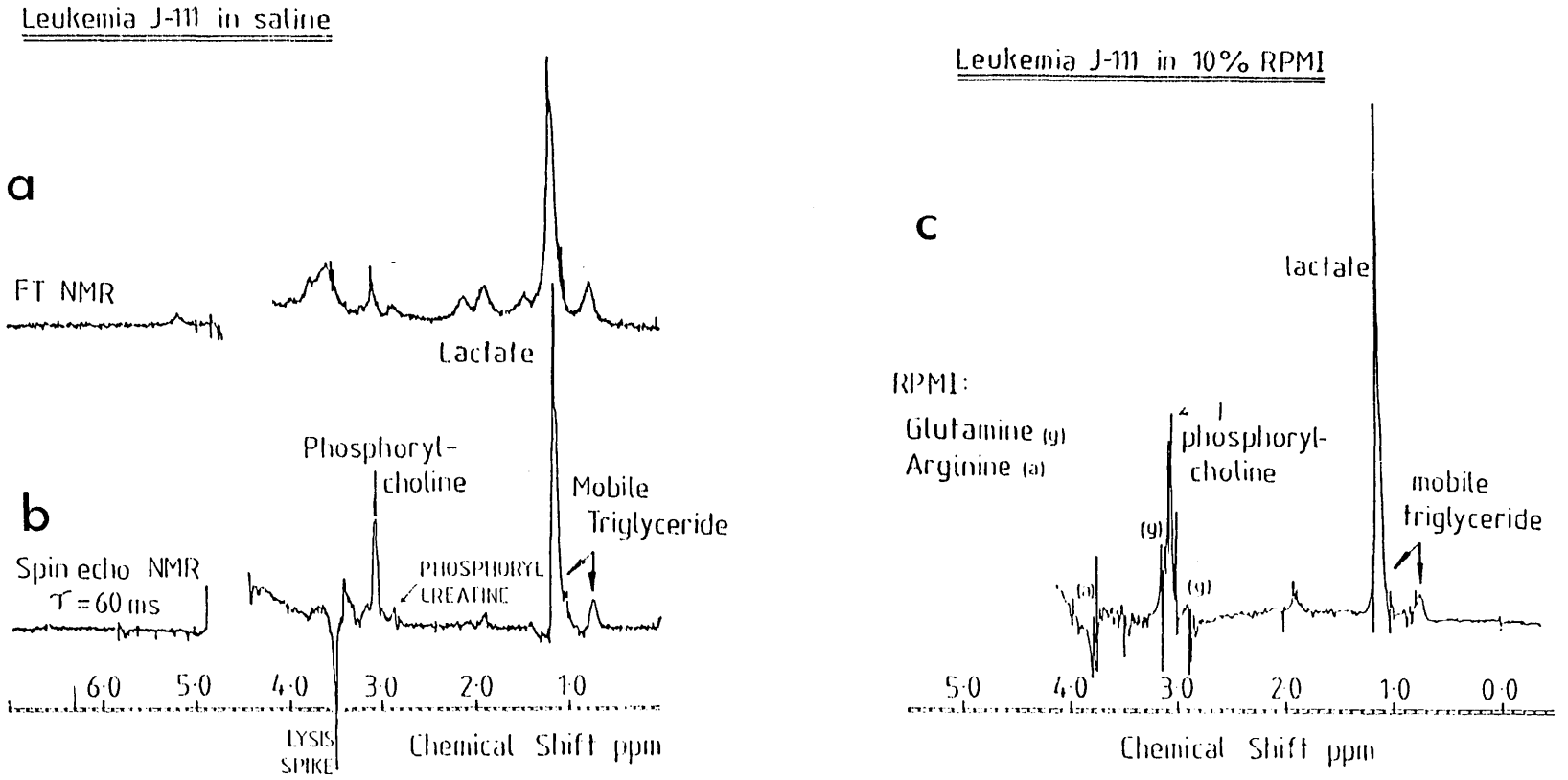


Figure 50 : A comparison between : a) The normal 250 MHz FT NMR in ^2H_2O / NaCl medium (400 scans) ; b) The Hahn spin echo NMR in ^2H_2O / NaCl medium (1000 scans, t = 60 ms) ; and c) The Hahn spin echo NMR in ^2H_2O, 10% RPMI 1640 medium (1000 scans, t = 60 ms) for the leukemia **J111** cells. The total sample size was 10^9 cells / 0.4 ml maintained at 20°C during data aquisation.

phosphorylcreatine, lactate and mobile triglyceride. These peak assignments are made on the basis of direct comparison with NMR studies as in (3.5.1.), on the intact and viable Friend leukaemia cell line (Agris and Campbell, 1982) and lysate from the RIF tumour line (Evanochko et al., 1984) as well as the known chemistry of this type of cell (Iyer, 1959). From these limited cell types it would seem that the species shown in figure 50 are the major NMR-active constituents in the cytosol of cancer cells. However, certain differences are observed between cell types.

The leukemia cell line was found to be non-viable within the time scale required for the metabolic studies using NMR at 250 MHz. The cells aggregated and severely reduced the signal which could be obtained, therefore a more complete medium, ^2H_2O / RPMI 1640 or ^2H_2O / NaCl 10% RPMI (10% RPMI) was used.

3.5.2.1. Study of Glycolysis :

Simple glycolysis was followed in the 10% RPMI. A typical spectrum is shown in figure 50 c where the contribution of the medium [mainly arginine (a) and glutamine (g)] is indicated. The lactate signals (Figure 51) indicate an initial aerobic phase during which the lactate resonance diminishes, followed by an anaerobic phase once the cells have utilised the available oxygen in the medium in which the lactate signal steadily increases.

In the presence of doxorubicin (30 μmoles) in 100% RPMI 1640 medium, there is a marked difference in glycolytic behavior. No aerobic phase can be seen within the time resolution (30 min) of the NMR experiment. The culture rapidly turns to anaerobic glycolysis to provide energy to combat the chemical stress of doxorubicin which involves free radical generation. Then the culture is rendered inert after 2 h at which time there is considerable difference in the amount of lactate generated by the culture.

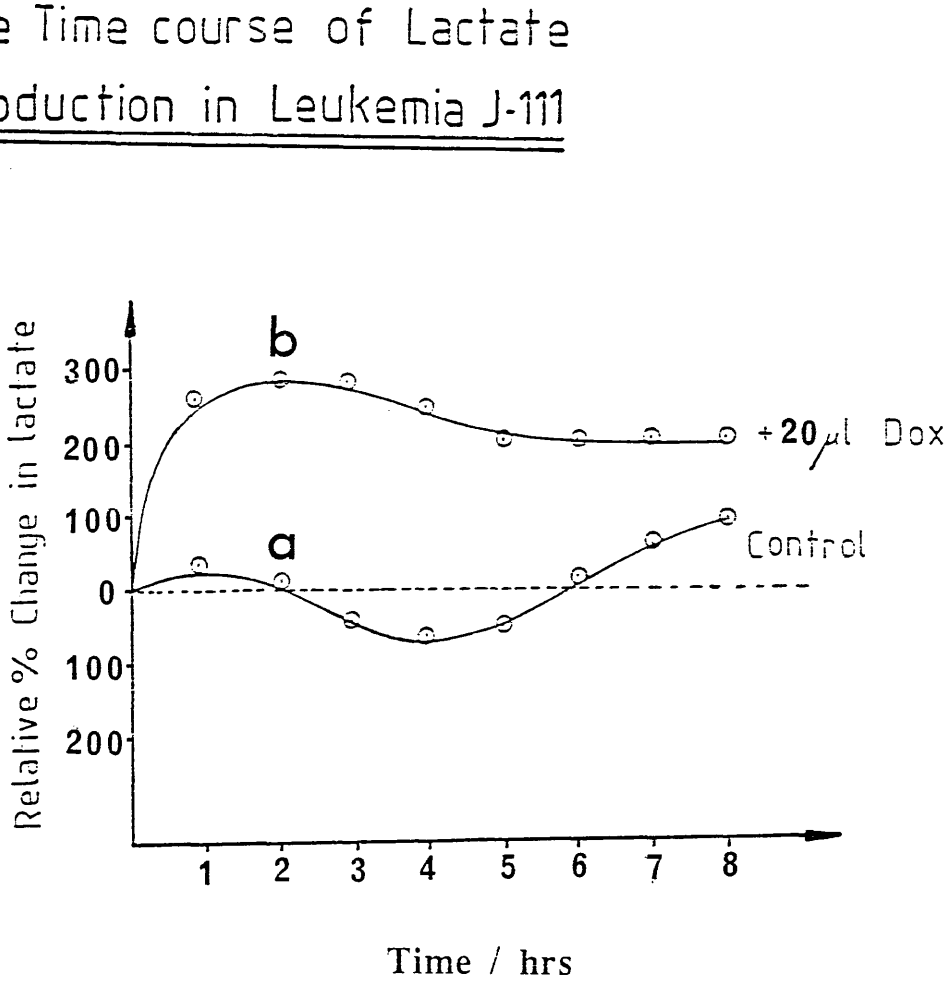


Figure 51 : A plot of the lactate signal intensity for : a) leukemia cells J111 in ^2H_2O / 10% RPMI 1640 and 5.5 µmoles glucose ; b) leukemia cells J111 in ^2H_2O / RPMI, 5.5 µmoles glucose and doxorubicin (30 nmol / 10^9 cells).

3.6. Glutathione-S-Transferase Activity Assay :

The standard curve for GST concentrations (Figure 52) indicated that it was linear over the range of (0 – 1) enzyme unit / ml ; each point was a mean of three different readings.

The results of this assay are summarized in table 4 which shows the GST Δ activity of standard human placental enzyme as one unit of the enzyme activity / ml (one international unit = 1 μmol of substrate converted per minute = 0.06 nkatal) and the related activities in the three tumour cells A549 and GLC_4 210 (S) and (R). The table also shows the GST activity of these cells after 24 hours pretreatment with doxorubicin at which point there is no much difference in responce between different tumour cell line. Each reading in the table is a mean of three different measurements of the same sample. The table shows that there is a difference in the basal enzyme activity in these three lines. A549 showed high GST activity, twice that of the other two cell lines.

GST activity in A549 tumour cells after treatment with doxorubicin seemed to be unaffected (Figure 53) even after eight hours of treatment with 3.5 μmol /l doxorubicin ; there was no clear difference between treated and related control.

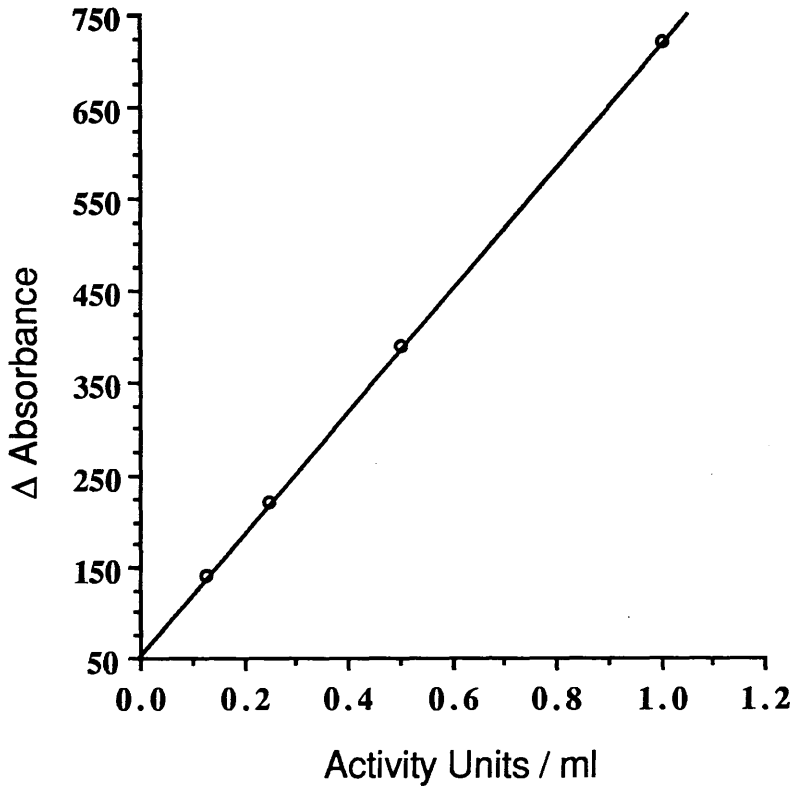


Figure 52 : Glutathione-S-transferase standard curve ; each point on the curve is a mean of three measurements.

Table 4 :

Summary of GST concentrations and the effect of doxorubicin in different tumour cells as measured using the ENCORE method. Each figure in the table is a mean of three readings.

Samples	Standard Human Placental GST		A549		GLC4 210 (S)		GLC4 210 (R)	
	Δ Absorbance	Enzyme Unit / ml	Δ Absorbance / 10^6	Enzyme Unit / 10^6 Cells	Δ Absorbance / 10^6	Enzyme Unit / 10^6 Cells	Δ Absorbance /10^6 Cells	Enzyme Unit / 10^6 Cells
Standard	**683**	**1**						
Control			**609**	**0.9**	**289**	**0.43**	**280**	**0.41**
24 hours Treatment			**517**	**0.8**	**280**	**0.41**	**277**	**0.41**

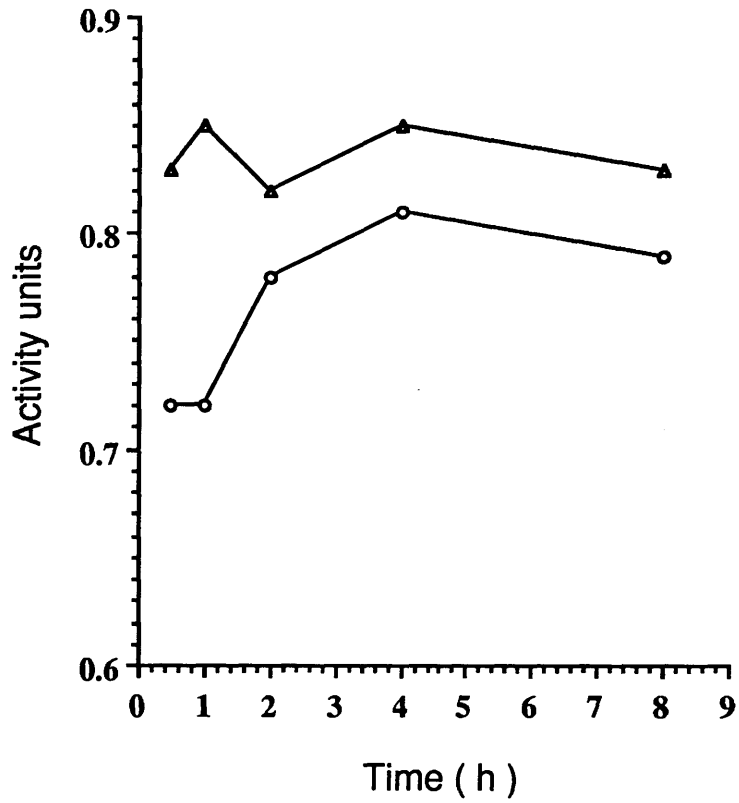


Figure 53 : Effect of pretreatment with 3.5 µmol / l doxorubicin on the GST activity of A549 cells ; Δ control no doxorubicin ; o doxorubicin treated cells.

3.7. Measurement of Lipid Peroxidation Due to Doxorubicin Treatment :

The Malondialdehyde standard curve is shown in figure 54, and as described above (2.10.) these experiments were designed to measure MDA as a product of membrane lipid peroxidation caused by doxorubicin free radical production. The time course of MDA production following incorporation of doxorubicin into Hela tumour and GLC_4 210 (S) cells is shown in figure 55 and 56. This resulted in the peroxidation of the membrane lipids as indicated by the increasing amounts of malondialdehyde produced with time of exposure to the drug. The addition of 10 µmol /l doxorubicin to the cell suspension increased the malondialdehyde level by 78 pmol / 10^6 cells over the control values in the case of Hela cells and 64 pmol / 10^6 cells in the case of GLC_4 210 (S) cells after five hours of exposure.

Treatment of Hela cells with N–acetylcysteine one hour prior to the addition of doxorubicin (10 µmol /l) did not result in any decrease of malondialdehyde within the first hour of the treatment, but in subsequent hours there was a slight reduction in the malondialdehyde measurement compared with cells treated with doxorubicin alone (Figure 55). NAC appeared to offer significant protection against doxorubicin-induced lipid peroxidation ($P < 0.05$, two way ANOVA).

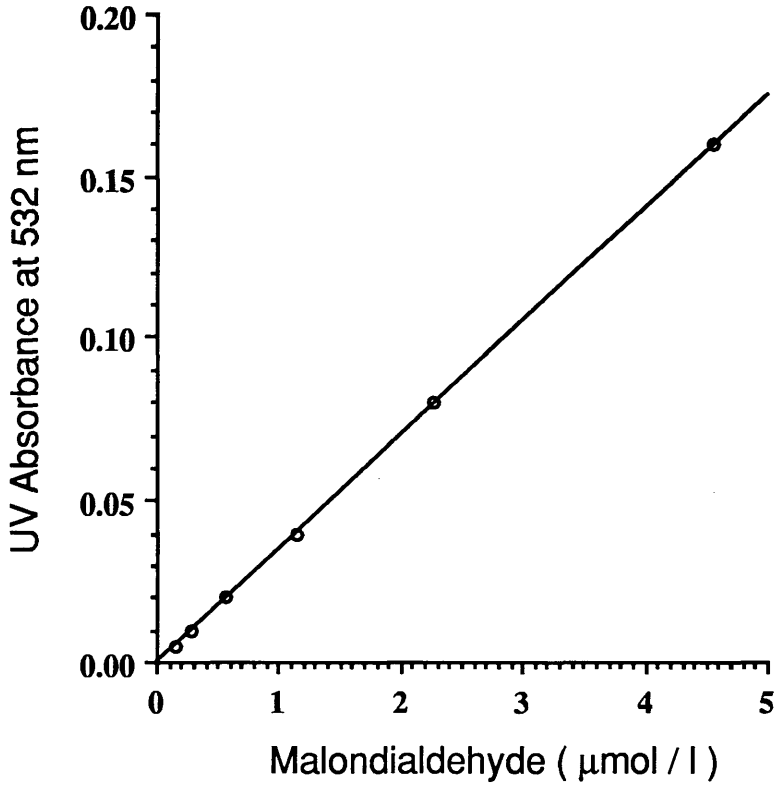


Figure 54 : Malondialdehyde standard curve ; each point on the curve is a mean of two measurements.

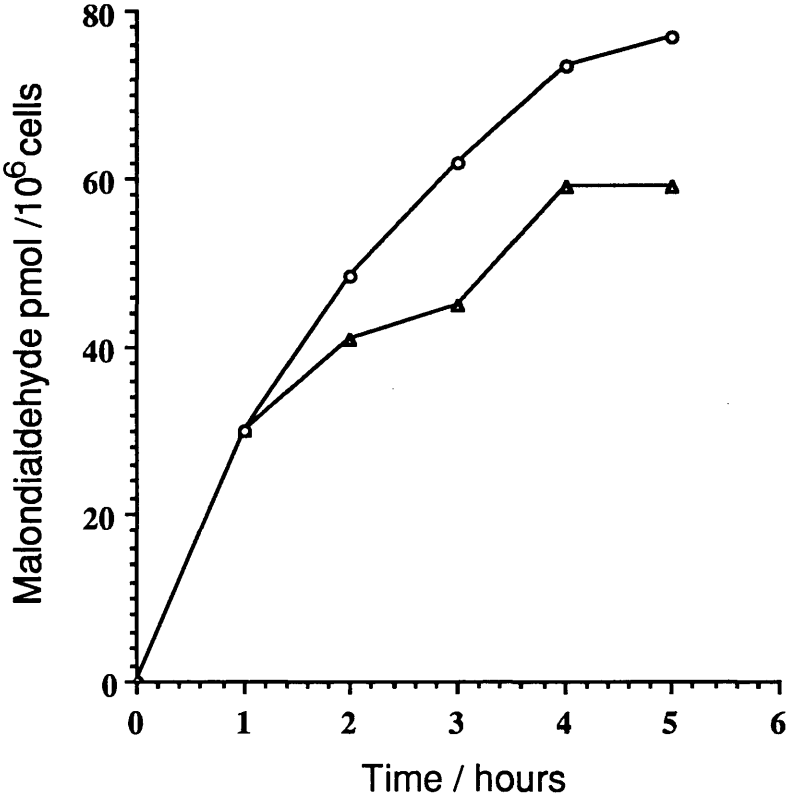


Figure 55 : The effect of NAC on MDA production due to doxorubicin in Hela cells. o MDA measurement using doxorubicin alone at 10 µmol /l ; Δ MDA result using NAC + doxorubicin.

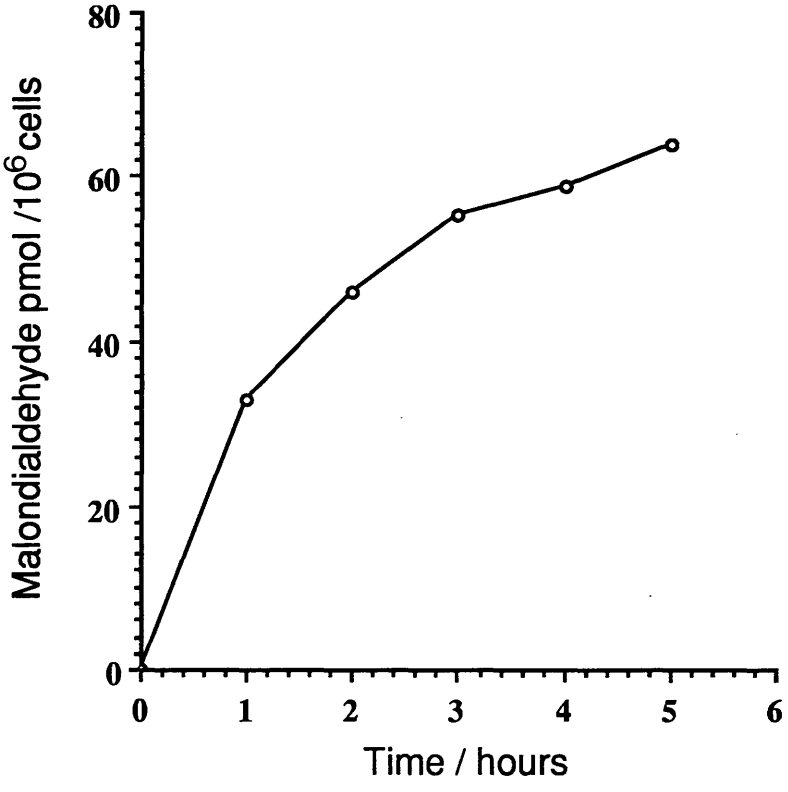


Figure 56 : MDA production due to doxorubicin (10 µmol /l) in GLC_4 210 (S) cells.

3.8. Tetrazolium Based Chemosensitivity Assay of Doxorubicin in Different Human Tumours :

The optimum concentrations of MTT used for the chemosensitivity assay were initially determined as 5 mg / ml for both A549 and Hela cell, and 3 mg / ml for GLC_4 cells (both sensitive and resistant) (Figure 57).

The measurement of cell kill by different doxorubicin concentrations showed the expected effects of these concentrations, with the sensitive cells (Hela and GLC_4 [S]) releasing the dye to a greater extent than the resistant lines (A549 and GLC_4 [R]).

Table 5 shown the results of a drug sensitivity assay which indicates that the LD_{50} for the four cell lines Hela, A549, GLC_4 210 (S) and (R) were 50, 80 , 30 – 45 and 100 – 250 nmol / l respectively. Duplicate determinations were carried out for each cell line in these assays and the mean of three absorbance reading ± standard errors were calculated in each case (Figures 58 – 61).

Statistical analysis of these data was carried out using Dunnett's test (1955) which is used for comparing several treatments with controls. The results showed significant ($P < 0.01$) differences in growth inhibition between the controls and treated cells, although the concentration of doxorubicin required was different for each cell line.

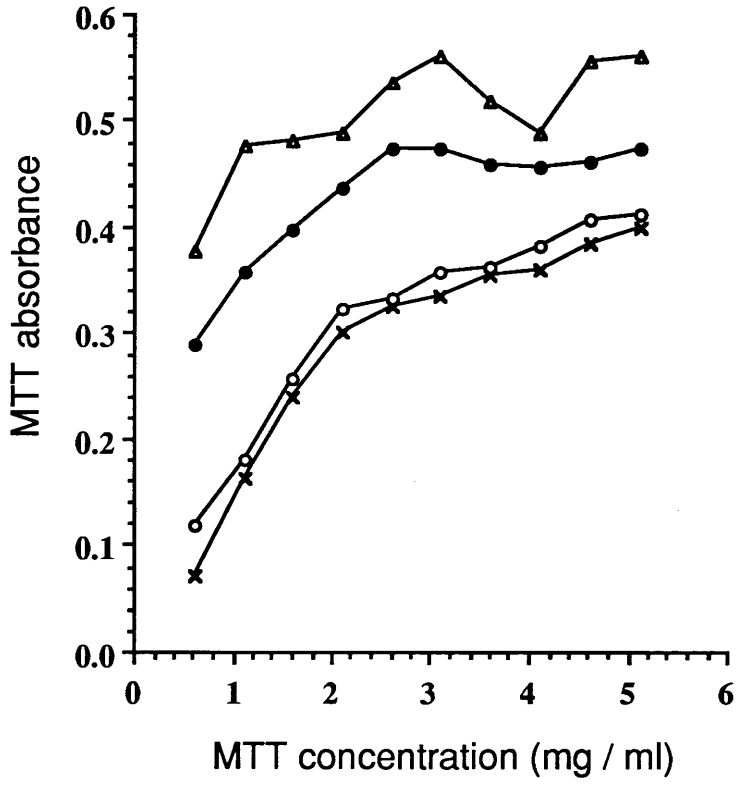


Figure 57 : Determination of the optimum concentration of MTT for the measurement of viability in the four cell lines. x Hela ; o A549 ; • GLC_4 210 (S) and Δ GLC_4 210 (R).

Table 5 :

Toxicity of Doxorubicin :

	Cell line			
	Hela	**GLC4 (S)**	**GLC4 (R)**	**A549**
LD_{50} (nmol / l)	**50**	**30 – 45**	**100 – 250**	**80**
Ratio of untreated / treated at 1075 nmol / l	**20.4**	**5.1 – 6.7**	**2.5 – 2.7**	**7.2 – 10**

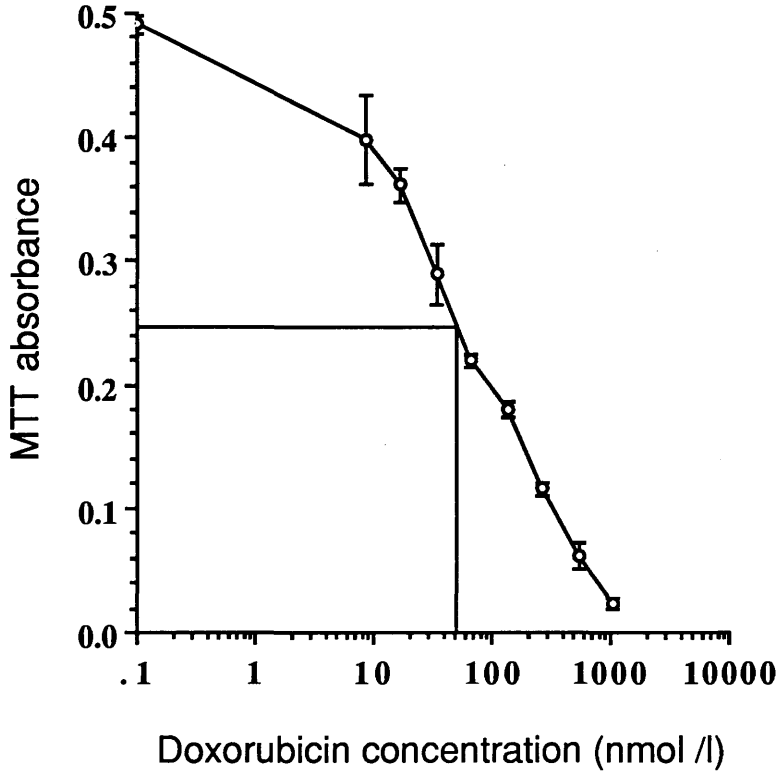


Figure 58 : Effect of doxorubicin on the viability of Hela cells, each point on the curve is a mean of three different measurements of cell viability from three wells. Bars = standard error.

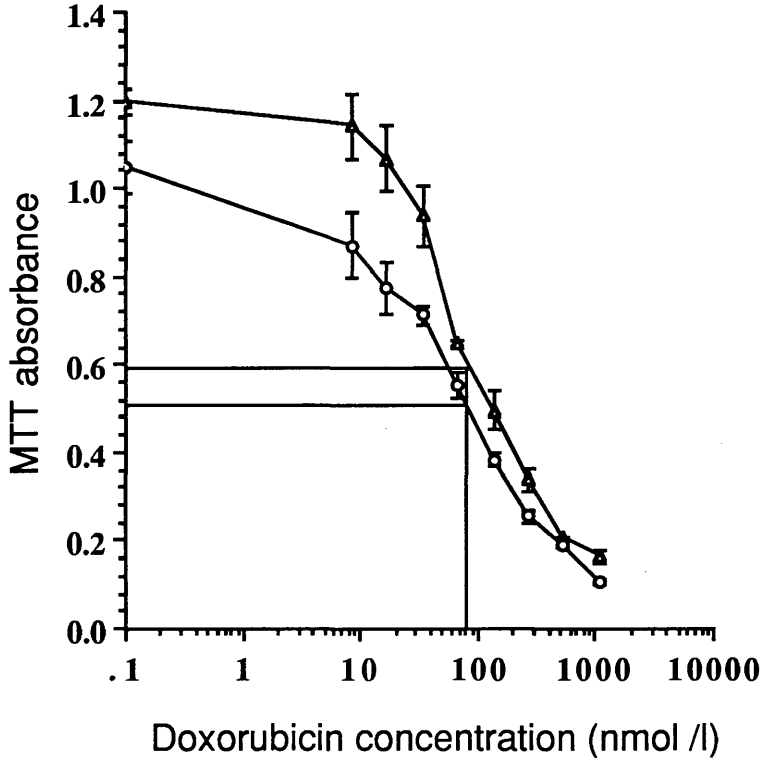


Figure 59 : Effect of doxorubicin on the viability of A549 cells, each point on the curve is a mean of three different measurement of the cell viability from three wells. The two curves are duplicate experiments. Bars = standard error.

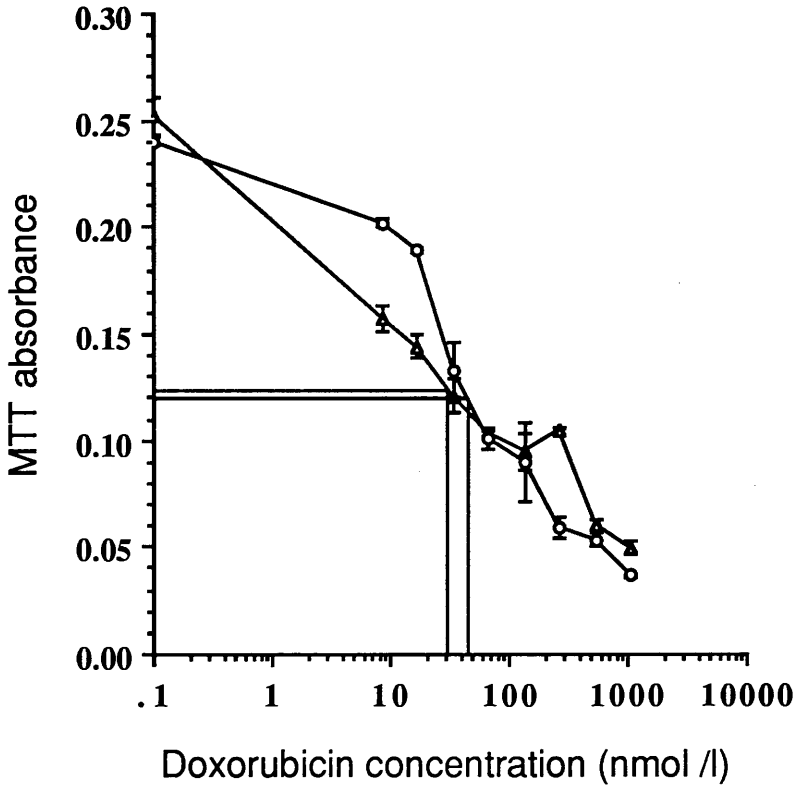


Figure 60 : Effect of doxorubicin in the viability of GLC_4 210 (S) cells, each point on the curve is a mean of three different measurement of cell viability from three wells. The two curves indicate duplicate experiments. Bars = standard error.

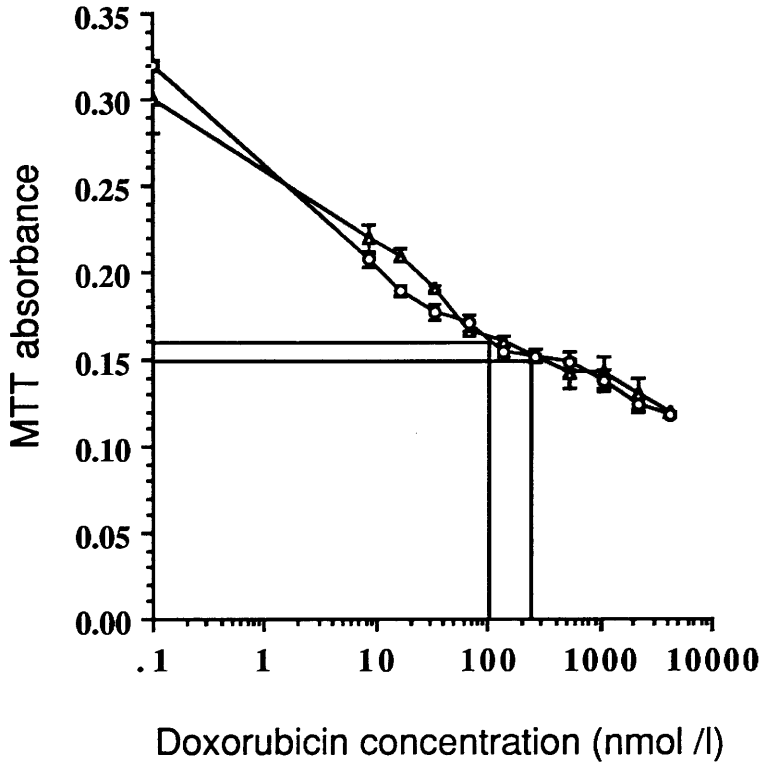


Figure 61 : Effect of doxorubicin on the viability of GLC_4 210 (R) cells, each point on the curve is a mean of three different measurements of cell viability from three wells. The two curves indicate duplicate experiments. Bars = standard error.

3.9. Effect of BSO Pretreatment on the Resistance of A549 Cell to Doxorubicin :

Figure 62 shows the effect of six hours pre-treatment of A549 resistant cells with BSO at a non-toxic concentration (2 mmol / l) as compared with cells treated with doxorubicin alone (control cells were grown in medium alone). Table 6 shows the difference in the growth inhibition (%) with the doxorubicin alone and doxorubicin plus 2 mmol / l BSO for each drug concentration used in this assay. 2 mmol / l BSO was found to be the best concentration, being both non toxic and giving statistically significant enhancement of doxorubicin toxicity. Therefore a dose-dependent effect of doxorubicin against the cells is enabled by the non-toxic BSO concentration used. Pretreatment with 0.2 mmol / l BSO gave no increase in the sensitivity of these cell lines, i.e. statistically non significant enhancement of doxorubicin cytotoxicity ($P > 0.05$) at the highest drug concentration (55.2 nmol /l).

Duplicate determinations were carried out in all cases and the mean of six absorbances ± standard error were calculated. Statistical analyses of these data was carried out using Students t -test. There was no significant difference from control at the three lowest doxorubicin concentrations, however cells pretreated with BSO 2 mmol /l showed a significant increase in sensitivity at all doxorubicin concentrations used ($P < 0.01$).

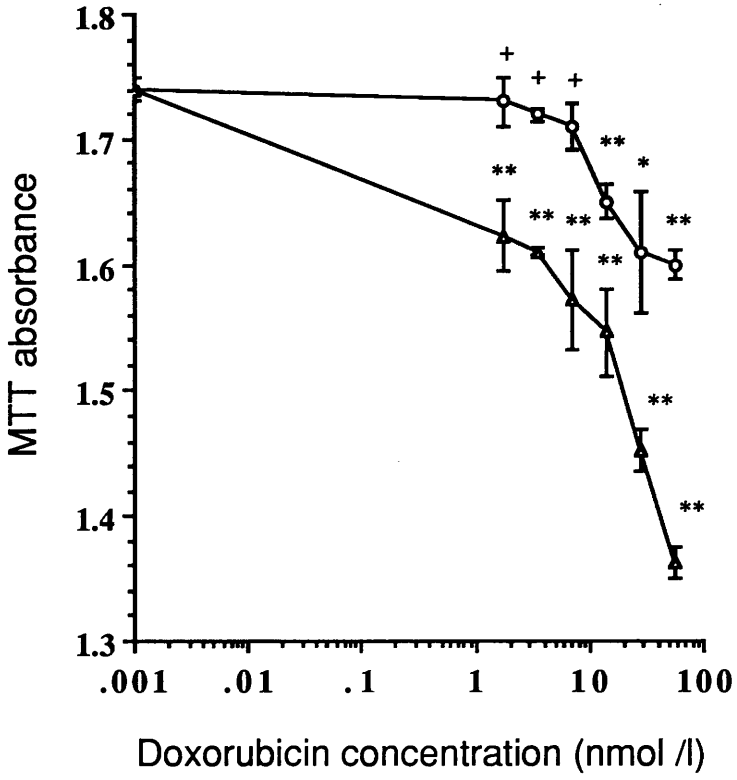


Figure 62 : The effect of BSO on the viability of A549 cells treated with different concentration of doxorubicin ; o treated with doxorubicin alone ; Δ pretreated with BSO. Bars = standard error ; + non significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$.

Table 6 :

Enhancement of Doxorubicin Cytotoxicity by BSO (2 mmol / l) in A549 (R) Cells.

Doxorubicin concentration (nmol / l)	% Inhibition of cells growth in relation to control			Significance (t-test)
	Doxorubicin only (a)	Doxorubicin + BSO (b)	Ratio (b / a)	
1.72	0.60	6.70	11.20	P < 0.01
3.45	1.15	7.50	6.50	P < 0.01
6.90	1.72	9.70	5.60	P < 0.01
13.80	4.60	11.20	2.40	P < 0.01
27.60	6.80	16.50	2.40	P < 0.01
55.20	7.80	21.80	2.80	P < 0.01

3.10. Enhancement of Doxorubicin Cytotoxicity using Amiodarone :

Circumvention of doxorubicin resistance in human non small cell lung carcinoma A549 was induced by one hour pretreatment of the cells with four different amiodarone concentrations (2 – 8 μmol / l). Amiodarone enhances the effect of doxorubicin on GSH in this type of tumour, since there was no cytotoxic effect when amiodarone used alone at 10 μmol /l (Chauffert et al., 1987). The effect of doxorubicin in these experiments was assayed by measuring both intracellular doxorubicin and glutathione. Intracellular amiodarone concentration were also measured.

3. 10.1. Drug Uptake Assay :

3.10.1.1. Qualitative Assay :

The result of a preliminary experiment in which the penetration of doxorubicin was examined in GLC_4 210 (S) and A549 is shown in figure 63. The penetration of doxorubicin into GLC_4 210 (S) was clearly greater than for A549 as indicated by the higher fluorescence observed in the sensitive cells.

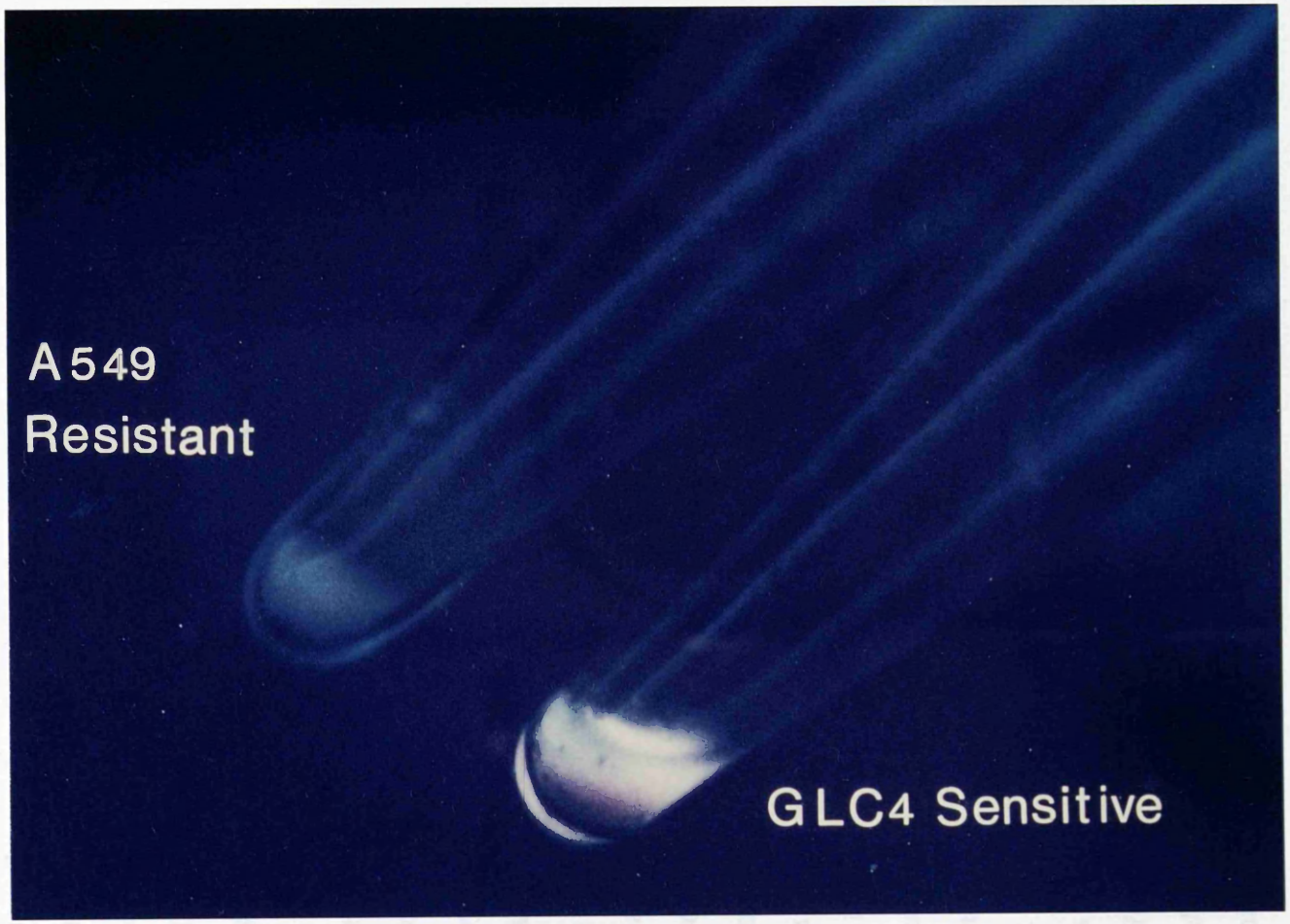


Figure 63 : Qualitative measurement of penetration of doxorubicin into GLC_4 210 sensitive and A549 resistant cells as observed under fluorescent light (340 nm).

3.10.1.2. Quantitative Assay :

In this assay the uptake of doxorubicin and amiodarone by the cells after two different methods of harvesting (Trypsinisation and scraping) was measured in order to determine whether trypsin caused leakage of drugs from the cells during harvesting. The results of the doxorubicin uptake experiment for A549 cells are shown in figure 64. Both free and DNA-bound doxorubicin are estimated in picomoles in this experiment. The general shapes of the curves in the two cases are typical and the plateau value of the doxorubicin % uptake in relation to the amiodarone concentration was reached at 2 μmol /l. Figure 65 shows the % uptake of amiodarone by the same cells related to its extracellular concentration following two methods of harvesting. The plateau level was reached at an extracellular amiodarone concentration of 2 μmol /l. Table 7 summarises the data obtained in the drug uptake experiments and the differences in doxorubicin concentrations at the different amiodarone concentration used. This table allows a comparison of the effect of the two methods of cells harvesting. These data indicate that an extracellular amiodarone concentration of 2 μmol /l is sufficient to increase intracellular doxorubicin accumulation in this type of tumour cell and may be able to induce cell sensitivity to the doxorubicin, but becomes more marked at 6 μmol /l and there may be a dose dependent trend for GSH depletion. Standard curves for doxorubicin and amiodarone are shown in figures 66 and 67. Typical chromatograms for doxorubicin and amiodarone as extracted from the cells are shown in figures 68 and 69. There was no statistical difference betwween the two methods of collection in the case of doxorubicin while in the case of amiodarone there was a significant difference ($P < 0.05$) using pair-difference t-test.

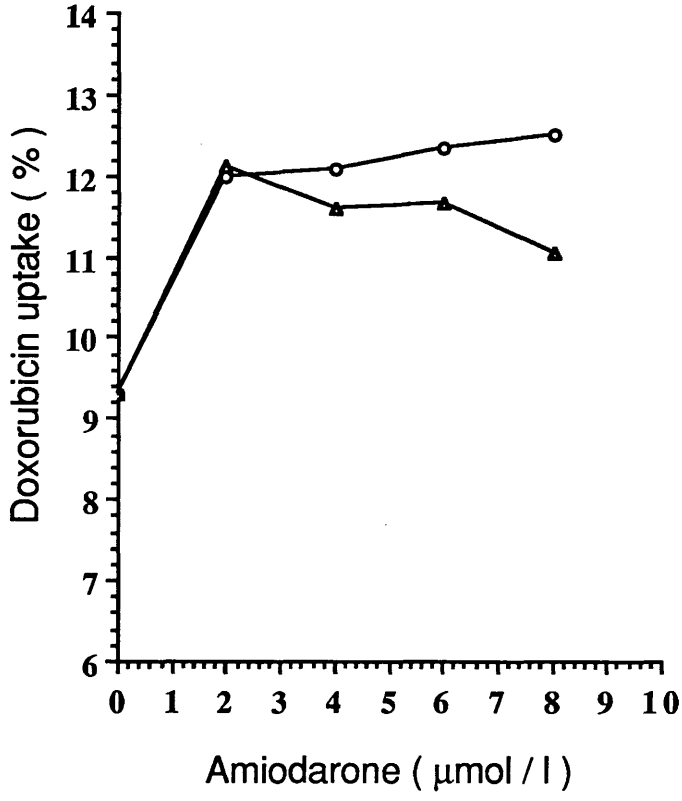


Figure 64 : Doxorubicin penetration (measured by HPLC) into A549 cells pretreated with different amiodarone concentrations and harvested by : o Trypsinized ; Δ scraped.

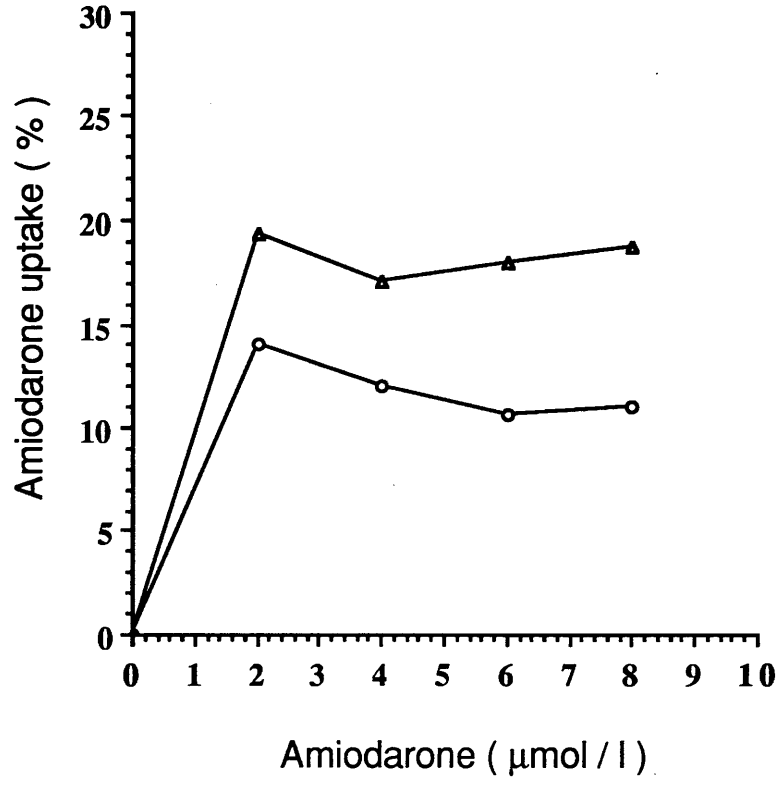


Figure 65 : HPLC measurement of amiodarone penetration into A549 cells treated with different concentration and harvested by : o Trypsinized ; Δ scraped.

Table 7 :

Enhancement of Doxorubicin. Effect on GSH reduction in A549 cells using Amiodarone

	AMIODARONE				DOXORUBICIN				GLUTATHIONE
Extracellular μM	Intracellular (nmoles / Flask)				Intracellular (nmoles / Flask)				(nmoles / Flask)
	Trypsinised Collection	Uptake %	Scraped Collection	Uptake %	Trypsinised Collection	Uptake %	Scraped Collection	Uptake %	Trypsinised Collection
Control (no DOX.)	–	–	–	–	–	–	–	–	108.3
Control (5μM DOX.)	–	–	–	–	2.3	9.3	2.3	9.3	95.3
2	1.4	14.0	1.9	19.4	3.00	12.0	3.0	12.1	84.5
4	2.4	12.0	3.4	17.1	3.02	12.1	2.9	11.6	82.3
6	3.2	10.6	5.4	18.0	3.10	12.3	2.9	11.7	48.0
8	4.4	11.0	7.5	18.7	3.12	12.5	2.8	11.1	40.3

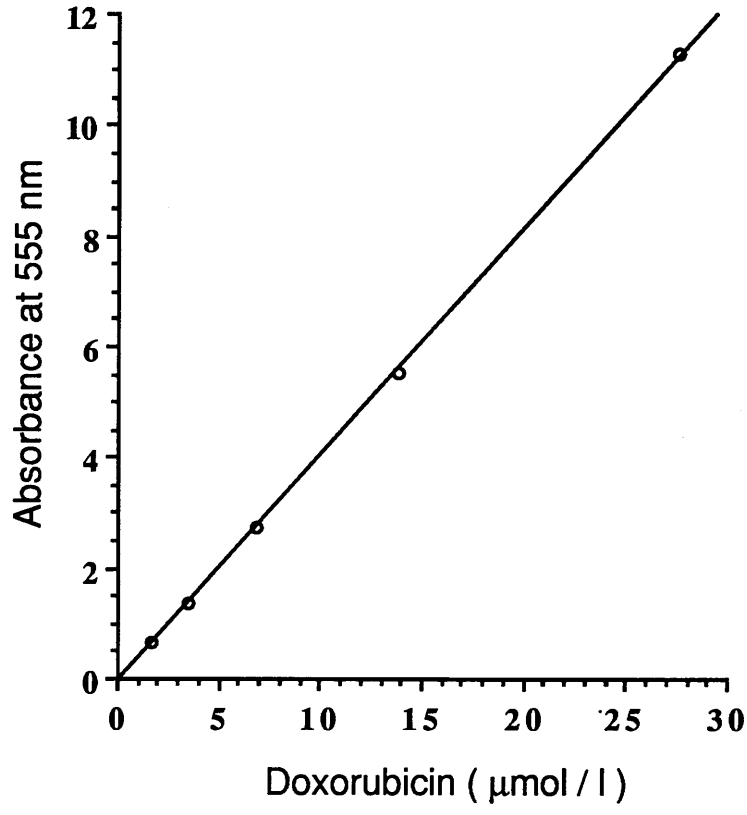


Figure 66 : Doxorubicin standard curve as measured by HPLC ; each point on the curve is a mean of two different measurements.

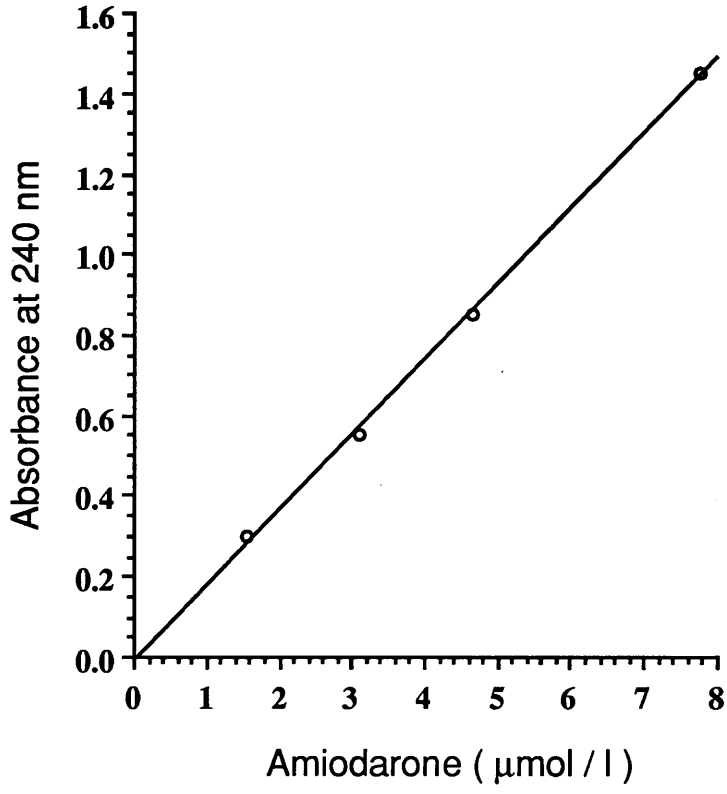


Figure 67 : Amiodarone standard curve as measured by HPLC ; each point on the curve is a mean of two different measurements.

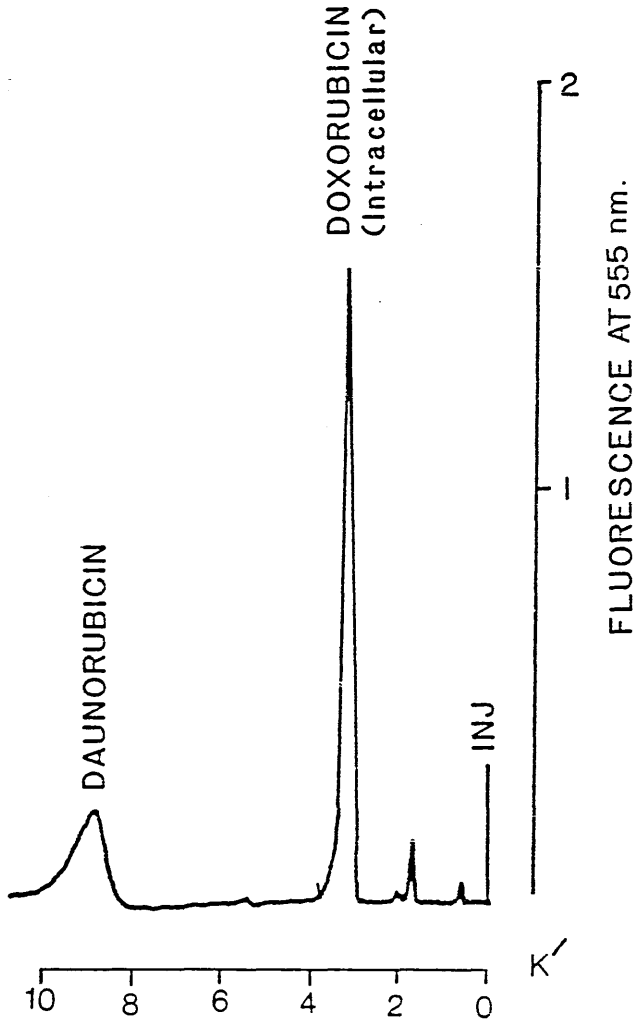


Figure 68 : HPLC profile of doxorubicin extracted from A549 cells with daunorubicin as internal standard.

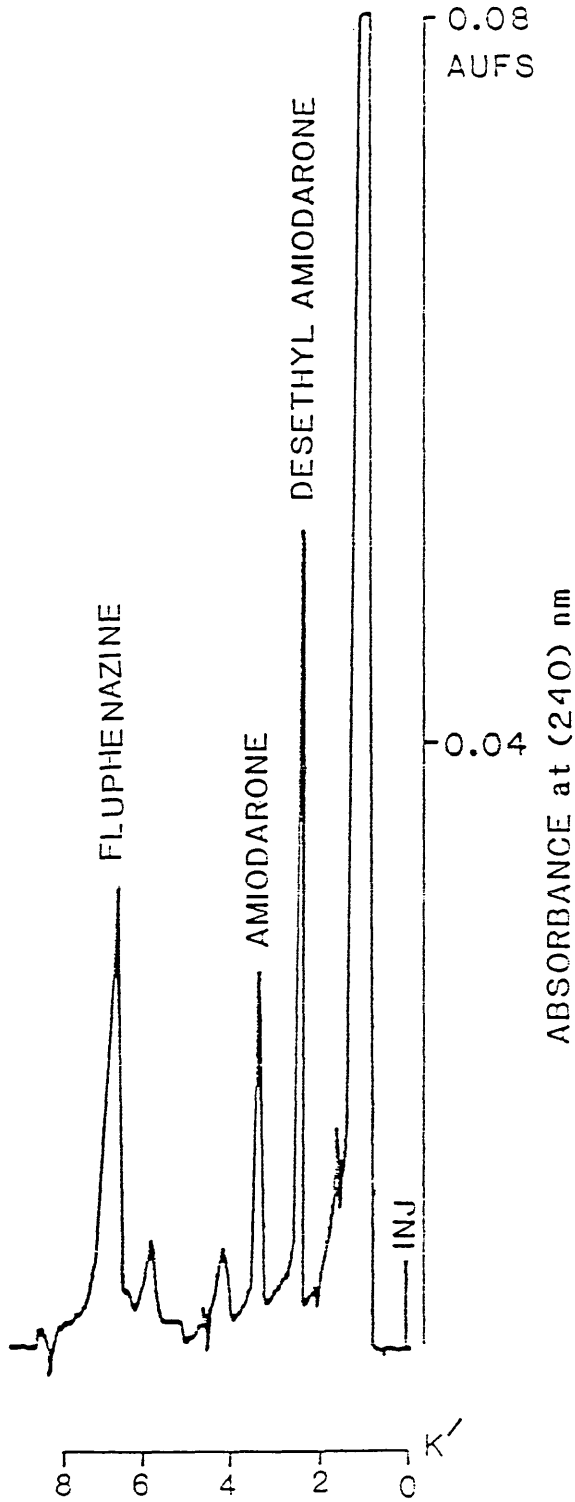


Figure 69 : HPLC profile of amiodarone and metabolite desethyl amiodarone extracted from A549 cells with the internal standard fluphenazine.

3.10.2. Glutathione Content of A549 Cells Treated with both Doxorubicin and Amiodarone :

As shown in Table 7 the glutathione content of A549 cells treated with doxorubicin fell as the concentration of amiodarone in the cellular environment increased up to 8 μmol / l. Glutathione depletion, compared with the control, was 12% for cells treated with 5 μmol / l doxorubicin and 62.8 % for cells also treated with amiodarone (8 μmol /l) (Figure 70). Comparison of cellular glutathione with the intracellular doxorubicin and amiodarone indicated that at maximum depletion intracellular doxorubicin was 12.5 % of the extracellular concentration (Figure 71) and amiodarone was 11 % of the extracellular concentration (Figure 72).

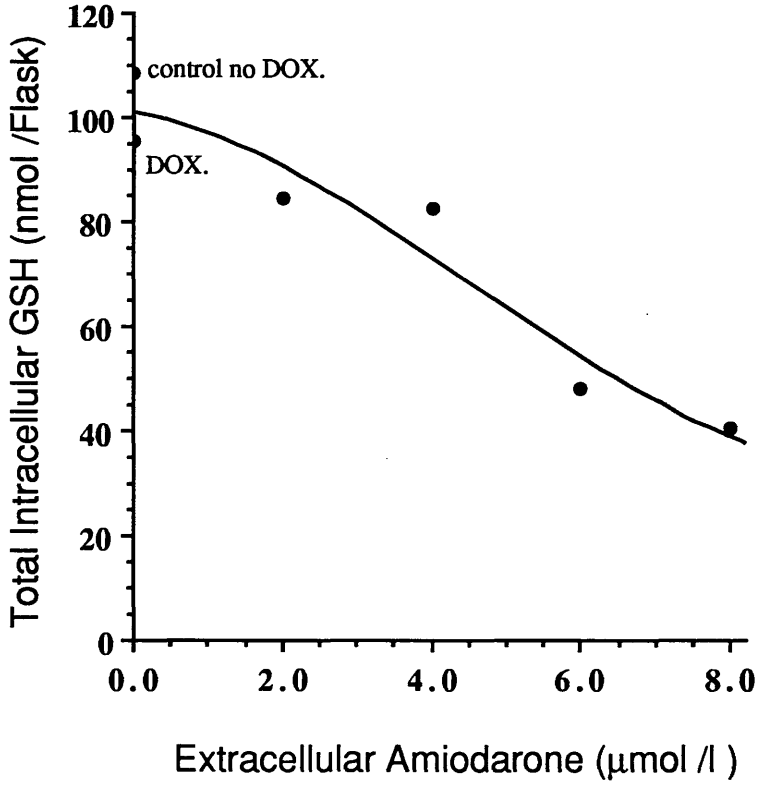


Figure 70 : Effect of extracellular amiodarone concentrations on the depletion of intracellular GSH in A549 cells treated with doxorubicin.

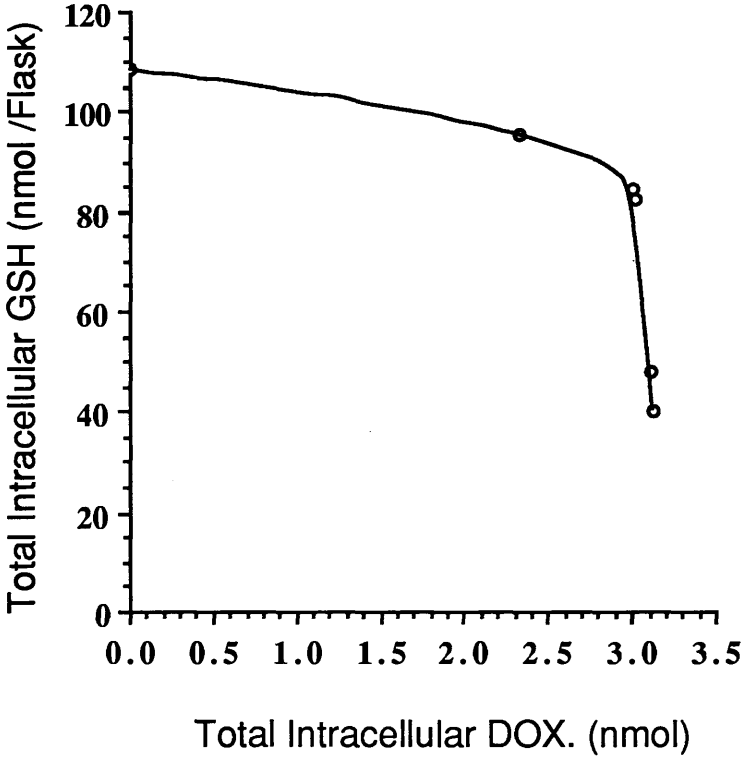


Figure 71 : Relationship of intracellular doxorubicin concentrations to GSH in A549 cells pretreated with 5 µmol /l doxorubicin and increasing amiodarone concentrations

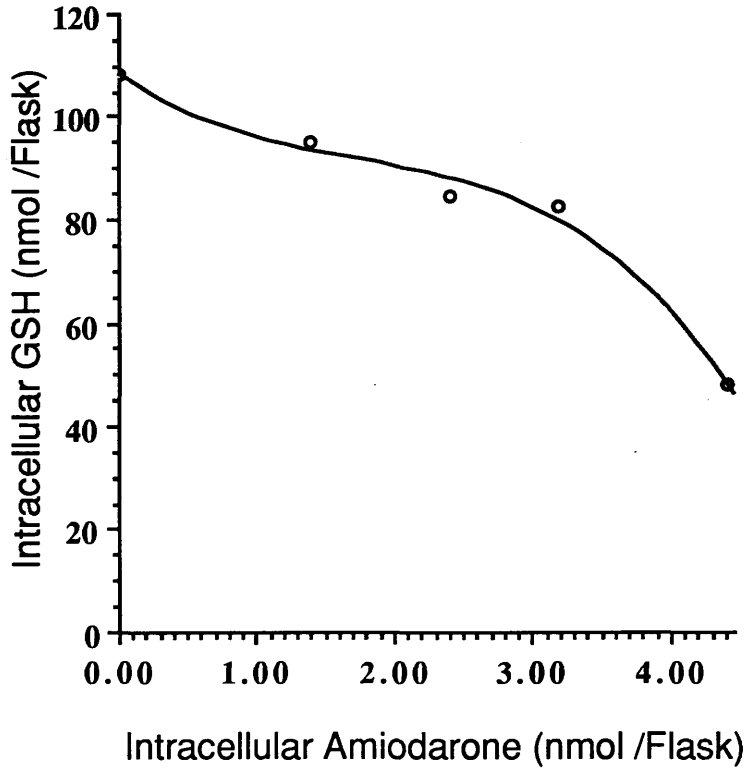


Figure 72 : Effect of intracellular amiodarone on the depletion of GSH by doxorubicin in A549 cells.

3.11. Chemosensitivity Assay of A549 cells Following Amiodarone Pretreatment :

Figure 73 shows the result of one hour pretreatment of A549 cells with four different amiodarone concentrations on the sensitivity to doxorubicin. The four amiodarone concentrations used in this assay were found to enhance the sensitivity of A549 cell proportionately, as measured by MTT absorbance, compared with the controls and doxorubicin treated cells. The plateau value was reached with the first amiodarone concentration used (2 μmol / l).

In this experiment the mean of four absorbance reading of four different wells of the same cell concentrations ± standard error were calculated. Statistical analyses was carried out using t -test, and the results were found to be significant ($P < 0.01$) indicating that amiodarone can increase cell kill when used along with doxorubicin.

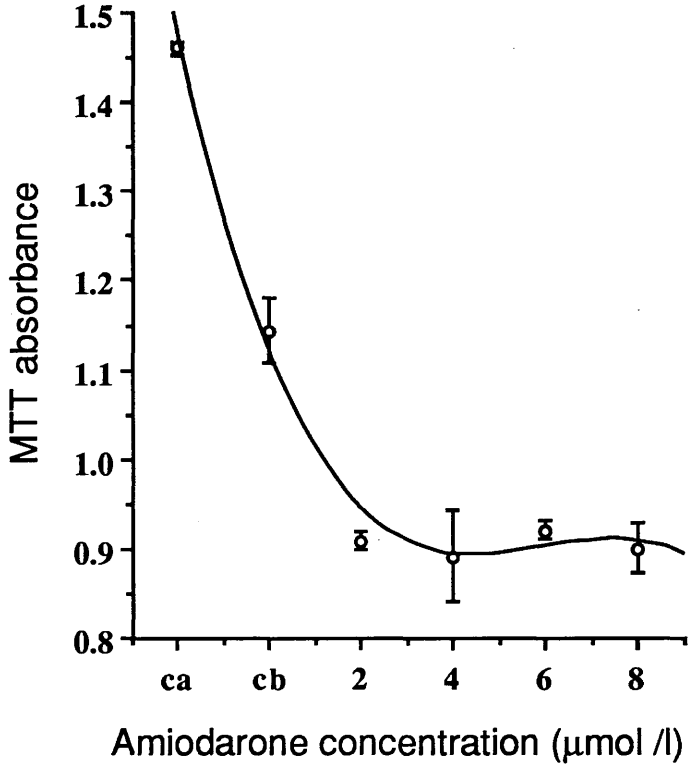


Figure 73 : The effect of amiodarone on the viability of A549 cells treated with doxorubicin ; ca control no doxorubicin ; cb control with doxorubicin alone. Bars = standard error.

IV. DISCUSSION AND CONCLUSION

4.1. Glutathione Measurement :

The importance of GSH in cancer chemotherapy and radiotherapy comes from its critical role in cellular defence against oxidative stress (Arrick and Nathan, 1984 ; Jordan et al., 1987). Thus the ability of GSH to antagonise the effect of cellular injury due to drugs and carcinogens such as quinone antibiotics has led many to use this tripeptide as a tool for controlling redox behavior due to these agents (Meister and Anderson, 1983 ; Murray et al., 1987). Modulation of cellular GSH levels is effective in improving the therapeutic efficacy of such antibiotic drugs especially when dealing with resistant tumours. In this situation, a reliable analytical method for GSH measurement is needed.

A study of methods for GSH determination suggests that such analyses are not always satisfactory. In early work, total nonprotein thiols were determined (Owens and Belcher, 1965 ; Cohn and Lyle, 1966 ; Wendell, 1970), methods used depending on the reaction of chemicals such as Ellman's reagent or o-phthaldehyde, however the reaction with primary amines producing the same sensitive conjugates (Benson and Hare, 1975). Since the enzymatic methods (Tietze, 1969 ; Koivusalo and Uotila, 1974 ; Davies et al., 1984) were convenient to determine total glutathione, estimation of oxidized glutathione generally required that reduced GSH be especially sequestered with N-ethylmaleimide or 2-vinylpyridine. HPLC methods (Fahey et al., 1981 ; Newton et al., 1981) were developed for GSH separation including recycling post column reaction (Reeve and Kuhlenkamp, 1980 ; Alpert and Gilbert, 1985 ; Burton and Aherne, 1986), however the detection required derivatization of thiols with fluorescent reagents. Amperomeric methods which involve electrochemical detector (Lunte and Kissinger, 1984 ; Stein et al., 1986) or coupled with coulometric detection (Harvey et al., 1989) were not easy due to the difficulty in the preparring and maintaining the electrodes and the cost of using such

equipments. Although there is no doubt about the sensitivity of these methods, they require time for sample preparation which may give problems with reproduciblity of results especially when dealing with the cell lysates.

4.1.1. Development of an HPLC Method for GSH Measurement :

In this work, the procedure described in section 2.4. gives direct measurement of GSH in the samples using cationic ion-pair HPLC. This method offers several features which are of some importance.

The time required is very short allowing reduced interference by disulphide and some cellular proteins which give inaccurate measurement (Harding, 1970). The time needed for sample preparation in this method (from cell lysis to injection into the HPLC system) can be reduced to a few minutes, and the complete measurement of GSH, including sample preparation, can be achieved within one hour when other lysate peaks are neglected. Where storage was required the stability of GSH in cell lysates was found to be more than half a day when kept in liquid nitrogen.

Another important advantage is the low cost, since no chemicals are involved in sample preparation or for derivatization. The sensitivity and accuracy of this procedure are adequate for quantitation of glutathione in small batches of cells.

Homogenization of cell samples may interfere with the resulting recovery (Meister and Anderson, 1983). Ultrasonic lysis of cells gave better recovery than freezing and thawing when examined using this method, perhaps due to the higher proportion of cells broken. This method was found to have acceptable sensitivity ($\cong$ 65 picomoles

on column) and precision (±1.89 % and ± 0.95 %) with the added advantage of rapidity when compared with the others ; where Harvey et al., (1989) quoted a best precision of ≅ ± 5 %.

The highest resolution of GSH was achieved at a buffer pH of 3.5 using orthophosphoric acid and was used routinely even though the low pH and high phosphate concentrations may reduce column life by degrading the silica. This effect can be minimised using a guard column of the same packing material as the analytical column. The resolution found at the above pH could be related to the carboxyl group (–COOH) in the glutathione molecule, the pKa values of which are 2.12 and 3.51 (Rabenstein and Fairhurst, 1975). Therefore separation of glutathione by this HPLC method was related to the degree of ionisation at pH 3.5. The mechanism of GSH seperation is the formation of ion-pairs between the negatively charged GSH molecules and the tetrabutyl ammonium hydroxide cations. The ion pairs are non-polar due to the large tetrabutyl groups, and are retained on the non-polar stationary phase.

The capacity ratio (k' = 7.75) which showed the best resolution for GSH in this method was reached at a concentration of 0.1 % of tetrabutyl ammonium hydroxide. At lower concentrations the number of analyte ions exceeds that of the ion-pair allowing elution of non ion-paired glutathione. At concentrations above 0.1 % of tetrabutyl ammonium hydroxide, miscellar ion-pairs are formed which are not retained (Watson et al., 1985). When the same sample was eluted in the absence of ion-pair, GSH was unretained due to its high polarity (Knox et al., 1978).

The use of HPLC grade methanol and water improved the base-line by reducing noise at the lower wavelength used (200 nm and 0.01 AUFS), while degassing the HPLC buffer was also useful in reducing noise at such a low UV wavelength.

Resorcinol was chosen as internal standard since it did not interfere with the peaks due to endogenous compounds. In addition it had high solubility and stability in the lysing buffer used, and high absorbance in the UV { E_o (274) = 923 A.U. and E_o (220) = 2329.7 A.U. }.

The resulting method, as developed, allowed the precise quantitation ($\cong \pm 2$ %) of GSH by direct injection of rapidly lysed cell homogenates, minimizing oxidative changes. This method can be adapted for measuring other cellular components including GSSG, GSH precursors and a variety of different amino acids, but this was not priority. Certain amino acids such as glycine, leucine, alanine, glutamine, arginine and phenylalanine have been identified as unretained or unresolved from each other during the preliminary experiments, this needs some additional work to obtain better resolution.

4.1.2. ^{1}H Spin Echo NMR as a Comparison Study to HPLC for Glutathione Analysis in Hela Cells :

Due to the instability of the active intermediates and of GSH itself, the analyses described above using cell homogenates are open to criticism. Ideally it would be better to study the effects of doxorubicin on GSH in the intact cell. ^{1}H spin echo NMR, detects signals arising from protons allowing the study of a wide range of intracellular compounds with higher sensitivity than ^{13}C and ^{31}P ; it is also suitable for membrane transport studies (Brown and Campbell, 1980 ; Agris and Campbell, 1982). This non invasive method is capable of detecting glutathione as well as some other compounds of intermediary metabolism. Assignments have been made by the procedure of standard addition to the cells. The disadvantage of this method, as noticed by Brown and Campbell (1980), is the resolution problem (i.e. many

resonances occur in a narrow range), but as it is clear from figure 42 there is much information with good resonances available from the protons of several intracellular molecules which are well resolved. Another disadvantage of using NMR technique alone is the low sensitivity due to the sample size requirement (10^8 to 10^9 cells) and the fact that, even with a calibration, results are at best semi-quantitative. The combination of HPLC and NMR allaws the best information for both techniques to be used together to study glutathione metabolism.

Reduced glutathione (GSH) is one of the cellular components that can be measured without any interference using this technique (Figure 42). Thus NMR provided a good opportunity to study in a non invasive manner the effects of doxorubicin and to compare the results with those from HPLC.

4.2. Effect of Doxorubicin Treatment on GSH in Sensitive and Resistant Human Tumour Cells Using HPLC :

The problem in the use of the promising antitumour antibiotic doxorubicin, a drug with wide pharmacological activity, is the production of a cumulative, dose-dependent form of cardiac toxicity which can be life threatening. This particular obstacle is thought to be due to the effect of free radical intermediate generation from quinone metabolism in the myocardium and oxygen consumption which can alter Ca^{2+} transport by cardiac sarcoplasmic reticulum (Doroshow, 1983 ; Harris and Doroshow, 1985).

The precise mechanism and the efficacy of thiol-containing scavengers such as glutathione in preventing damage to the Ca^{2+} pump is confirmed by their ability to inhibit free radical related oxidation induced cardiac toxicity (Thornalley and Dodd, 1985). Thiol compounds also detoxify radicals such as the potent oxidizing species the hydroxyl radical ($^{\cdot}OH$) generated by doxorubicin metabolism in heart myocardium and leading to membrane lipid peroxidation (Thornalley and Dodd, 1985 ; Russo et al., 1986 ; Thomas and Girotti, 1989).

Normal mammalian tissues contain variable glutathione concentrations ranging from those just adequate for cell survival (0.1 mmol / l) (Kosower, 1976) to high concentrations (10 mmol / l) in tissues such as liver (Doroshow et al., 1979). It has been suggested that in the case of breast tissue, elevation of such thiol-containing compounds of the apocrine epithelium indicate pre-neoplasmic changes ; in tumour tissues glutathione concentrations often are higher than those of the normal tissues (Murray et al., 1987).

This section was concerned with the study of glutathione concentration and the effect of doxorubicin at different concentrations in four different human tumour cell lines as models of sensitive and resistant tumours in order to understand the exact situation of glutathione in the neoplastic tissues and its importance in case of cancer chemotherapy. The results reported here, using a method for estimation of glutathione which is unaffected by artifacts due to cell disruption, indicates that the basal glutathione concentration in the four different cancer cells (Hela, A549, GLC_4 210 [S] and [R]) differed from one another, being high in Hela cells and in inherently resistant cells (A549) as compared with GLC_4 210, either the initially sensitive line or those with acquired resistant cells. These findings are in agreement with those of Meister, (1983) who used an enzymatic method and Murray et al., (1987), both of whom demonstrated that tumours may be found with low, moderate and high intracellular glutathione concentrations. Their statements that certain tumours may have glutathione concentrations close to the minimum required for cell survival are confirmed by my findings in GLC_4 210 (R) in which there was a very low level of

glutathione which was unaffected even by high doxorubicin concentrations. This may be the minimum requirement for survival in cells grown under the stress of doxorubicin and which have acquired resistance to this drug. Cytoplasmic glutathione may be depleted with only the vital mitochondrial pool remaining ; there is evidence that cells will not die until this pool is depleted (Gaetjens et al., 1984). Cells which adapt themselves to doxorubicin following long term treatment at low drug concentrations (Kaye and Merry, 1985), are not affected by this drug and their minimum glutathione content seems also not to be affected due to continous stimulation of new GSH synthesis as explained by Meijer et al., (1987).

Of the two species of tumour cells sensitive to doxorubicin, one, (Hela) showed a high glutathione content and the other a low content of glutathione (GLC_4 210 [S]). In both of them glutathione was reduced after doxorubicin treatment. Responses similar to this have been reported, using HPLC, for the hepatic glutathione levels in rats using the GSH lowering drug azathioprine (Kaplowitz, 1977) and DL-buthionine-S-R-sulfoximine as a selective irreversible inhibitor of γ-glutamyl cysteine synthetase (Somfai-Relle et al., 1984 ; Crook et al., 1986 ; Kable et al., 1989). Glutathione depletion seems to be increased with doxorubicin dose, perhaps due to increased cellular penetration of the drug, with the intracellular metabolism of the drug to the active radicals mentioned in section 1.2.3.4.

The other two tumour cell lines, resistant to doxorubicin, showed very different results. One (GLC_4 210 [R]), which developed resistance after continuous growth at low doxorubicin doses, had very low glutathione levels. The glutathione in this cell line was reduced through the culturing procedure in the prescence of doxorubicin (Meijer et al., 1987), as these cells were reverted from GLC_4 210 (S) cells. The resulting minimal level of glutathione was necessary for cell survival which was reduced only at a very high drug concentrations. The other resistant tumour line (A549), inherently resistant to doxorubicin, had a high glutathione content which has been suggested as contributing to their resistance (Arrick and Nathan, 1984 ;

Hamilton et al., 1985 ; Russo and Mitchell, 1985). This glutathione is not reduced by low doxorubicin concentrations, however high concentrations are effective due to increased penetration into the cells (Gigli et al., 1989). BSO as a selective inhibitor of GSH, by inhibiting γ-glutamyl cysteine synthetase, could be adjunct to therapy with doxorubicin (see 1.3.3. and 4.8.).

The effect of doxorubicin on glutathione reduction in the different tumour cell lines used showed that, in case of A549 tumour cells a doxorubicin concentration was required which was about eleven times that necessary for Hela cells, and four times more than required for GLC_4 210 (S) cells. The minimal reduction of glutathione in case of GLC_4 210 (R) and A549 cells even at the higher doxorubicin concentrations used may be either due to the low penetrability of doxorubicin into these cells (Kessel and Wilberding, 1985 ; Supino et al., 1988) because of the changes in membrane structure, or that doxorubicin, having penetrated the cells is exported by an enhanced efflux mechanism diminishing the opportunity for metabolism and oxidative radical attack on its intracellular targets (Kaye and Merry, 1985 ; Vrignaud et al., 1986). This explanation is quite plausible in the case of A549 cells in which very little doxorubicin appears to be retained by the cells. Moreover these cells contain high glutathione concentrations which can easily detoxify doxorubicin intermediates and free radicals formed resulting from this drug metabolism. This finding was confirmed by Supino et al., (1986) and Merry et al., (1987) that different mechanisms have to be responsible for drug resistance.

The same doxorubicin concentrations which were effective in the case of sensitive cells gave no clear effects on A549 cells, however in case of sensitive tumours (Hela and GLC_4 210 [S]) doxorubicin seems to cross the cellular membrane and be metabolized to the active intermediate, utilizing most of the available glutathione.

4.3. Effect of Doxorubicin on Hela cells Glutathione Content using NMR :

The NMR method used detected a rapid dose-related depletion of the cytosolic glutathione pool, confirming the HPLC results. Although the NMR measurements are only semiquantitative, the changes observed in the NMR experiments can be conclusively assigned to cellular activity in the intact cells by doxorubicin. The effect of doxorubicin on the glutathione pool took two forms. At high doses the depletion was very rapid suggesting that glutathione is acting as a primary sink for doxorubicin free radicals ; cellular stress as indicated by an increase in lactate did not occur till this primary defence had gone. This is consistent with the recent report that glutathione levels may correlate with the cellular resistance to doxorubicin (Romine and Kessel, 1986). The lower doxorubicin doses depleted the glutathione pool in a slow manner with the added feature of a lag phase. Such findings may be explained either as slow intracellular accumulation of doxorubicin (Gigli et al., 1989) or that the response to doxorubicin free radicals initiation is not observed until sufficient free radicals have been generated to deplete the final 10% of glutathione (mitochodrial component) essential for cell survival (Gaetjens et al., 1984).

In order to eliminate the lack of precursors as a cause of reduced synthesis of glutathione, Hela tumour cells were treated with the primary constituents of glutathione while they were in the NMR tube in the presence of low doxorubicin concentrations. The three amino acids glycine, glutamate and cysteine showed simple transport characteristics, passing across the cell membrane barrier into the cytosol. Glycine passed most rapidly and completely and accumulated intracellularly, confirming the findings of Righetti et al., (1971). Glutamate and cysteine showed a fall after entry which may be due to metabolism and synthesis into glutathione (Issels and Nagele, 1989). However reduction of the resonance intensity of cysteine could also be due to its function as free radical scavenger due to the abundant electrons on

its -SH group, since Doroshow et al., (1981) concluded that non-protein sulfhydryl content of several organs increased after L-cysteine administration.

4.4. Studies of Glycolysis in Hela and J111 Human Leukaemia Cell Lines :

Study of cellular metabolism by NMR methods depends on the ability to maintain cell viability while the experiment is running. Hela tumour cells were found to be viable in simple physiological saline solution (^2H_2O / NaCl, 0.154 mol / 1) for periods long enough to study the doxorubicin and inhibition effects. This environment is quite stringent for cells in that much more complex nutrient media are usually used to increase life time but it does give a time scale in which experiments can be carried out with viable cells. Leukaemia cells J111 were found to be non-viable within the time scale required for metabolic studies in the above medium using NMR at 250 MHz. The cells aggregated, and this severely reduced the signals which could be obtained. Therefore in this case a more complete medium, ^2H_2O / RPMI 1640 and ^2H_2O / NaCl 10% RPMI 1640 was used.

During cellular carcinogenesis and due to the changes of enzyme patterns, glycolytic capacity of malignant cells in both aerobic and anaerobic conditions is increased in order to meet the energy requirements for enhanced nucleic acid and phospholipid synthesis (Kallinowski et al., 1988). At the advanced tumour stage in vivo glucose uptake is expected to be reduced due to the restriction in the blood flow, however Kallinowski et al., (1987) concluded that in case of the hypoxic tumours glycolytic rate increased with size so long as glucose supply was maintained.

Cancer cells, which may be poorly oxygenated, derive the bulk of their biochemical energy from the production of lactate rather than the complete degradation of glucose via the tricarboxylic acid cycle ; consequently the kinetics of lactate production can be used to assess the hypoxic stress of the cells in the culture or tumour following glucose addition since a direct relationship between glucose consumption and lactate production has been found (Reglinski et al., 1987 ; Kallinowski et al., 1988). The sugar is metabolized efficiently to produce a variety of products, dominant amongst which is lactate. In vitro this lactate mainly arises from the α anomer of the glucose (Ugurbil et al., 1978). The results shown above indicate that the rate of cellular uptake of sugar and production of lactate can be seen to be linked in Hela cells with the lag phase in lactate production during the maximum rate of uptake of glucose. In the absence of added glucose the lactate profile in untreated Hela cells is flat over a period of 8 hours.

In the case of leukaemia (J111) cells the lactate profile indicates an initial aerobic phase during which the lactate resonance diminishes, followed by an anaerobic phase once the cells have utilised the available oxygen in the medium, in which the lactate signal steadily increases. This is similar to what was observed for Hela cells where a short aerobic period was evident as a lag phase in the glycolysis plot.

In Hela cells in the presence of doxorubicin there was a marked difference in glycolytic behavior, with a clear relationship between glutathione concentration and rate of glycolysis. The maximum rate of glycolysis did not seem to occur until after depletion of glutathione to a low level (Figures 46 and 47) (T = 3h). This seems to confirm that glutathione is the main oxygen radical scavenger in the cells, since at the maximum glutathione reduction level the anaerobic state was established. This may be due to the abundance of free radical and intermediates of doxorubicin metabolism after GSH depletion, acting as oxygen utelising agents, converting cells to anaerobic state at which the maximum rate of glycolysis took place (Al-Kabban et al., 1988 ; Kallinowski et al., 1988). These findings could be confirmed by the fact that the

lactate profile in cysteine-treated cells exposed to doxorubicin showed no stress when compared with unprotected cells (Figures 47 and 49) ; cysteine being a thiol-containing compound which protects against doxorubicin free-radicals produced.

In the case of leukaemia cells, no aerobic phase was seen in the presence of doxorubicin and the culture rapidly turned to anaerobic metabolism. This may be due to the fact that this type of cell contains a minimum (almost undetectable) amount of glutathione. Doxorubicin is thus reduced to its active metabolite consuming the available oxygen in the NMR tube turning the culture directly to the anaerobic state (Yoda et al., 1986).

4.5. Protection of Cellular Glutathione from Doxorubicin Effects by N-acetylcysteine and Cysteine :

A method of increasing cellular glutathione levels might be beneficial in protecting cells against active radical intermediates. Accordingly treatment of Hela tumour cells with cysteine or NAC one hour prior to a previously effective dose of doxorubicin was investigated, and resulted in a decreased effect on the cellular glutathione. This is thought to be due to the activity of these thiol-containing compounds in concert with glutathione as radical scavengers (due to the availability of electrons on the sulphur molecules) or that NAC could be deacetylated to yield cysteine as a GSH precursor or acting as a delivery system for cystine sulphur from the medium outside the cells which then converted to cysteine (Issels et al., 1988) . NAC was seen to protect against high doxorubicin doses with lactate stress not observed until intracellular

NAC concentrations fell to insignificant levels. This finding compares with the glycolysis study in which the maximum rate of lactate production did not occur until after depletion of glutathione to a very low level. NAC mimics the protective role of glutathione, but has the advantage of direct penetration into cells, increasing the thiol concentration. Similar responses have been reported using NAC or cysteine to protect rats against the side effects of cyclophosphamide without interference with its antitumour activity (Berrigan et al., 1982).

NAC at low concentrations shows no interference with the therapeutic efficacy of doxorubicin, however this study indicates that such thiol-containing compounds could be toxic to the cells when used at high concentrations. GSH depletion in this experiment could be due to increased cell death (see section 3.8.). The experiment reported here indicated that 1.4 μmol NAC / 10^6 cells seems to be the best non toxic effective concentration for protection of Hela cells.

In the case of cysteine the protective action was confirmed by the amino acid transport experiment using NMR and the finding of Issels and Nagele, (1989) in which cysteine was found to pass across the cell membrane, showing a later fall which could be due either to its synthesis into glutathione or metabolism following action as a free radical scavenger in its own right as a thiol containing compound (Doroshow et al., 1981). Meister (1984) showed that glutathione may be increased by cysteine supply and recently Issels and Nagele, (1989) added that the cysteine moiety of newly synthesised glutathione is derived from the extracellular pool of cystine.

The plateau level of glutathione was reached with a single dose of cysteine, confirming the direct and easy penetration of cysteine into the cell ; excess cysteine may be metabolized and excreted (Meister, 1983). For activity as a glutathione precursor it must be present at similar concentrations to the other two amino acids (glycine and glutamate) and given in a combination with them.

4.6. GST Relationship to the Intracellular GSH Level :

Glutathione-S-transferase (GST) has a major role in catalysing the conjugation of reduced glutathione with a wide spectrum of electrophiles (Ketterer et al., 1982 ; Mannervik, 1985) of which doxorubicin may be one. Hence it might be expected that cells with a high GSH content may also have high GST activity (Evans et al., 1987). The results here indicate that there is relationship between the glutathione content of different lung tumour cells and their GST activity which is mainly of the acidic type (Beckett and Hayes, 1987). Glutathione content in the three cell lines used was in the order A549 > GLC_4 210 (S) > GLC_4 210 (R) ; the GST activity was high in the cells of high GSH content (A549) compared with the other two cell lines of lower GSH content (GLC_4 210 [S and R]). Similar findings were reported by Scott and Wright (1980) who suggested that GST activity may be elevated in case of inherent elevation of glutathione content such as was detected in A549 cells. Recently Smith et al., (1989) concluded that certain GST classes could be elevated and take part in the resistance of tumour toward cancer chemotherapy.

Twenty four hours pretreatment of these cells with effective doxorubicin concentrations gave no clear reduction in the GST activity. A reduction would be expected due to the reduction in cell numbers as a result of doxorubicin-induced cell killing as shown in section (3.8.). These results, although only few in number suggest that GST has a high degree of stability and is membrane-bound. The fall in GSH in the detoxification process of doxorubicin whould therefore seen to be unrelated to any change in GST activity. It therefore seems that although some authors have related GST to the level of cell resistance or sensitivity to 1,3-Bis (2-chloroethyl)-1-nitosourea (BCNU) and nitrogen mustard (Evans et al., 1987 ; Smith et al., 1989), it is not a useful marker for examining the effect of doxorubicin on cells in vitro. This is in agreement with Meijer et al., (1987) that there was no detected changes in GST activity due to developement of acquired resistance in GLC_4-Adr line to doxorubicin.

4.7. Lipid Peroxidation Induced by Doxorubicin :

As a biological term lipid peroxidation refers to the destructive effect on the normal living tissue caused by active oxygen radicals. Transformation of normal tissue into malignant cells can render them less susceptable to peroxidation (Dormandy, 1988). Accordingly there might be a role in cancer eradication for the induction of lipid peroxidation in cancer cells (Thomas and Girotti, 1989). Free radicals transform the unsaturated lipid into lipid radicals of high activity ending in lipid peroxy-radical (see 1.2.6.3). Doxorubicin has been found to establish this sort of reaction as one of its mechanisms of tissue damages (Mimnaugh et al., 1985 ; Niki, 1987).

Although thiobarbituric acid reaction is not the ideal method for lipid peroxidation measurement in different sample origins due to the lack of specificity, it is still widely used in both laboratory animal and human sudies. It gives broad brush information about the induction of lipid peroxidation when the results compared with controls. The thiobarbituric acid reaction with other substances, such as saturated aldehydes, may be not important in in vitro studies, but must be recognized when dealing with patient speciments (Knight et al., 1988).

In this study two sensitive tumour cell lines were chosen (Hela and GLC_4 210 [S]) treated with an effective doxorubicin concentration. MDA was measured as an indicator of lipid peroxidation induced by doxorubicin free radicals. The two lines showed high activity of doxorubicin on the unsaturated fatty acids of the cellular membrane as indicated by increased MDA accumulation. In these two cell lines there is high doxorubicin retention and fast GSH utilization (3.3.1. and 3.3.3.), and that are the main reasons for lipid peroxidation (Thomas and Girotti, 1989). Llesuy et al., (1985) have shown that there seems to be a direct relationship between lipid peroxidation and intracellular doxorubicin concentration.

The use of N-acetylcysteine to circumvent lipid peroxidation produced support for the activity of such a thiol-containing compound as an antioxidant agent when added prior to the cytotoxic drug. It seems to have no effect within the first hour of administration, but subsequently produced some protective effect against doxorubicin by minimizing lipid peroxidation. A similar finding has been noticed by Myers et al., (1977) who found that lipid peroxidation as measured by MDA could be blocked by tocopherol, reducing the cardiotoxicity of doxorubicin in mice. Accordingly combination of these results with these described in section 3.4.1. may indicate potential benefits in using NAC as a protective agent to guard normal heart tissue against the side effects of doxorubicin. Doroshow et al., (1981) found that treatment of experimental animals with pharmacological doses of N-acetylcysteine, selectively rescued heart tissue but not others from the toxicity of doxorubicin as detected by lipid peroxidation. They postulated that NAC can break the free radical chain reaction by acting as a hydroxyl radical scavenger, enhancing the ability of heart muscle to withstand doxorubicin exposure.

4.8 Chemosensitivity Measurements of Doxorubicin in different Tumours of Human Source and Effect of BSO Pretreatment:

The MTT experiments were carried out using the standard protocol of 10^3 cells treated over 24 h. These indicate relative resistance, but MTT uptake measured cell metabolism rather than time viability. The Trypan blue experiments, (Appendix) showed adequate viability for HeLa and A549 lines at Doxorubicin concentrations of the same order as those used for the HPLC experiments.

The results of the chemosensitivity assay for the four cell lines gives baseline data about the behavior of these human tumours when treated with doxorubicin. The two sensitive cell lines (Hela and GLC_4 210 [S]) showed a major reduction in dye absorbance with a reduction in viability proportional to drug concentration. Viability was reduced 20 and 6 times respectively when compared with controls at the highest doxorubicin concentration used (1075 nM), however in the case of the acquired

resistance line (GLC_4 210 [R]) the viability was reduced by only 2.5 times that of the control. For inherently resistant cells (A549), for which the viability of the basal non treated cells was high compared with the others (twice that of Hela and more than four times that of the other two lines), the reduction in viability at the above doxorubicin concentration was about 8 times that of the related control. These results indicate that for each cell line the resistance to doxorubicin and its activity are dependent on the drug concentration outside the cells (Bellamy et al., 1988 b) and that may desided the intracellular incorporation and distribution. Similar behavior was described by Vrignaud et al., (1986) who measured cloning efficiency.

Resistance of tumour cells toward doxorubicin could at least in part be developed due to the difference in the capabilities of DNA repair (Meijer et al., 1987 ; Bellamy et al., 1988 b) by the catalytic activity of ATP-dependent enzyme DNA topoisomerase II. DNA intercalation is necessary but not sufficient for the antitumour activity of doxorubicin, this drug induces protein-linked DNA breaks in a mechanism independent of radical production in the presence of DNA topoisomerase II, but not in the absence of this enzyme (Tewey et al., 1984). Therefore in resistant tumours, increasing ability of DNA repair may be explained by genetic reduction of DNA topoisomerase II production preventing DNA-DOX complex formation.

The results here paralleled the effect of different doxorubicin concentrations on measured GSH. The LD_{50} of doxorubicin in these four cell lines differed, in the order GLC_4 210 (R) > A549 > Hela > GLC_4 210 (S). The fact that the cells with acquired resistance showed the highest LD_{50} while having a lower GSH concentration than the others could be explained as being due to increased synthesis of membrane glycoproteins P-170 – P-180, resulting in increased doxorubicin efflux (Garman and Center, 1982). In the case of A549 cells, doxorubicin resistance could be due both to membrane glycoproteins (P-170 – P-180) induction and also to the high glutathione content (Suzukake et al., 1982 ; Arrick and Nathan, 1984 ; Hamilton et al., 1985 ; Russo et al., 1986 ; Dusre et al., 1989). Certainly the viability of these two cell lines is only slightly affected by doxorubicin when

compared with the two sensitive lines. The high level of resistance shown by GLC_4 210 (R) cells compared with A549 agrees with the findings of Shen et al., (1986) that cells with induced resistance are more resistant than wild type tumour cells to certain cytotoxic drugs.

The high GSH concentration in Hela cells can be rapidly reduced after treatment with doxorubicin confirming high penetration and accumulation of the drug in this type of cell, as in GLC_4 210 (S) cells with low GSH concentrations. It is suggested that in the case of sensitive cells there are little or no P-170 – P-180 glycoproteins to facilitate the efflux of doxorubicin. Therefore there is enough time for the drug be metabolised and react with GSH. The remaining doxorubicin intermediate could be intercalated with the DNA base pairs after GSH depletion, inducing cell killing (see 1.2.3.1.1.). It is interesting that in case of GLC_4 210 (S) cells with a low GSH content, the effect much more severe than in Hela cells of high GSH content. These results would confirm other findings (Russo et al., 1986 ; Jordan et al., 1987), that glutathione is protective against cytotoxic agents.

Because of the importance of glutathione in the protection of cells against free radical formation due to certain cytotoxic agents such as doxorubicin, depletion of intracellular GSH in order to increase the sensitivity of the resistant cells is theoretically a valid method of therapy (Arrick et al., 1982 ; Russo et al., 1986 ; Lee et al., 1987 ; Jordan et al., 1987). The experiments carried out above on a human lung tumour cell lines, showed that when GSH was depleted with sub-toxic BSO concentrations in A549 cells 6 hours prior to exposure to doxorubicin, cell resistance was reduced. This finding confirms that viability and intracellular glutathione depletion are linked, and support the recent study of Dusre et al., (1989) who found that toxicity of doxorubicin could be enhanced by an increase of $^{\cdot}OH$ formation as an indirect activity of BSO by decreasing GSH level. It is therefore possible that there may be potential benefits of agents such as BSO as adjuncts to therapy with doxorubicin.

4.9. Enhancement of Doxorubicin Cytotoxicity Using Amiodarone :

In all of the reported cases of doxorubicin resistance enhancement of active drug efflux is a major problem, in addition to the low penetration and distribution which is effected by the hydrophobic / hydrophilic (membrane / cytoplasmic) properties (Hindenburg et al., 1987). Several trials have been carried out to overcome this problem, using calcium channel blockers such as verapamil and norverapamil. Although the exact mechanism of the use of calcium channel blockers to overcome cellular resistance is not yet understood, Merry et al., (1987 ; 1989) indicated that these antiarrythmic drugs can increase the ratio of bound to unbound doxorubicin. This is consistent with the above finding of Hindenburg et al., (1987) that these agents are able to displace drug from the hydrophobic into the hydrophilic compartment of the cell (drug within the hydrophilic componant is less available than lipid associated drug), therefore doxorubicin ditribution is expected to be increased.

In this study another antiarrythmic drug, amiodarone, was used to circumvent doxorubicin resistance in the inherently resistant human cell line A549. The resistance of these cells to doxorubicin can be reverted by verapamil, associated with increased intracellular doxorubicin accumulation (Merry et al., 1987), and I also observed drug accumulation and its effect on the already high glutathione content of these cells. The ability of calcium channel blockers to restore the sensitivity of doxorubicin-resistant tumours is then due to blockage of enhanced drug efflux and alteration of drug distribution (Bellamy et al., 1988 a) followed by enhanced DNA damage (Cairo et al., 1989).

The overall mechanism of action of amiodarone appears to be the same as that of the calcium channel blocker verapamil. Although amiodarone has never been reported as a calcium channel blocking agent, its antiarrhythmic effect could be similar to that of the β-blocking agent (Chauffert et al., 1986). These drugs may also act through

the superphosphorylation of plasma membrane glycoprotein P-170 – P-180, thus blocking the exodus of doxorubicin. The increased cytotoxicity of doxorubicin after amiodarone treatment can thus be explained as due to intracellular doxorubicin accumulation, distribution and binding to its target.

For drug estimation the cells were harvested in two different ways, trypsinization and scraping, in order to exclude drug release from protein binding during incubation with trypsin. Cells were left in the trypsin solution and drugs extracted after lysing cells in the same buffer. The results showed no difference in doxorubicin retention between the two methods of harvesting at all amiodarone concentrations used. There is no leakage in doxorubicin during incubation with trypsin (15 minutes). Doxorubicin retention was increased at all amiodarone concentrations used to circumvent resistance, 2 μM amiodarone being an acceptable concentration for increasing doxorubicin uptake in such type of tumour cells. Similar work has been performed by Merry et al., (1986) to circumvent human glioma cells using verapamil. Recently Merry et al., (1987 ; 1989) found that 6.6 μM is the best concentration of verapamil at which there is no interference with the therapeutic effecacy of doxorubicin with significant decrease of cellular resistance at $P < 0.05$ of A549 and Murine tumour cells toward doxorubicin. In this study 2 μM of amiodarone gave a highly significant ($P < 0.01$) reduction in resistance of A549 tumour cells to doxorubicin. Chauffert et al., (1986 ; 1987) reported that amiodarone, a relatively non toxic antiarrhythmic agent is able to restore sensitivity to anthracyclines in naturally resistant rat colon cancer by enhancing cytotoxic effects in the same manner as verapamil but more effectively. This difference in activity should be related to the drug half-life which is short in the case of verapamil (5 hours) as compared with that of amiodarone (25 ± 12 days). This may offer the chance of amiodarone being more effective than verapamil, in addition to which the effect is achieved in vivo at plasma amiodarone concentrations which are similar to those obtained when using it as an anti-arrhythmic agent (Chauffert et al, 1986).

Penetration of amiodarone itself seems to be proportional to its extracellular

concentration, and levels were found to be higher in cells harvested by scraping. This could be due to its high efflux and adherence to flask walls during the incubation time with trypsin buffer leading to losses during harvesting. Another explanation could be competition between doxorubicin and amiodarone for efflux transport systems during the incubation. Such an interaction has been shown for verapamil (Kessel and Wilberding 1984) formed the bases of one of the hypothesis about the action of such agents in increasing intracellular doxorubicin levels. The same authors added that verapamil promotes anthracycline acumulation via competition for exodus. When cells were harvested by scraping in frozen solution of PBS there was little chance and time for leakage and, since cells were directly lysed and extracted, less release from protein binding occurred.

At low amiodarone concentrations (2 μM) the percent amiodarone retained was higher than at the other extracellular concentrations, possibly due to limitation in the membrane intake binding sites which may be saturated at the high amiodarone concentrations, independent of sites concerned with transport of the drugs out of the cells. Center (1985) has postulated that membrane glycoprotein P-180 acts as a channel which is capable of bringing about the efflux of a variety of compounds ; the activity of this channel could be modulated by channel blockers due to superphosphorylation of this protein. This could also explain the differences in doxorubicin penetration and accumulation at high amiodarone concentrations ; as a result of increasing of superphosphorylation of high molecular weight glycoproteins at high amiodarone concentrations these channels could be blocked completely and doxorubicin retained in the cells.

As it is known that intracellular GSH is increased in some forms of anthracycline resistance, and in these cells reduction of GSH, renders them sensitive (Hamilton et al., 1985), it would seem that the high concentration of GSH in A549 tumour cells is an indication of their resistance to doxorubicin. Measurement of GSH in A549 cells after amiodarone and doxorubicin treatment gives strong support for the mode of action of amiodarone against resistance toward doxorubicin. Furthermore

combination of the results of drug accumulation and GSH metabolism in the same experiment gives an indication of the resensitising of such cells to anthracycline, confirming the finding of others such as Hamada et al., (1987) and Chauffert et al., (1987). In this experiment GSH showed a sharp reduction which was related to the intracellular doxorubicin and the intra- as well as extra- cellular amiodarone (Table7) ; the reduction in GSH content was minimal in cells treated with doxorubicin alone.

The cytotoxicity of doxorubicin with or without amiodarone as shown by cellular viability as MTT absorbance give a further confirmation for the above results, showing that amiodarone enhances cell killing by doxorubicin. This result showed that induction of cell killing is a function of time and extracellular doxorubicin concentration (Gigli et al., 1989) after blocking of drug efflux using amiodarone. The plateau level of cell killing was reached at a concentration of 2 μM amiodarone, indicating that A549 tumour cells can be sensitised toward doxorubicin at this concentration.

4.10. Conclusion :

The combination of HPLC and ^1H spin echo NMR for analysis of cell composition and analyte identities is valuable, both because they provide complementary information in some cases and because the NMR technique has the advantage that chemical processes in the living cell can be maintained in situ. The techniques have different sampling requirements since large cell numbers are required for NMR whilst HPLC is more sensitive. This combination of methods allows monitoring of intact cell metabolism and more complete investigation of different cellular components.

The HPLC method developed here is specific for GSH measurement and is of a good sensitivity and precision. It is also fast compared with others, which makes it possible to use it for the measurement of glutathione for clinical purposes (Meister, 1983). The method also measures other cellular components some of which are amino acids that absorb in the low UV wave length.

The ^1H spin echo NMR method is clearly a very powerful one in that it enables specific metabolites to be monitored in the living cell without the need for the addition of probe molecules or high energy sources which can alter the chemical processes. It is therefore ideal as a model system for the studying the action of drug and natural product uptake by the whole cell.

My results confirm, using the reliable and compatible HPLC and NMR methods, that the rapid and significant effect of doxorubicin on intracellular glutathione concentration in intact cultured cells is not an artifact of cell disruption. The initial response of the cell to free radical attack is expressed directly through the glutathione system, there is, after a lag, a dose-related rise in anaerobic glycolysis, supplementation with small thiols delays the onset of this effect. Pretreatment with cysteine or N-acetylcysteine can avert radical damage and the protection is related to the intracellular thiol concentration, but may also involve radical quenching at the lipid

bilayer, minimizing lipid peroxidation.

The lactate signal in spin echo NMR can be used to monitor glycolysis in intact and viable cancer cells and the increased stress in the living cell caused by the widly used drug, doxorubicin. The culture density in the NMR tube is necessarily high, and it is therefore a reasonable model for solid tumours, where the cells are also of a high density and are served by a restricted flow of nutrients and oxygen. Such cells eventually rely heavily on anaerobic glycolysis and finally become necrotic. The NMR method is a reasonable in vitro model for the assessment of potential therapeutic agents which are expected to have an effect on cellular energetic in tumour.

The results of viability studies in the different tumour cell lines used confirm the result of non-artefactural intracellular glutathione depletion, and both studies illuminate the mode of action of BSO in reducing the high GSH content of resistant cells. Glutathione-S-transferase activity in these different tumour cells gave clear evidence of its use as a good marker for doxorubicin resistant tumours, but not as an indicator of the effect of doxorubicin. The application of these methods to the study of known tumour lines showing resistance, sensitivity and acquired resistance to doxorubicin further confirms the importance of glutathione concentration in resistance to doxorubicin and the potential benefits of agents such as BSO as adjuncts to therapy with doxorubicin.

The association of amiodarone with doxorubicin could be useful in therapeutic trials of tumour resistance to doxorubicin, as it is effective at non-toxic concentrations. This agent, which is effective in reversing tumour resistance toward doxorubicin without interference with the therapeutic efficacy of such drug and is also used in human medicine in its own right, could be an excellent adjunct to therapy with doxorubicin. The overall studies mentioned here indicate that doxorubicin, one of the best chemotherapeutic agents in use, may have its unwanted side effects controlled. N-acetylcysteine, cysteine and amiodarone can be used safely in vivo in the concentrations mentioned through this work and may improve doxorubicin

chemotherapy used alone or possibly in combination, since they work in different ways. More work especially on the clinical side, is needed to confirm the continuing potential of this drug in control of cancer.

4.11. Future Work :

For the continuity of this work some areas need to be extended. In particular the relationship of tumour resistance to membrane fluidity which has already been addressed using NMR technique. The enhancement or maintenance of intracellular GSH levels using substances other than NAC and cysteine has also to be further explained. For example the use of oxothiazolidine as a cysteine delevery agent in combination with BSO as a GSH reducing compound and with amiodarone. Glutathione monomethyl or methyl esters are also worthy of study using this model since they are rapidly taken up into the cell.

The findings on lipid peroxidation in this thesis are at a basic level only. This effect of doxorubicin needs to be examined more carefuly, using methods other than MDA. Suggestion are diene conjugate species and lipid hydroperoxidation.

In conclusion, a model now exists for the detailed study of the effect of a number of antineoplastic drugs and co-factors on both GSH metabolism and the integrity of the cell membrane. The extension of these studies, and their possible transfer to the in vivo situation offers an exciting field for further work.

V. REFERENCES

AGRIS, P.F. and CAMPBELL, I.D. (1982) Proton nuclear magnetic resonance of intact Friend leukemia cells: Phosphorylcholine increase during differentiation. Science, 216, 1325 – 1327.

AL-KABBAN, M., WATSON, I.D., STEWART, M.J., REGLINSKI, J., SMITH, W. E. and SUCKLING, C.J. (1988) The use of ^{1}H spin echo NMR and HPLC to confirm doxorubicin induced depletion of glutathione in the intact Hela cell. British Journal of Cancer, 57, 553 – 558.

ALBERTS, D.S., PENG, Y., MOON, T.E. and BRESSLER, R. (1978) Carnitine prevention of adriamycin toxicity in mice. Biomedicine, 29, 265 – 268.

ALLISON, R.D. and MEISTER, A. (1981) Evidence that transpeptidation is a significant function of γ-glutamyl transpeptidase. Journal of Biological Chemistry, 256, 2988 – 2992.

ALPERT, A.J. and GILBERT, H.F. (1985) Detection of oxidized and reduced glutathione with a recycling postcolumn reaction. Analytical Biochemistry, 144, 553 – 562.

ANDERSON, M.E. and MEISTER, A. (1983) Transport and direct utilization of γ-glutamylcyst(e)ine foe glutathione synthesis. Proceedings of the National Academy of Science, 80, 707 – 711.

ARCAMONE, F., FRANCESCHI, G., PENCO, S. and SELVA, A. (1969) Adriamycin (14-hydroxydaunomycin) a novel antitumour antibiotic. Tetrahedron Letters, 13, 1007 – 1010.

ARRICK, B.A. and NATHAN, C.F. (1984) Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer Research, 44, 4224 – 4232.

ARRICK, B.A., NATHAN, C.F., GRIFFITH, O.W. and COHN, Z.A. (1982) Glutathione depletion sensitizes tumour cells to oxidative cytolysis. The Journal of Biological Chemistry, 257, 1231 – 1237.

AUST, S.D. and SVINGEN, B.A. (1982) The role of iron in the enzymatic lipid peroxidation. In Free Radicals in Biology, ed. Pryor, W. A. 5, pp. 1 – 28.

AVERBUCH, S.D., GAUDIANO, G., KOCH, T.H. and BACHUR, N.R. (1986) Doxorubicin-induced skin necrosis in the swine model : Protection with a novel radical dimer. Journal of Clinical Oncology, 4, 88 – 94.

BACHMANN, E. and ZBINDEN, G. (1979) Effect of doxorubicin and rubidazone on respiratory function and calcium transport in rat heart mitochondria, Toxicology Letters, 3, 29 – 34.

BACHUR, N.R., GORDEN, S.L. and GEE, M.V. (1977) Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. Molecular Pharmacology, 13, 901 – 910.

BACHUR, N.R., GORDON, S.L. and GEE, M.V. (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Research, 38, 1745 – 1750.

BACHUR, N.R., GORDON, S.L., GEE, M.V. and et al. (1979) NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proceedings of the National Academy of Science, 76, 954 – 957.

BACHUR, N.R., STEELE, M., MERIWETHER, W.D. and HILDEBRAND, R.C. (1976) Cellular pharmacodynamics of several anthracycline antibiotics. Journal of Midicinal Chemistry, 19, 651 – 654.

BARLOGIE, B., GOHDE, W., JOHNSTON, D.A. and et al. (1978) Determination of ploidy and proliferative characteristics of human solid tumours by pulse cytophotometry. Cancer Research, 38, 3333 – 3339.

BARRANCO, S. C. (1986) Cellular and molecular effects of adriamycin on dividing and nondividing cells. In Cell Cycle Effects of Drugs, ed. DETHLEFSEN, L. A. Ch. 9, PP. 251 – 268.

BARRANCO, S.C. (1975) Review of the survival and cell kinetics effects of adriamycin (NSC-123127) on the mammalian cells. Cancer Chemotherapy Reports, 6, 147 – 152.

BARRANCO, S.C., GERNER, E.W., BURK, K.H. and HUMPHREY, R.M. (1973) Survival and cell kinetics effects of adriamycin on mammalian cells. Cancer Research, 33, 11 – 16.

BARRANCO, S.C., HO, D.H.W., DREWINKO, B., ROMSDAHL, M.M. and HUMPHREY, R.M. (1972) Differential sensitivities of human melanoma cells grown *in vitro* to arabinosylcytosine. Cancer Research, 32, 2733 – 2736.

BARRANCO, S.C., NOVAK, J.K. and HUMPHREY, R.M. (1975) Studies on recovery from chemically induced damage in mammalian cells. Cancer Research, 35, 1194 – 1204.

BARRANCO, S.C., TOWNSEND, C.M., COSTANZI, J.J. and et al. (1982) Use of 1,2 : 5,6-dianhydrogalactitol in studies on cell kinetics-directed chemotherapy schedules in human tumours *in vivo*. Cancer Research, 42, 2899 – 2905.

BATES, D.A. and WINTERBOURN, C.C. (1982) Deoxyribose breakdown by the adriamycin semiquinone and H_2O_2 : evidence for hydroxyl radical participation. FEBS Letters, 145, 137 – 142.

BAUER, W. and VINOGRAD, J. (1970) Interaction of closed circular DNA with intercalative dyes. II. The free energy of superhelix formation in SV40 DNA. Journal of Molecular Biology, 47, 419 – 435.

BECKETT, G.J. and HAYES, J.D. (1987) Plasma gutathione S-transferase measurements and liver disease in man. Journal of Clinical Biochemistry and Nutrition, 2, 1 – 24.

BELLAMY, W.T., DALTON, W.S., KAILEY, J.M. and et al. (1988 a) Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and

association with drug accumulation and DNA damage. Cancer Research, 48, 6303 – 6308.

BELLAMY, W.T., DORR, R.T., DALTON, W.S. and ALBERTS, D.S.(1988 b) Direct relation of DNA lesions in multidrug-resistant human myeloma cells to intracellular doxorubicin concentration. Cancer Research, 48, 6360 – 6364.

BELLI, J.A. and PIRO, A.J. (1977) The interaction between radiation and adriamycin damage in mammalian cells. Cancer Research, 37, 1624 – 1630.

BENJAMIN, J.L. (1981) Cell transformation by polyoma virus. In Cancer, ed. BURCHENAL, J. H. and OETTGEN, H.F. Vol. 1, pp. 101 – 110. New york : Grune and stratton.

BENJAMIN, R.S., RIGGS, C.E. and BACHUR, N.R. (1973) pharmacokinetics and metabolism of adriamycin in man. Clinical Pharmacology and Therapeutics,14, 592 – 600.

BENJAMIN, R.S., RIGGS, C.E. and BACHUR, N.R. (1977) Plasma Pharmacokinetics of adriamycin and its metabolites in humans with normal hepatic and renal function. Cancer Research, 37, 1416 – 1420.

BENSON, J.R. and HARE, P.E. (1975) O-phthalaldehyde: Fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrine. Proceedings of the National Academy of Science, 72, 619 – 622.

BERALDO, H., GARNIER-SUILLEROT, A., TOSI, L. and LAVELLE, F. (1985) Iron(III)-adriamycin and iron(III)-daunomycin complexes: Physiochemical characteristics, interaction with DNA, and antitumour activity. Biochemistry, 24, 284 – 289.

BERRIGAN, M.J., MARINELLO, A.J., PAVELIC, Z., WILLIAMS, C.J., STRUCK, R.F. and GURTOO, H.L. (1982) Protective role of thiols in cyclophosphamide-induced urotoxicity and depression of hepatic drug metabolism. Cancer Research, 42, 3688 – 3695.

BETT, W. R. (1957) Historical aspects of cancer. In Cancer, ed. RAVEN, R. W. Vol. 1. Part 1. pp. 1 – 5. London : Butterworth and Co. LTD.

BHUYAN, B.K., McGoVERN, J.P. and CRAMPTON, S.L. (1981) Intracellular uptake of 7-con-O-methylonogarol and adriamycin by cells in culture and its relationship to cell survival. Cancer Research, 41, 882 – 887.

BIAGLOW, J.E., VARNES, M.E., CLARK, E.P. and EPP, E.R. (1983) The role of thiols in cellular response to radiation and drugs. Radiation Research, 95, 437 – 455.

BIEDLER, J. L., RIEHM, H., PETERSON, R. H. F. and SPENGLER, B. A. (1975) Membrane-medicated drug resistance and phenotypic reversion to normal growth behavior of Chinese hamster cells. Journal of the National Cancer Institute, 55, 671 – 680.

BILLINGHAM, M.E., BRISTOW, M.R., GLATSTEIN, E., MASON, J.W., MASEK, M.A. and DANIELS, J.R. (1977) Adriamycin cardiotoxicity: Endomyocardial biopsy evidence of enhancement by irradiation. American Journal of Surgical Pathology, 1, 17 – 22.

BIRD, R.P., HUNG, S.S.O., HADLEY, M. and DRAPER, H.H. (1983) Determination of malonaldehyde in biological materials by high–pressure liquid chromatography. Analytical Biochemistry, 128, 240 – 244.

BORN, R. and EICHHOLTZ-WIRTH, H. (1981) Effect of different physiological conditions on the action of adriamycin on Chinese hamster cells *in vitro*. British Journal of Cancer, 44, 241 – 246.

BRENNER, D.E., GALLOWAY, S., COPPER, J., NOONE, R. and HANDE, K.R. (1985) Improved high-performance liquid chromatography assay of doxorubicin: detection of circulating aglycones in human plasma and comparison with thin-layer chromatography. Cancer Chemotherapy and Pharmacology, 14, 139 – 145.

BRISTOW, M.R. (1982) Evaluation and prevention of anthracycline cardiotoxicity. In Anthracycline Cardiotoxicity, 37, 627 – 637. Academic Press.

BRISTOW, M.R., BILLINGHAM, M.E., MASON, J.W. and DANIELS, J.R. (1978) Clinical spectrum of anthracycline antibiotic cardiotoxicity. Cancer Treatment Reports, 62, 873 – 879.

BROCKMEIER, F.K., ROSLAND, G.A. and FINNE, P.H. (1984) Cardiomyopathy induced by anthracycline-derivatives. Acta Paediatrica Scandinavica, 73, 387 – 391.

BROWN, F.F. and CAMPBELL, I.D. (1980) NMR studies of red cells. Philosophical Transactions- Royal Society London, B289, 395 – 406.

BROWN, F.F., CAMPBELL, I.D., KUCHEL, P.W. and RABENSTEIN, D.C. (1977) Human erythrocyte metabolism studies by ^{1}H spin echo NMR. FEBS Letters, 82, 12 – 16.

BULKLEY, G.B. (1987) Free radical-mediated reperfusion injury: A sselsctive review. British Journal of Cancer, 55, 66 – 73.

BURKE, T.G. and TRITTON, T.R. (1985) Structural basis of anthracycline selectivity for unilamellar phosphatidylcholine vesicles: an equilibrium binding study. Biochemistry, 24, 1768 – 1776.

BURTON, N.K. and AHERNE, G.W. (1986) Sensitive measurement of glutathione using isocratic high-performance liquid chromatography with fluorescence detection. Journal of Chromatography, 382, 253 – 257.

CAIRO, M.S., SIEGEL, S., ANAS, N. and SENDER, L. (1989) Clinical trial of continuous infusion verapamil, bolus vinblastine, and continuous infusion VP-16 in drug-resistant pediatric tumour. Cancer Research, 49, 1063 –1066.

CALABRESI, P. and PARKS, R.E. (1985) Antiproliferative agents and drugs used for immunosuppression. In The Pharmacological Basis of Therapeutics, ed. GILMAN,

A.G., GOODMAN, L.S., RALL, T.W. and MURAD, F. Seventh ed. Ch. 55, pp. 1283 – 1285. New york : Macmillan publishing Co.

CALMAN, K.C., SMYTH, J.F. and TATTERSALL, M.H.N. (1980) Basic Principle of Cancer Chemotherapy. London. The Macmillan Press LTD.

CAPRANICO, G., DASDIA, T. and ZUNINO, F. (1986) Comparison of doxorubicin-induced DNA damage in doxorubicin-sensitive and -resistant P388 murine leukemia cells. International Journal of Cancer, 37, 227 – 231.

CAPRANICO, G., ISABELLA, P.D., PENCO, S., TINELLI, S. and ZUNINO, F. (1989) Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin, and 4-demethyl-6-deoxydoxorubicin in murine leukemia P388 cells. Cancer Research, 49, 2022 – 2027.

CARMICHAEL, J., ADAMS, D.J., ANSELL, J. and WOLF, C.R. (1986) Glutathione and glutathione transferase levels in mouse granulocytes following cyclophosphamide administration. Cancer Research, 46, 725 – 739.

CENTER, M. S. (1983) Evidence that adriamycin resistance in chinese hamster lung cells is regulated by phosphorylation of a plasma membrane glycoprotein. Biochemical and Biophysical Research Communications, 115, 159 – 166.

CENTER, M.S. (1985) Mechanisms regulating cell resistance to adriamycin : Evidance that drug accumulation in resistant cells is modulated by phosphorylation of a plasma membrane glycoprotein. Biochemical Pharmacology, 34, 1471 – 1476.

CHAUFFERT, B., MARTIN, M.S., HAMMANN, A., MICHEL, M.F. and MARTIN, F. (1986) Amiodarone-induced enhancement of doxorubicin and 4'deoxydoxorubicin cytotoxicity to rat colon cancer cell *in vitro* and *in vivo*. Cancer Research, 46, 825 – 830.

CHAUFFERT, B., REY, D., COUDERT, B., DUMAS, M. and MARTIN, F. (1987) Amiodarone is more efficient than verapamil in reversing resistance to anthracyclines in tumour cells. British Journal of Cancer, 56, 119 – 122.

CHEN, C., KNOP, R.H. and COHEN, J.S. (1983) Adriamycin inhibits the B to Z transition of poly (dGm^{5}dC). poly (dGm^{5}dC). Biochemistry, 22, 5468 – 5471.

COHN, V.H. and LYLE, J. (1966) A fluorometric assay for glutathione. Analytical Biochemistry, 14, 434 – 440.

COLOFIORE, J.R., ARA, G., BERRY, D. and BELLI, J.A. (1982) Enhanced survival of adriamycin-treated Chinese hamster cells by 2-deoxy-D-glucose and 2,4-dinitrophenol. Cancer Research, 42, 3934 – 3940.

CROOK, T.R., SOUHAMI, R.L., WHYMAN, G.D. and McLEAN, A.E.M. (1986) Glutathione depletion as a determinant of sensitivity of human leukemia cells to cyclophosphamide. Cancer Research, 46, 5035 – 5038.

CROOP, J.M., GROS, P. and HOUSMAN, D.E. (1988) Genetics of multidrug resistance. Journal of Clinical Investigation, 81, 1303 – 1309.

CUMMINGS, J. and McARDLE, C. S. (1986) Studies on the *in vivo* disposition of adriamycin in human tumours which exhibit different responces to the drug. British Journal of Cancer, 53, 835 – 838.

CUMMINGS, J., MERRY, S. and WILLMOTT, N. (1986) Disposition kinetics of adriamycin, adriamycinol and their 7–deoxyaglycones in AKR mice bearing a sub-cutaneously growing ridgway osteogenic sarcoma (ROS). European Journal of Cancer and Clinical Oncology, 22, 451 – 460.

DABROWIAK, J.C. (1980) Metal binding to antitumor antibiotics. In Metal Ions in Biological Systems, ed. SIGEL, H. Ch. 7 . Marcel Dekker, N.Y.

DALMARK, M. (1981) Characteristics of doxorubicin transport in human red blood cells. Scandinavian Journal of Clinical and Laboratory Investigation, 41, 633 – 639.

DALMARK, M. and HOFFMANN, E.K. (1983) Doxorubicin (Adriamycin) transport in Ehrlich ascites tumour cells: comparison with transport in human red blood cells. Scandinavian Journal of Clinical and Laboratory Investigation, 43, 241 – 248.

DALMARK, M. and STORM, H.H. (1981) A fickian diffusion transport process with features of transport catalysis: Doxorubicin transport in Human red blood cells. The Journal of General Physiology, 78, 349 – 364.

DAVIES, M.H., BIRT, D.F. and SCHNELL, R.C. (1984) Direct enzymatic assay for reduced and oxidized glutathione. Journal of Pharmacological Methods, 12, 191 – 194.

DECORTI, G., KLUGMANN, F.B., CANDUSSIO, L., FURLANI, A., SCARCIA, V. and BALDINI, L. (1989) Uptake of adriamycin by rat and mouse mast cells and correlation with histamine release. Cancer Research, 49, 1921 – 1926.

DEDRICK, R.L., MYERS, C.E., BUNGAY, P.M. and DEVITA, V.T. (1978) Phamacokinetic rationale for peritoneal drug administration in the treatment of ovarian cancer. Cancer Treatment Reports, 62, 1 – 11.

DETHLEFSEN, L.A. (1980) In quest of the quaint quiescent cells. In Radiation Biology in Cancer Research, ed. MEYN, R.E. and WITHERS, H.R. pp. 415 – 435. New York : Raven Press.

DiMARCO, A. (1975) Adriamycin (NSC- 123127) : mode and mechanism of action. Cancer Chemotherapy Reports, 6, 91 – 106.

DiMARCO, A. and ARCAMONE, F. (1975) DNA complexing antibiotics: Daunomycin, adriamycin and their derivatives, Arzneimittel-Forschung (Drug Research), 25, 368 – 375.

DORMANDY, T.L. (1988) In praise of peroxidation. The Lancet, 12. 1126 – 1128.

DOROSHOW, J.H. (1983) Effect of anthracycline antibiotics on oxygen radical formation in rat heart. Cancer Research, 43, 460 – 472.

DOROSHOW, J.H. and REEVES, J. (1981) Daunorubicin-stimulated reactive oxygen metabolism in cardiac sarcosomes. Biochemical pharmacology, 30, 259 – 262.

DOROSHOW, J.H., LOCKER, G.Y., BALDINGER, J. and MYERS, C.E. (1979) The effect of doxorubicin on hepatic and cardiac glutathione. Research Communications in Chemical Pathology and Pharmacology, 26, 285 –295.

DOROSHOW, J.H., LOCKER, G.Y., IFRIM, I. and MYERS, C.E. (1981) Prevention of doxorubicin cardiac toxicity in the mouse by n-acetylcysteine. Journal of Clinical Investigation, 68, 1053 – 1064.

DORR, R.T. and ALBERTS, D.S. (1982) Pharmacology of doxorubicin. In The Current Concept in the Use of Doxorubicin Chemotherapy, ed. JONES, S.E. pp. 3 – 20. U.S.A.

DUNNETT, C. W. (1955) A multiple composition procedure for comparing several treatments with a control. American Statistical Association Journal, 50, 1096 – 1121.

DURAND, R.E. and OLIVE, P.L. (1981) Flow cytometry studies of intracellular adriamycin in single cells *in vitro*. Cancer Research, 41, 3489 – 3494.

DUSRE, L., MIMNAUGH, E.G., MYERS, C.E. and SINHA, B.K. (1989) Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumour cells. Cancer Research, 49, 511 – 515.

ELLMAN, G.L. (1959) Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, 82, 70 – 77.

ERICKSON, L.C., BRADLEY, M.O., DUCORE, J.M., EWIG, R.A.G. and KOHN, K.W. (1980) DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. Proceedings of the National Academy of Science, 77, 467 – 471.

ESTERBAUER, H. (1982) Aldehydeic products of lipid peroxidation. In Free Radicals Lipid Peroxidation and Cancer, ed. SLATER, T.F. and MCBRIEN, D. pp. 102 – 128. Academic Press.

EVANOCHKO, W.T., SAKAI, T.T., NG, T.C. and et al. (1984) NMR study of *in vivo* RIF-1 tumours. Analysis of perchloric acid extracts and identification of ^{1}H, ^{31}P and ^{13}C resonances. Biochemica et Biophysica acta, 805, 104 – 116.

EVANS, C.G., BODELL, W.J., TOKUDA, K., DOANE-SETZER, P. and SMITH, M.T. (1987) Glutathione and related enzymes in rat brain tumour cell resistance to 1,3- bis(2-chloroethyl)-1-nitrosourea and nitrogen mustard. Cancer Research, 47, 2525 – 2530.

EVANS, F.E. and KAPLAN, N.O. (1977) ^{31}P nuclear magnetic resonance studies of Hela cells. Proceedings of the National Accademy of Science, 74, 4909 – 4913.

FABREGAT, I., SATRUSTEGUI, J. and MACHADO, A. (1984) Interaction with protein SH groups could be involved in adriamycin cardiotoxicity. Biochemical Medicine, 32, 289 – 295.

FAHEY, R.C., NEWTON, G.L., DORIAN, R. and KOSOWER, E.M. (1981) Analysis of biological thiols: Quantitative determination of thiols at the picomole level based upon derivatization with monobromobimanes and separation by cation-exchange chromatography. Analytical Biochemistry, 111, 357 – 365.

FAIRCHILD, C.R., IVY, S.P., KAO-SHAN, C-S. and et al. (1987) Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells. Cancer Research, 47, 5141 – 5148.

FERRERO, M.E., FERRERO, E., GAJA, G. and BERNELLI-ZAZZERA, A. (1975) Adriamycin: Energy metabolism and mitochondrial oxidations in the heart of treated rabbits. Biochemical Pharmacology, 25, 125 – 130.

FIDLER, I.J. and HART, I.R. (1982) Principles of cancer biology : Biology of cancer metastases. In Cancer Principles and Practice of Oncology, ed. DeVITA, V.T. HELLMAN, S. and ROSENBERG, S.A. Lippincott.

FOLKERS, K., LIU, M., WATANABE, T. and PORTER, T.H. (1977) Inhibition by adriamycin of the mitochondrial biosynthesis of coenzyme Q_{10} and implication for the cardiotoxicity of adriamycin in cancer patients. Biochemical and Biophysical Research Communication, 77, 1536 – 1542.

FORMELLI, F., ZEDECK, M.S., STERNBERG, S.S. and PHILIPS, F.S. (1978) Effects of adriamycin on DNA synthesis in mouse and rat heart. Cancer Research, 38, 3286 – 3292.

FRIDOVICH, I. (1978) The biology of oxygen radicals. Science, 201, 875 – 880.

FRIDOVICH, S.E. and PORTER, N.A. (1981) Oxidation of archidonic acid in micelles by superoxide and hydrogen peroxide. The Journal of Biological Chemistry, 256, 269 – 265.

GAETJENS, E.C., CHEN, P. and BROOME, J.D. (1984) L1210 (A) mouse lymphoma cells depleted of glutathione with L-Buthionine-S-R-Sulfoximine proliferate in tissue culture. Biochemical and Biophysical Research Communications, 123, 626 – 632.

GARMAN, D. and CENTER, M. S. (1982) Alterations in cell surface membranes in chinese hamster lung cells resistant to adriamycin. Biochemical and Biophysical Reasearch Communications, 105, 157 – 163.

GARMAN, D., ALBERS, L. and CENTER, M. S. (1983) Identification and characterization of a plasma membrane phosphoprotein which is present in chinese hamster lung cells resistant to adriamycin. Biochemical Pharmacology, 32, 3633 – 3637.

GHALIOUNGUI, P. (1984) Malignancy in ancient Egypt. In IV Mediterranean Congress of Chemotherapy, ed. DAIKOS, G. K. and GIAMARELLOU, H. Vol. 4, Sec. 11, pp. 1068 – 1069. Rhodos and Greece : Chemioterapia.

GIANNI, L., CORDEN, B.J. and MYERS, C.E. (1983) The biochemical basis of anthracycline toxicity and antitumour activity. In Reviews in Biochemical Toxocology, ed. HODGSON, E., BEND, J.R. and PHILPOT, R.M. 5, pp. 1 – 82. New York. Elsevier Biomedical.

GIANNI, L., ZWEIER, J.L., LEVY, A, and MYERS, C.E. (1985) Characterization of the cycle of iron-mediated electron transfer from adriamycin to molecular oxygen. The Journal of Biological Chemistry, 260, 6820 – 6826.

GIGLI, M., RASOANAIVO, T.W.D., MILLOT, J-M. and et al. (1989) Correlation between growth inhibition and intranuclear doxorubicin and 4'-deoxy-4'-iododoxorubicin quantitated in living K562 cells by microspectrofluorometry. Cancer Research, 49, 560 – 564.

GOORMAGHTIGH, E. and RUYSSCHAERT, J.M. (1984) Anthracycline glycoside-membrane interactions. Biochimica et Biophysica acta, 779, 271 – 288.

GOORMAGHTIGH, E., CHATELAIN, P., CASPERS, J. and RUYSSCHAERT, J.M. (1980 a) Evidence of a complex between adriamycin derivatives and cardiolipin: Possible role in cardiotoxicity. Biochemical Pharmacology, 29, 3003 – 3010.

GOORMAGHTIGH, E., CHATELAIN, P., CASPERS, J. and RUYSSCHAERT, J.M. (1980 b) Evidence of specific complex between adriamycin and negatively-charged phospholipid. Biochimica et Biophysica acta, 597, 1 – 14.

GRAVES, D.E. and KRUGH, T.R. (1983) Adriamycin and daunorubicin bind in a cooperative manner to deoxyribonucleic acid. Biochemistry, 22, 3941 – 3947.

GRIFFITH, O.W. (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Analytical Biochemistry, 106, 207 – 212.

GRIFFITH, O.W. and MEISTER, A. (1978) Differential inhibition of glutamine and γ-glutamyl cysteine synthetase by α-alkyl analogs of methionine sulfoximine that induce convulsion. Journal of Biological Chemistry, 253, 2333 – 2338.

GRIFFITH, O.W. and MEISTER, A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl-homocysteine sulfoximine). The Journal of Biological Chemistry, 254, 7558 – 7560.

GRIFFITH, O.W. and TATE, S.S. (1980) The apparent glutathione oxidase activity of γ-glutamyl transpeptidase. The Journal of Biological Chemistry, 255, 5011 – 5014.

GRIFFITH, O.W., ANDERSON, M.E. and MEISTER, A. (1979) Inhibition of glutathione biosynthesis by prothionine sulfoximine (S-n-propyl-homocysteine sulfoximine), a selective inhibitor of γ-glutamyl cysteine synthetase. Journal of Biological Chemistry, 254, 1205 – 1210.

GRIFFITH, O.W., BRIDGES, R.J. and MEISTER, A. (1981) Formation of γ-glutamylcyst(e)ine *in vivo* is catalyzed by γ-glutamyl transpeptidase. Proceedings of the National Academy of Science, 78, 2777 – 2781.

GRISWOLD, D.P., CORBETT, T.H. and SCHABEL, F.M. (1981) Clonogenicity and growth of experimental tumours in relation to developing resistance and therapeutic failure. Cancer Treatment Reports, 65, 51 – 54.

GUTTERIDGE, J.M.C. (1984) Ferrous ion-EDTA-stimulated phospholipid peroxidation. Biochemical Journal, 224, 697 – 701.

HALL, R.L., WILKE, W.L. and FETTMAN, M.J. (1986) The progression of adriamycin-induced nephrotic syndrome in rats and the effect of captopril. Toxocology and Applied Pharmacology, 82, 164 – 174.

HAMADA, H., HAGIWARA. K., NAKAJIMA, T. and TSURUO, T. (1987) Phosphorylation of the M_r 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumour cells: Effects of verapamil, trifluoperazine, and phorbol esters. Cancer Research, 47, 2860 – 2865.

HAMILTON, T.C., WINKER, M.A., LOUIE, K.J. and et al. (1985) Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochemical Pharmacology, 34, 2583 – 2586.

HARDING, J.J. (1970) Free and protein-pound glutathione in normal and cataractous human lenses. Biochemical Journal, 117, 957 – 960.

HARRIS, P.A., GARAI, A.S. and VALENZUELA, M.A. (1975) Reduction of doxorubicin (adriamycin) bone marrow toxicity. Journal of Pharmaceutical Sciences, 64, 1474 – 1576.

HARRIS, R.N. and DOROSHOW, J.H. (1985) Effect of doxorubicin-enhanced hydrogen peroxide and hydroxyl radical formation on calcium sequestration by cardiac sarcoplasmic reticulum. Biochemical and Biophysical Research Communications, 130, 739 – 745.

HARVEY, P.R.C., ILSON, R.G. and STRASBERG, S.M. (1989) The simultaneous determination of oxidized and reduced glutathiones in liver tissue by ion pairing reverse phase high performance liquid chromatography with a coulometric electrochemical detector. Clinica Chimica Acta, 180, 203 – 212.

HAZELTON, G.A. and LANG, C.A. (1980) Glutathione contants of tissues in the aging mouse. Biochemical Journal, 188, 25 – 30.

HENRY, N., FANTINE, E.O., BOLARD, J. and GARNIER-SUILLEROT, A. (1985) Interaction of adriamycin with negatively charged model membranes: Evidence of two types of binding sites. Biochemistry, 24, 7085 – 7092.

HEPPNER, G.H., DEXTER, D.L., DENUCCI, T., MILLER, F.R. and CALABRESI, P. (1978) Heterogeneity in drug sensitivity among tumour cell subpopulations of a single mammary tumour. Cancer Reseach, 39, 3758 – 3763.

HERMAN, E.H., FERRANS, V.J., MYERS, C.E. and VAN VLEET, J.F. (1985) Comparison of the effectiveness of (±)-1,2-bis(3,5-dioxopiperazinyl-l-yl)propan (ICRF-187) and n-acetylcysteine in preventing chronic doxorubicin cardiotoxicity in beagles. Cancer Research, 45, 276 – 281.

HIBBS, J.B., TAINTOR, R.R. and VAVRIN, Z. (1984) Iron depletion: Possible cause of tumor cell cytotoxicity induced by activated macrophages. Biochemical and Biophysical Research Communications, 123, 716 – 723.

HINDENBURG, A.A., BAKER, M.A., GLEYZER, E., STEWART, V.J., CASE, N. and TAUB, R.N. (1987) Effect of verapamil and other agents on the distribution of anthracyclines and on reversal of drug resistance. Cancer Research, 47, 1421 – 1425.

HINO, Y., YOO, S.B., KAJIYAMA, K., KAGIYAMA, A. and OGURA, R. (1985) Effect of riboflavin-butyrate on cardiac glutathione reductase affected by adriamycin. Journal of Nutritional Science and Vitaminology, 31, 139 – 145.

HISSIN, P.J. and HILF, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry, 74, 214 – 226.

HIXON, S. G., ELLIS, C. N. and DAUGHERTY, J. P. (1981) Heart mitochondrial DNA synthesis: preferential inhibition by adriamycin. Journal of Molecular and Cellular Cardiology, 13, 855 – 860.

HOPKINS, F.G. (1921) On an autoxidisable constituent of the cell. Biochemical Journal, 15, 286 – 305.

HUBER, K.R., SCHMIDT, W.F., THOMPSON, E.A., FORSTHOEFEL, A.M., NEUBERG, R.W. and ETTINGER, R.S. (1989) Effect of verapamil on cell cycle transit and c-*myc* gene expression in normal and malignant murine cells. British Journal of Cancer, 59, 714 – 718.

HYNDS, S.A. (1986) Cancer Chemotherapy: Use of Low Density Lipoproteins as Targeting Vehicles for Treatment. PhD Thesis, University of Glasgow.

INABA, M., KOBAYASHI, H., SAKURAI, Y. and JOHNSON, R. K. (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Research, 39, 2200 – 2203.

ISSELS, R.D. and NAGELE, A. (1989) Promotion of cystine uptake, increase of glutathione biosynthesis, and modulation of glutathione status by S-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR-2721) in Chinese hamster cells. Cancer Research, 49, 2082 – 2086.

ISSELS, R.D., NAGELE, A., ECKERT, K-G. and WILMANNS, W. (1988) Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. Biochemical Pharmacology, 37, 881 – 888.

IYER, G.Y.N. (1959) Free amino acids in leukocytes from normal and leukemic cells. Journal of Laboratory and Clinical Medicine, 54, 229 – 231.

JAIN, S.K. (1984) The accumulation of malonyldialdehyde, a product of faty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. Journal of Biological Chemistry, 259, 3391 – 3394.

JENSEN, R.A. (1986) Doxorubicin cardiotoxicity: Contractile changes after long-term treatment in the rat. The Journal of Pharmacology and Experimental Therapeutics, 236, 197 – 203.

JONES, C., BURTON, M.A. and GRAY, B.N. (1989) Enhanced *in vivo* activity of adriamycin incorporated into controlled release microspheres. British Journal of Cancer, 59, 743 – 745.

JONES, R.L., LANIER, A.C., KEEL, R.A. and WILSON, W.D. (1980) The effect of ionic strength on DNA-ligand unwinding angles for acridines and quinoline derivatives. Nucleic Acids Research, 8, 1613 – 1624.

JORDAN, J., DOHERTY, M.D. and COHEN, G.M. (1987) Effect of glutathione depletion on the cytotoxicity of agents toward a human colonic tumour cell line. British Journal of Cancer, 55, 627 – 631.

KABLE, E.P.W., FAVIER, D. and PARSONS, P.G.(1989) Sensitivity of human melanoma cells to L-dopa and DL-buthionine (S,R)-sulfoximine. Cancer Research, 49, 2327 – 2331.

KALLINOWSKI, F., RUNKEL, S., FORTMEYER, H.P., FOERSTER, H. and VAUPEL, P. (1987) L-glutamine: a major substrate for tumour cells *in vivo* ? Journal of Cancer Research and Clinical Oncology, 113, 209 – 215.

KALLINOWSKI, F., VAUPEL, P., RUNKEL, S. and et al. (1988) Glucose uptake, lactate release, ketone body turnover, metabolic micromilieu, and pH distributions in human breast cancer xenografts in nude rats. Cancer Research, 48, 7264 – 7272.

KALYANARAMAN, B., SEALY, R.C. and SINHA, B.K. (1984) An electron spin resonance study of the reduction of peroxides by anthracycline semiquinones. Biochimica et Biophysica acta, 799, 270 – 275.

KAPLOWITZ, N. (1977) Interaction of azathioprine and glutathione in the liver of the rat. The Journal of Pharmacology and Experimental Therapeutics, 200, 479 – 486.

KAPLOWITZ, N., KUHLENKAMP, J., GOLDSTEIN, L. and REEVE, J. (1980) Effet of salicylates and phenobarbital on hepatic glutathione in the rat. The Journal of Pharmacology and Experimental Therapeutics, 212, 240 – 245.

KAPPUS, H., MUIRAWAN, H. and SCHEULEN, M.E. (1980) *In vivo* studies on adriamycin induced lipid peroxidation and effect of ferrous ions. In Mechanism of Toxicity and Hazard Evaluation, ed. HOLMSTEDT, B., LAUWERYS, R., MERICER, M. and ROBERFROID, M. pp. 635. Elsevier / North Holland.

KARCZMAR, G.S. and TRITTON, T.R. (1979) The interaction of adriamycin with small unilamellar vesicle liposomes. Biochimica et Biophysica acta, 557, 306 – 319.

KAYE, S.B. and MERRY, S. (1985) Tumour cell resistance to anthracyclines – A review. Cancer Chemotherapy and Pharmacology, 14, 96 – 103.

KENNEDY, K.A., SIEGFRIED, J.M., SARTORELLI, A.C. and TRITTON, T.R. (1983) Effect of anthracyclines on oxygenated and hypoxic tumor cells. Cancer Research, 43, 54 – 59.

KESSEL, D. and WILBERDING, C. (1984) Mode of action of calcium antagonists which alter anthracycline resistance. Biochemical Pharmacology, 33, 1157 – 1160.

KESSEL, D. and WILBERDING, C. (1985) Anthracycline resistance in P388 murine leukemia and its circumvention by calcium antagonists. Cancer Research, 45, 1687 – 1691.

KETTERER, B., BEALE, D. and MEYER, D. (1982) The structure and multiple functions of glutathione transferases. Biochemical Society Transactions, 10, 82 – 84.

KIM, S.H. and KIM, J.H. (1972) Lethal effects of adriamycin on the division cycle of Hela cells. Cancer Research, 32, 323 – 325.

KIMLER, B.F. and CHENG, C.C. (1982) Comparison of the effects of dihydroxy-anthraquinone and adriamycin on the survival of cultured Chinese hamster cells. Cancer Research, 42, 3631 – 3636.

KLEIN, P. and ROBBINS, E. (1970) An altrasensitive assay for soluble sulfhydryl and its application to the study of glutathione levels during the Hela life cycle. The Journal of Cell Biology, 46, 165 – 168.

KNIGHT, J.A., PIEPER, R.K. and McCLELLAN, L. (1988) Specificity of the thiobarbituric acid reaction : Its use in studies of lipid peroxidation. Clinical Chemistry, 34, 2433 – 2438.

KNOX, J.H., DONE, J.N., FELL, A.F., GILBERT, M.T., PRYDE, A. and WALL, R.A. (1978) High-Performance Liquid Chromatography. 6, pp. 52 – 67. Edinburgh University Press.

KOIVUSALO, M. and UOTILA, L. (1974) Enzymic method for the quantitative determination of reduced glutathione. Analytical Biochemistry, 59, 34 – 45.

KORNBRUST, D. J. and MAVIS, R.D. (1980) Microsomal lipid peroxidation : characterization of the role of iron and NADPH. Molecular Pharmacology, 17, 400 – 407.

KOSOWER, E.M. (1976) Chemical properties of glutathione. In Glutathione: Metabolism and Function, ed. ARIAS, I.M. and JAKOBY, W.B. Vol. 6, Ch. 1. New York : Raven Press.

LAMPIDIS, T.J., JOHNSON, L.V. and ISRAEL, M. (1981) Effect of adriamycin on rat heart cells in culture: Increased accumulation and nucleoli fragmentation in cardiac muscle v. non-muscle cells. Journal of Molecular and Cellular Cardiology, 13, 913 – 924.

LARSSON, A., ORRENIUS, S., HOLMGREN, A. and MANNERVIK, B. (1983) Metabolism and transport of glutathione and other γ-glutamyl compounds. In Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects. pp. 1 – 22. New York : Raven.

LEDWOZYW, A., MICHALAK, J., STEPIEN, A. and KADZIOLKA, A. (1986) The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. Clinica Chimica Acta, 155, 275 – 284.

LEE, F.Y.F., ALLALUNIS-TURNER, M.J. and SIEMANN, D.W. (1987) Depletion of tumour *versus* normal tissue glutathione by buthionine sulfoximine. British Journal of Cancer, 56, 33 – 38.

LEGHA, S.S., BENJAMIN, R.S., MACKAY, B. and et al. (1982) Reduction of doxorubicin cardiotoxicity by prolonged continuous intravenous infusion. Annals of Internal Medicine, 96, 133 – 139.

LEPAGE, G.A., JUNGA, I.G. and BOWMAN, B. (1964) Biochemical and carcinostatic effects of 2'-deoxythioguanosine. Cancer Research, 24, 835 – 840.

LEVINE, S. (1960) Effect of manipulation on 32P loss from tissue culture cells. Experimental Cell Research, 19, 220 – 227.

LEVITT, P.M., GURALINCK, E.S., KAGAN, A.R. and GILBERT, H. (1979) The Cancer Reference Book. Direct and Clear Answer to Everyone's Questions. New York and London : Paddington press LTD.

LINDON, J.C. (1986) NMR spectroscopy: analytical applications from chemistry to the clinic. Journal of Pharmaceutical and Biomedical Analysis, 4, 137 – 145.

LLESUY, S.F., MILEI, J., MOLINA, H., BOVERIS, A. and MILEI, S. (1985) Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4'- epiadriamycin in mice. Tumori, 71, 241 – 249.

LOUIE, K.G., HAMILTON, T.C., WINKER, M.A. and et al. (1986) Adriamycin accumulation and metabolism in adriamycin-sensitive and resistant human ovarian cancer cell lines. Biochemical Pharmacology , 35, 467 – 472.

LOWN, J.W., SIM, S., MAJUMDAR, K.C. and CHANG, R. (1977) Strand scission of DNA by bound adriamycin and daunorubicin in the presence of reducing agents. Biochemical and Biophysical Research Communications, 76, 705 – 710.

LUNTE, S.M. and KISSINGER, P.T. (1984) The use of liquid chromatography with dual-electrode electrochemical detection in the investigation of glutathione oxidation during benzene metabolism. Journal of Chromatography, 317, 579 – 588.

MAILER, K. and PETERING, D.H. (1976) Inhibition of oxidative phosphorylation in tumor cells and mitochondria by daunomycin and adriamycin. Biochemical Pharmacology, 25, 2085 – 2089.

MALATESTA, V., PENCO, S., SACCHI, N., VALENTINI, L., VIGEVANI, A. and ARCAMONE, F. (1984) Electrochemical deglycosidation of anthracyclines: stereoelectronic requirements. Canadian Journal of Chemistry, 62, 2845 – 2850.

MANFAIT, M. and THEOPHANIDES, T. (1983) Fourier transform infrared spectra of cells treated with the drug adriamycin. Biochemical and Biophysical Research Communications, 116, 321 – 326.

MANFAIT, M., ALIX, A.J.P., JEANNESSON, P., JARDILLIER, J. and THEOPHANIDES, T. (1982) Interaction of adriamycin with DNA as studied by resonance raman spectroscopy. Nucleic Acids Research, 10, 3803 – 3816.

MANNERVIK, B. (1985) The isoenzyme of glutathione transferase. In Advances in Enzymology, ed. MEISTER, A. Vol. 57, PP. 357 – 417. New york. John Wiley and Sons.

MARKLUND, S.L., WESTMAN, N.G., LUNDGREN, E. and ROOS, G. (1982) Copper- and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Research, 42, 1955 – 1961.

MARSH, W. and CENTER, M. S. (1985) *In vitro* phosphorylation and the identification of multiple protein changes in membranes of chinese hamster lung cells resistant to adriamycin. Biochemical Pharmacology, 34, 4180 – 4184.

MASON, R.P. (1979) Free radical metabolites of forign compounds and their toxocologic significance. Reviews in Biochemical Toxocology, 2, 151 – 200.

MAY, G.L., WRIGHT, L.C., HOLMES, K.T. and et al. (1986) Assignment of methylene proton resonances in NMR spectra of embryonic and transformed cells to plasma membrane triglyceride. The Journal of Biological Chemistry, 261, 3048 – 3053.

McFALLS, E.O., PAULSON, D.J., GILBERT, E.F. and SHUG, A.L. (1986) Carnitine protection against adriamycin-indused cardiomyopathy in rats. Life Sciences, 38, 497 –505.

McKAY, C.N.N., BROWN, D.H., REGLINSKI, J., SMITH, W.E., CAPELL, H. and STURROCK, R.D. (1986) Changes in glutathione in intact erythrocytes during incubation with penicillamine as detected by ^{1}H spin echo NMR spectroscopy. Biochimica et Biophysica acta, 888, 30 – 35.

McQUAID, S., McCANN, S., DALY, P., LAWLOR, E. and HUMPHRIES, P. (1989) Observations on the transcriptional activity of the glutathione-S-transferase π gene in human haematological malignancies and in the peripheral leucocytes of cancer patients under chemotherapy. British Journal of Cancer, 59, 540 – 543.

MEIJER, C., MULDER, N.H., TIMMER-BOSSCHA, H., ZIJISTRA, J.G. and DE VRIES, E.G.E. (1987) Role of free radicals in an adriamycine-resistant human small cell lung cancer cell line. Cancer Research, 47, 4613 – 4617.

MEISTER, A. (1974) Glutathione synthesis. In The Enzymes, ed. Boyer, P.D. Vol. 10, pp. 671 – 697. New York : Academic.

MEISTER, A. (1981) Metabolism and functions of glutathione. Trends in Biochemical Sciences, 6, 231 – 234.

MEISTER, A. (1983) Selective modification of glutathione metabolism. Science, 220, 472 – 477.

MEISTER, A. (1984) New aspect of glutathione biochemistry and transport: selective altration of glutathione metabolism. Federation Proceedings, 43, 3031 – 3042.

MEISTER, A. and ANDERSON, M. E. (1983) Glutathione. Annual Review of Biochemistry, 52, 711 – 760.

MERRY, S., COURTNEY, E.R., FETHERSTON, C.A., KAYE, S.B. and FRESHNEY, R.I. (1987) Circumvention of drug resistance in human non-small cell lung cancer *in vitro* by verapamil. British Journal of Cancer, 56, 401 – 405.

MERRY, S., FETHERSTIN, C.A., KAYE, S.B., FRESHNEY, R.I. and PLUMB, J.A. (1986) Resistance of human glioma to adriamycin *in vitro*: The role of membrane transport and its circumvention with verapamil. British Journal of Cancer, 53, 129 – 135.

MERRY, S., FLANIGAN, P., SCHLICH, E., FRESHNEY, R.I. and KAYE, S.B. (1989) Inherent adriamycin resistance in a murine tumour line: circumvention with verapamil and norverapamil. British Journal of Cancer, 59, 895 – 897.

MERRY, S., KAYE, S.B. and FRESHNEY, R.I. (1984) Cross–resistance to cytotoxic drugs in human glioma cell lines in culture. British Journal of Cancer, 50, 831 – 835.

MIKELENS, P. and LEVINSON, W. (1978) Metal ion participation in binding of daunomycinone, daunomycin, and adriamycin to nucleic acids. Bioinorganic Chemistry, 9, 441 – 452.

MIMNAUGH, E. G., KENNEDY, K. A., TRUSH, M. A. and SINHA, B. K. (1985) Adriamycin-enhanced membrane lipd peroxidation in isolated rat Nuclei. Cancer Research, 45, 3296 – 3304.

MIMNAUGH, E.G., SIDDIK, Z.H., DREW, R., SIKIC, V.I. and GRAM, T.E. (1979) The effects of α-tocopherol on the toxicity, disposition, and metabolism of adriamycin in mice. Toxocology and Applied Pharmacology, 49, 119 – 126.

MIMNAUGH, E.G., TRUSH, M.A., GINSBURG, E., HIROKATA, Y. and GRAM, T.E. (1981) The effect of adriamycin *in vitro* and *in vivo* on hepatic microsomal drug-metabolizing enzymes: role of microsomal lipid peroxidation. Toxicology and Applied Pharmacology, 61, 313 – 325.

MINOW, R.A., BENJAMIN, R.S. and GOTTLIEB, J.A. (1975) Adriamycin (NSC-123127) cardiomyopathy: An overview with determination of risk factors. Cancer Chemotherapy Reports, 6, 195 – 201.

MINOW, R.A., BENJAMIN, R.S., LEE, E.T. and GOTTLIEB, J.A. (1977) Adriamycin cardiomyopathy-risk factors. Cancer, 39, 1397 – 1402.

MITCHELL, J.B. and RUSSO, A. (1987) The role of glutathione in radiation and drug induced cytotoxicity. British Journal of Cancer, 55, 96 – 104.

MOMPARLER, R.L., KARON, M., SIEGEL, S.E. and AVILA, F. (1976) Effect of adriamycin on DNA, RNA and protein synthesis in cell-free systems and intact cells. Cancer Research, 36, 2891 – 2895.

MOORE, H.W. (1977) Bioactivation as a model for drug design bioreductive alkylation. Science, 197, 527 – 532.

MOORE, H.W. and CZERNIAK, R. (1981) Naturally occurring quinones as potential bioreductive alkylating agents. Medicinal Research Revews, 1, 249 – 280.

MOORE, M.A.S. (1975) Biology of the malignant cell. In Cancer Today, ed. KIRK, R.I. and McCULLAGH, P.J. Ch. 1, pp 13 – 26. Australia : The John Curtin School of Medical Research.

MOUNTFORD, C.E., GROSSMAN, G., REID, G. and FOX, R.M. (1982) Characterisation of transformed cells in tumours by ^{1}H NMR. Cancer Research, 42, 2270 – 2276.

MUINDI, J.R.F., SINHA, B.K., GIANNI, L. and MYERS, C.E. (1984) Hydroxyl radical production and DNA damage induced by anthracycline-iron complex. FEBS Letters, 172, 226 – 230.

MUINDI, J.R.F., SINHA, B.K., GIANNI, L. and MYERS, C.E. (1985) Thiol-dependent DNA damage produced by anthracycline-iron complexes: The structure-

activity relationships and molecular mechanisms. Molecular Pharmacology, 27, 356 – 265.

MURPHREE, S.A., CUNNINGHAM, L.S., HWANG, K.M. and SARTORELLI, A.C. (1976) Effects of adriamycin on surface properties of sarcoma 180 ascites cells. Biochemical Pharmacology, 25, 1227 – 1231.

MURRAY, G.I., BURKE, M.D. and EWEN, S.W.B. (1987) Glutathione isolation in benign and malignant human breast lesions. British Journal of Cancer, 55, 605 – 609.

MYERS, C. E., McGUIRE, W. P., LISS, R. H., IFRIM, I., GROTZINGER, K. and YOUNG, R. C. (1977) Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. Science, 197, 165 – 167.

MYERS, C.E., GIANNI, L., SIMONE, C.B., KLECKER, R. and GREENE, R. (1982) Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin iron complex. Biochemistry, 21, 1707 – 1713.

NAKATA, Y. and HOPFINGER, A.J. (1980 a) Predicted mode of intercalation of doxorubicin with dinucleotide dimers. Biochemical and Biophysical Research Communications, 95, 583 – 588.

NAKATA, Y. and HOPFINGER, A.J. (1980 b) An extended conformational analysis of doxorubicin, FEBS Letters, 117, 259 – 264.

NEIDLE, S. (1978) Interaction of daunomycin and related antibiotics with biological receptors, Topics in Antibiotic Chemistry, 2, 240 – 278.

NEIDLE, S. (1979) The molecular basis for the action of some DNA-binding drugs. Progress in Medicinal Chemistry, 16, 151 – 221.

NEWTON, G.L., DORIAN, R. and FAHEY, R.C. (1981) Analysis of biological thiols: Derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography. Analytical Biochemistry, 114, 383 – 387.

NICHOLSON, J.K., O'FLYNN, M.P. and SADLER, P.J. (1984) Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. Biochemical Journal, 217, 365 – 375.

NICKERSON, W.J., FALCONE, G., and STRAUSS, G. (1963) Studies on quinone-thioethers. I. Mechanism of formation and properties of thiodione. Biochemistry, 2, 537 – 543.

NIKI, E. (1987) Lipid antioxidants: How they may act in biological systems. British Journal of Cancer, 55, 153 – 157.

OGURA, R., TOYAMA, H., SHIMADA, T. and MURAKAMI, M. (1979) The role of ubiquinone coenzyme Q_{10} in preventing adriamycin–induced mitochondrial disorders in rat heart. Journal of Applied Biochemistry, 1, 325 – 335.

OKAMOTO, K. and OGURA, R. (1985) Effects of vitamins on lipid peroxidation and suppression of DNA synthesis induced by adriamycin in Ehrlich cells. Journal of Nutritional Science and Vitaminology, 31, 129 – 137.

OKANO, C., HOKAMA, Y. and CHOU, S.C. (1984) Inhibition of acetate incorporation into lipids by adriamycin. Research Communication in Chemical Pathology and Pharmacology, 46, 293 – 296.

OLSON, R.D., MAcDONALD, J.S., VAN BOXTEL, C.J. and et al. (1980) Regulatory rol of glutathione and soluble sulfhydryl groups in the toxicity of adriamycin. Journal of Pharmacological and Experimental Therapeutics, 215, 450 – 454.

OWENS, C.W.I. and BELCHER, R. V. (1965) A colorimetric micro-method for the determination of glutathione. Biochemical Journal, 94, 707 – 711.

OZOLS, R.F., LOCKER, G.Y., DOROSHOW, J.H., GROTZINGER, K.R., MYERS, C.E. and YOUNG, R.C. (1979) Pharmacokinetic of adriamycin and tissue penetration in murine ovarian cancer. Cancer Research, 39, 3209 – 3214.

PAN, S. and BACHUR, N.R. (1980) Xanthine oxidase catalyzed reductive cleavage of anthracycline antibiotics and free radical formation. Molecular Pharmacology, 17, 95 – 99.

PÁN, S., REDERSEN, L. and BACHUR, N.R. (1981) Comparative flavoprotein catalysis of anthracycline antibiotic reductive cleavage and oxygen consumption. Molecular Pharmacology, 19, 184 – 186.

PANIKER, N. V. and BEUTLER, E. (1972) The effect of methylene blue and diaminodiphenylsulfone on red cell reduced glutathione synthesis. Journal of Laboratory and Clinical Medicine, 80, 481 – 487.

PATEL, D.J., KOZLOWSKI, S. A. and RICE, J. A. (1981) Hydrogen bonding, overlap geometry, and sequence specificity in anthracycline antitumour antibiotic-DNA complexes in solution. Proceedings of the National Academy of Science, 78, 3333 – 3337.

PHILLIPS, D.R. and CARLYLE, G.A. (1981) The effect of physiological levels of divalent metal ions on the interaction of daunomycin with DNA: Evidence of a ternary daunomycin-Cu^{2+}-DNA complex. Biochemical Pharmacology, 30, 2021 – 2024.

PHILLIPS, D.R. and ROBERTS, G.C.K. (1980) Proton nuclear magnetic resonance study of self-complementary hexanucleotide $d(pTpA)_3$ and its interaction with daunomycin. Biochemistry, 19, 4795 – 4801.

PIETRONEGRO, D.P., McGINNESS, J.E. and KOREN, M.G. (1974) Spontaneous generation of adriamycin semiquinone radicals at phsiologic pH. Physiological Chemistry and Physics and Medical NMR, 11, 405 – 414.

PIGRAM, W.J., FULLER, W. and HAMILTON, L.D. (1972) Stereochemistry of intercalation: interaction of daunomycin with DNA. Nature New Biology, 235,17 – 19.

PITOT, H. C. (1981) Fundamentals of Oncology. second ed. Ch. 5. pp. 80 – 102. New york and Basel: Marcel dekker, INC.

PORUMB, H. (1978) The solution spectroscopy of drugs and the drug-nucleic acid interactions. Progress in Biophysics and molecular Biology, 34, 175 – 195.

POTMESIL, M., ISRAEL, M. and SILBER, R. (1984) Two mechanisms of adriamycin-DNA interaction in L1210 cells. Biochemical Pharmacology, 33, 3137 – 3142.

PRAGA, C., BERETTA, B., VIGO, P.L. and et al. (1979) Adriamycin cardiotoxicity: A survey of 1273 patients. Cancer Treatment Reports, 63, 827 – 834.

PURI,, R.N. and MEISTER, A. (1983) Transport of glutathione as γ-glutamyl-custeinylglycyl ester into liver and kidney. Proceedings of the National Academy of Science, 80, 5258 – 5260.

QUIGLEY, G.J., WANG, A.H.-J., UGHETTO, G., MAREL, G.V.D., BOOM, J.H.V. and RICH, A. (1980) Molecular structure of an anticancer drug-DNA complex: Daunorubicin plus d (Cp Gp Tp Ap Cp G). Proceedings of the National Accademy of Science, 77, 7204 – 7208.

RABENSTEIN, D.L. (1978) Pulsed fourier trasform nuclear magnetic resonance spectroscopy. Analytical Chemistry, 50, 1265 – 1276.

RABENSTEIN, D.L. and FAIRHURST, M.T. (1975) Nuclear magnetic resonance studies of the solution chemistry of metal complex. XI. The binding of methylmercury by sulfhydryl-containing amino acids and by glutathione. Journal of the American Chemical Society, 16, 2086 – 2092.

RABENSTEIN, D.L. and NAKASHIMA, T. T. (1979) Spin- echo fourier transform nuclear magnetic resonance spectroscopy. Analytical Chemistry, 51, 1465 – 1474.

RABENSTEIN, D.L., BROWN, D.W. and McNEIL, C.J. (1985) Determination of glutathione in intact and haemolysed erythrocytes by titration with tert-butyl hydroperoxide with end point detection by ^{1}H nuclear magnetic resonance spectroscopy. Analytical Chemistry, 57, 2294 – 2299.

RAHMAN, A., KESSLER, A., MORE, N. and et al. (1980) Liposomal protection of adriamycin-indused cardiotoxicity in mice. Cancer Research, 40, 1532 – 1537.

RAHMAN, A., MORE, N. and SCHEIN, P. S. (1982) Doxorubicin-induced chronic cardiotoxicity and its protection by liposomal administration. Cancer Research, 42, 1817 – 1825.

RAIJMAKERS, R., SPETH, P., WITTE, T., LINSSEN, P., WESSELS, J. and HAANEN, C. (1987) Infusion-rate independent cellular adriamycin concentrations and cytotoxicity to human marrow clonogenic cells (CFU-GM). British Journal of Cancer, 56, 123 – 126.

REEVE, J. and KUHLENKAMP, J. (1980) Estimation of glutathione in rate liver by reversed-phase high-performance liquid chromatography: Separation from cysteine and γ-glutamylcysteine. Journal of Chromatography, 194, 424 – 428.

REGLINSKI, J. and SMITH, W.E. (1986) nuclear magnetic resonance in living systems. Trends in Analytical Chemistry, 5, 190 – 195.

REGLINSKI, J., HOEY, S., SMITH, W.E. and STURROCK, R.D. (1988 a) Cellular response to oxidative stress at sulfhydryl group receptor sites on the erythrocyte membrane. The Journal of Biological Chemistry, 263, 12360 – 12366.

REGLINSKI, J., SMITH, W.E., SUCKLING, C. J., AL-KABBAN, M., STEWART, M.J. and WATSON, I.D. (1988 b) Doxorubicin-induced altered glycolytic patterns in the leukemic cell studied by proton spin echo NMR. Clinica Chimica Acta, 175, 285 – 290.

REGLINSKI, J., SMITH, W.E., SUCKLING, C.J., AL-KABBAN, M., WATSON, I.D. and STEWART, M.J. (1987) A ^{1}H spin echo NMR study of the Hela tumour cell. FEBS Letters, 214, 351 – 356.

REINERT, K.E. (1983) Anthracycline-binding induced DNA stiffening, bending and elongation, sterochemical implications from viscometric investigations. Nucleic Acids Research, 11, 3411 – 3430.

REVIS, N.W. and MARUSIC, N. (1978) Glutathione peroxidase activity and selenium concentration in the hearts of doxorubicin-treated rabbits. Journal of Molecular and Cellular Cardiology, 10, 945 – 951.

REVIS, N.W. and MARUSIC, N. (1979) Sequestration of $^{45}Ca^{+2}$ by mitochondria from rabbit heart, liver and kidney after doxorubicin or digoxin / doxorubicin treatment. Experimental Molecular Pathology, 31, 440 – 451.

RICHMAN, P. and MEISTER, A. (1975) Regulation of γ-glutamyl-cysteine synthetase by non-allosteric feedback inhibition of glutathione. Journal of Biological Chemistry, 250, 1422 – 1426.

RIGGS, C.E., BENJAMIN, R.S., SERPICK, A. and BACHUR, N.R. (1977) Biliary disposition of adriamycin. Clinical Pharmacology and Therapeutics, 22, 234 – 238.

RIGHETTI, P., LITTLE, E.P. and WOLF, G. (1971) Reutilization of amino acids in protein synthesis in Hela cells. The Journal of Biological Chemistry, 246, 5724 – 5732.

RIORDAN, J.R., DEUCHARS, K., KARTNER, N., ALON, N., TRENT, J. and LING, V. (1985) Amplification of P-glycoprotein genes multidrug-resistant mammalian cell lines. Nature, 316, 817 – 819.

ROGAN, A.M., HAMILTON, T.C., YOUNG, R.C., KLECKER, R.W. and OZOLS, R.F. (1984) Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science, 224, 994 – 996.

ROMINE, M.T. and KESSEL, D. (1986) Intracellular glutathione as a determinant of responsiveness to antitumour drugs. Biochemical Pharmacology, 35, 3323 – 3326.

ROSS, W.E. and SMITH, M.C. (1982) Repair of deoxyribonucleic acid lesions caused by adriamycin and ellipticine. Biochemical Pharmacology, 31, 1931 – 1935.

ROWLEY, D.A. and HALLIWELL, B. (1982) Superoxide–dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts. FEBS Letters, 142, 39 – 41.

RUSSO, A. and MITCHELL, J.B. (1985) Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. Cancer Treatment Reports, 69, 1293 – 1296.

RUSSO, A., DeGRAFF, W., FRIEDMAN, N. and MITCHELL, J.B. (1986) Selective modulation of glutathione levels in human normal *versus* tumour cells and subsequent differential response to chemotherapy drugs. Cancer Research, 46, 2845 – 2848.

SALLAN, S.E. and CLAVELL, L.A. (1984) Cardiac effects of anthracyclines used in the treatment of childhood acute lymphoblastic leukemia: A 10 - year experience. Seminars in Oncology, 11, 19 – 21.

SATOH, K. (1978) Serum lipid peroxide in cerebrovascular disorders determinated by a new colorimetric method. Clinica Chemica Acta, 90, 37 – 43.

SAWYER, D.T. and VALENTINE, J.S. (1981) How super is superoxide ? Accounts of Chemical Research, 14, 393 – 400.

SCHIMKE, R.T., ALT, F.W., KELLEMS, R.E., KAUFMAN, R.J. and BERTION, J.R. (1978) Amplification, of dihydrofolate reductase genes in methotrexate-resistant cultured mouse cells. Cold Spring Harbor Symposia on Quantitative Biology, 42, 649 – 657.

SCHLAGER, S.I. and OHANIAN, S.H. (1979 a) A role for fatty acid composition of complex cellular lipids in the susceptibility of tumor cells to humoral immune killing. Journal of Immunology, 123, 146 – 152.

SCHLAGER, S.I. and OHANIAN, S.H. (1979 b) Physical and chemical composition of subcellular fractions from tumor cells treated with metabolic inhibitors or hormones. Cancer Research, 39, 1369 – 1376.

SCHUMANN, J., ZANTE, J. and GOHDE, W. (1978) Aneuploidies in solid tumors. In: Third International Symposium on Pulse Cytometry, ed. LUTZ, D. pp. 447 – 457, European Press Medikon, Ghent, Belgium.

SCHWARTZ, H.S. (1973) A fluorometric assay for daunomycin and adriamycin in animal tissues. Biochemical Medicine, 7, 396 – 404.

SCHWARTZ, H.S. and KANTER, P.M. (1981) DNA damage by anthracycline drugs in human leukemia cells. Cancer Letters, 13, 309 – 313.

SCOTT, E.M. and WRIGHT, R.C. (1980) Variability of glutathione S-transferase of human erythrocytes. American Journal of Human Genetics, 32, 115 – 117.

SEDDON, A.P., LI, L. and MEISTER, A. (1984) Resolution of 5-oxo-L-prolinase into 5-oxo-L-proline-dependent ATPase and a coupling protein. Journal of Biological Chemistry, 259, 8091 – 8094.

SEVANIAN, A. and HOCHSTEIN, P. (1985) Mechanisms and consequences of lipid peroxidation in biological systems. Annul Review of Nutrition, 5, 365 – 390.

SEVANIAN, A., HACKER, A. D. and ELSAYED, N. (1982) Influence of vitamin E and nitrogen dioxide on lipid peroxidation in rat lung and liver microsomes. Lipids, 17, 269 – 277.

SHAPIRO, J.R., YUNG, W.K.A. and SHAPIRO, W.R. (1981) Isolation, karyotype, and clonal growth of heterogeneous subpopulations of human malignant gliomas. Cancer Research, 41, 2349 – 2359.

SHEA, T.C., KELLEY, S.L. and HENNER, W.D. (1988) Identification of an anionic form of glutathione transferase present in many human tumours and human tumour cell lines. Cancer Research, 48, 527 – 533.

SHEARMAN, C.W. and LOEB, L.A. (1983) On the fidelity of DNA replication, specificity of nucleotide substitution by intercalating agents. The Journal of Biological Chemistry, 258, 4477 – 4484.

SHEARMAN, C.W., FORGETTE, M.M. and LOEB, L.A. (1983) On the fidelity of DNA replication, mechanism of misincorporation by intercalating agents. The Journal of Biological Chemistry, 258, 4478 – 4491.

SHEN, D., CARDARELLI, C., HWANG, J. and et al. (1986) Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. The Journal of Biological Chemistry, 261, 7762 – 7770.

SHINOZAWA, S., ETOWO, K., YASUNORI, A. and ODA, T. (1984) Effects of Coenzyme Q_{10} on the survival time and lipid peroxidation of adriamycin (doxorubicin) treated mice. Acta Medica Okayama, 38, 57 – 63.

SHOEMAKER, R.H., CURT, G.A. and CARNEY, D.N. (1983) Evidence for multidrug-resistant cells in human tumor cell populations. Cancer Treatment Reports, 67, 883 – 888.

SIEGFRIED, J.M., BURKE, T.G. and TRITTON, T.R. (1985) Cellular transport of anthracyclines by passive diffusion. Biochemical Pharmacology, 34, 593 – 598.

SIEGFRIED, J.M., SARTORELLI, A.C. and TRITTON, T.R. (1983) Evidence for the lack of relationship between inhibition of nucleic acid synthesis and cytotoxicity of adriamycin. Cancer Biochemistry Biophysics, 6, 137 – 142.

SIKIC, B.I., EHSAN, M.N., HARKER, W.G., FRIEND, N.F. and BROWN, B.W. (1985) Dissociation of antitumor potency from anthracycline cardiotoxicity in a doxorubicin analog. Science, 228, 1544 – 1546.

SINHA, B.K. and GREGORY, J.L. (1981) Role of one-electron and two-electron reduction products of adriamycin and daunomycin in deoxyribonucleic acid binding. Biochemical Pharmacology, 30, 2626 – 2629.

SINHA, B.K. and SIK, R.H. (1980) Binding of [^{14}C]-adriamycin to cellular macromolecules *in vivo* . Biochemical Pharmacology, 29, 1867 – 1868.

SINHA, B.K., TRUSH, M.A., KENNEDY, K.A. and MIMNAUGH, E.G. (1984) Enzymatic activation and binding of adriamycin to nuclear DNA. Cancer Research, 44, 2892 – 2896.

SMITH, E., STRATFORD, I.J. and ADAMS, G.E. (1980) Cytotoxicity of adriamycin on aerobic and hypoxic Chinese hamster V79 cells *in vitro*. British Journal of Cancer, 41, 568 – 573.

SMITH, M.T., EVANS, C.G., DOANE-SETZER, P., CASTRO, V.M., TAHIR, M.K. and MANNERVIK, B. (1989) Denitrosation of 1.3-bis(2-chloroethyl)-1-nitrosourea by class mu glutathione transferases and its role in cellular resistance in rat brain tumour cells. Cancer Research, 49, 2621 – 2625.

SNOKE, J.E. and BLOCH, K. (1954) Biosynthesis of glutathione. In Glutathione. ed. COLOWICK, S., LAZAROW, A., RACKER, E., SCHWARZ, D.R., STADTMAN, E. and WAELSCH, H. pp. 129 – 137. New York : Academic.

SOMFAI-RELLE, S., SUZUKAKE, K., VISTICA, B. P. and VISTICA, D.T. (1984) Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumour cells resistant to L-phenylalanine mustard. Biochemical Pharmacology, 33, 485 – 490.

SOUHAMI, R. and TOBIAS, J. (1986) Cancer and It's Management. Oxford, London. Ch. 6, pp. 78 – 107. Blackwell Scientific Publications.

STATES, B. and SEGAL, S. (1969) Thin-layer chromatographic separation of cysteine and the n-ethylmaleimide adducts of cysteine and glutathione. Analytical Biochemistry, 27, 323 – 329.

STATHOPOULOS, G. (1975) Kanam mandibles tumour. The Lancet, 1, 165.

STEIN, A.F., DILLS, R.L. and KLAASSEN, C.D. (1986) High-performance liquid chromatographic analysis of glutathione and its thiol and disulfide degradation products. Journal of Chromatography, 381, 259 – 270.

STOCKMAN, P.K., BECKETT, G.J. and HAYES, J.D. (1985) Identification of a basic hybrid glutathione-S-transferase from human liver. Biochemical Journal, 227, 457 – 465.

STOREY, G.C.A., HOLT, D.W., HOLT, P. and CURRY, V.L. (1982) High-performance liquid chromatographic measurement of amiodarone and its desethyl metabolite: Methodology and preliminary observations. Therapeutic Drug Monitoring, 4, 385 – 388.

SUGIOKA, K., NAKANO, H., NOGUCHI, T., TSUCHIYA, J. and NAKANO, M. (1981) Decompostion of unsaturated phospholipid by iron-ADP-adriamycin co-ordination complex. Biochemical and Biophysical Research Communications, 100, 1251 – 1258.

SUPINO, R., MARIANI, M., CAPRANICO, G., COLOMBO, A., and PARMIANI, G. (1988) Doxorubicin cellular pharmacokinetics and DNA breakage in multidrug resistant B16 melanoma cell line. British Journal of Cancer, 57, 142 – 146.

SUPINO, R., PROSPERI, E., FORMELLI, F., MARIANI, M. and PARMIANI, G. (1986) Characterization of a doxorubicin–resistant murine melanoma line : Studies on cross-resistance and its circumvention. British Journal of Cancer, 54, 33 – 42.

SUZUKAKE, K., PETRO, B.J. and VISTICA, D.T. (1982) Reduction in glutathione contant of L-PAM resistant L1210 cells confers drug sensitivity. Biochemical Pharmacology, 31, 121 – 124.

SUZUKI, T., KANDA, H., KAWAI, Y. and et al. (1979) Cardiotoxicity of anthracycline antineoplastic drugs: Clinicopathological and experimental studies. Japanese Circulation Journal, 43, 1000 – 1008.

TACCA , M.D., DANESI, R., DUCCI, M., BERNARDINI, C. and ROMANINI, A. (1985) Might adriamycinol contribute to adriamycin-induced cardiotoxicity? Pharmacological Research Communications, 17, 1073 – 1085.

TANNOCK, I. and GUTTMAN, P. (1981) Response of Chinese hamster overy cells to anticancer drugs under aerobic and hypoxic conditions. British Journal of Cancer, 43, 245 – 248.

TANNOCK, I.F. (1968) The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. British Journal of Cancer, 22, 258 – 273.

TEICHER, B.A., LAZO, J.S. and SARTORELLI, A.C. (1981) Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Research, 41, 73 – 81.

TERASAKI, T., IGA, T., SUGIYAMA, Y. and HANANO, M. (1984) Pharmacokinetic study on the mechanism of tissue distribution of doxorubicin: Interorgan and interspecies variation of tissue-to-plasma partition coefficients in rats, rabbits, and guinea pigs. Journal of Pharmaceutical Science, 73, 1359 – 1363.

TEWEY, K.M., COWE, T.C., YANG, L., HALLIGAN, B.D. and LIU, L.F. (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science, 226, 466 – 468.

THAYER, W.S. (1977) Adriamycin stimulated superoxide formation in submitochondrial particles. Chemico- Biological Interactions, 19, 265 – 278.

THEISS, J.C., STONER, G.D. and SHIMKIN, M.B. (1977) Murine pulmonary adenoma bioassay of potentially effective agents against slow-growing solid tumors. Cancer Research, 37, 305 – 309.

THERASSE, J. and LEMONNIER, F. (1987) Determination of plasma lipoperoxides by high-performance liquid chromatography. Journal of Chromatography, 413, 237 – 241.

THOMAS, J.P. and GIROTTI, A.W. (1989) Role of lipid peroxidation in hematoporphyrin derivative-sensitized photokilling of tumour cells: Protective effects of glutathione peroxidase. Cancer Research, 49, 1682 – 1686.

THOMPSON, G.A. and MEISTER, A. (1975) Utilization of L-cystine by the γ-glutamyl transpeptidase–γ-glutamylcyclotransferase pathway. Proceedings of the National Academy of Science, 72, 1985 – 1988.

THOR, H., SMITH, M.T., HARTZELL, P., BELLOMO, G., JEWELL, S.A. and ORRENIUS, S.(1982) The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. The Journal of Biological Chemistry, 257, 12419 – 12425.

THORNALLEY, P.J. and DODD, N.J.F. (1985) Free radical production from normal and adriamycin-treated rat cardiac sarcosomes. Biochemical Pharmacology, 34, 669 – 674.

TIETZE, F. (1969) Enzymic method for quantitative determination of nanogram amount of total and oxidized glutathione: Applications to mammalian blood and other tissues. Analytical Biochemistry, 27, 502 – 522.

TOBIAS, J.S. and GRIFFITHS, C.F. (1976) Management of ovarian carcinoma: Current concept and future prospects. New England Journal of Medicine, 294, 818 – 823 and 877 – 882.

TOKES, Z.A., ROGERS, K.E. and REMBAUM, A. (1982) Synthesis of adriamycin-coupled polyglutaraldehyde microspheres and evaluation of their cytostatic activity. Proceedings of the National Academy of Science, 79, 2026 – 2030.

TRITTON, T.R. and YEE, G. (1982) The anticancer agent adriamycin can be actively cytotoxic without entering cells. Science, 217, 248 – 250.

TRITTON, T.R., MURPHREE, S.A. and SARTORELLI, A.C. (1978) Adriamycin: A proposal on the specificity of drug action. Biochemical and Biophysical Research Communications, 84, 802 – 808.

TRITTON, T.R., YEE, G. and WINGARD, L.B. (1983) Immobilized adriamycin: a tool for separating cell surface from intracellular mechanisms. Federation Proceedings, 42, 284 – 287.

TSURUO, T., LIDA, H., NAGANUMA, K., TSUKAGOSHI, S. and SAKURAI, Y. (1983) Promotion by verapamil of vincristine responsiveness in tumor cell lines inherently resistant to the drug. Cancer Research, 43, 808 – 813.

TWENTYMAN, P.R., FOX, N.E. and WHITE, D.J.G. (1987) Cyclosporin A and its analogues as modifiers of adriamycin and vincristine resistance in a multi-drug resistant human lung cancer cell line. British Journal of Cancer, 56, 55 – 57.

UGURBIL, K., BROWN, T.R., DEN HOLLANDER, J.A., GLYNN, P. and SHULMAN, R.G. (1978) High-resolution ^{13}C nuclear magnetic resonance studies of glucose metabolism in Escherichia coli. Proceedings of the National Accademy of Science, 75, 3742 – 3746.

UNVERFERTH, B.J., MAGORINE, R.D., BALCERZAK, S.P., LEIER, C.V. and UNVERFERTH, D.V. (1983) Early changes in human myocardial nuclei after doxorubicin. Cancer, 52, 515 – 521.

UNVERFERTH, D.V., MAGORIEN, R.D., LEIER, C.V. and BALCERZAK, S.P. (1982) Doxorubicin cardiotoxicity. Cancer Treatment Reviews, 9, 149 – 164.

VAN DER WERF, P., ORLOWSKI, M. and MEISTER, A. (1971) Enzymatic conversion of 5-oxo-L-proline (L-pyrrolidone carboxylate) to L-glutamate coupled with ATP cleavage to ADP: a reaction in the γ-glutamyl cycle. Proceedings of the National Academy of Science, 68, 2982 – 2985.

VAN VLEET, J.F. and FERRANS, V.J. (1980) Evaluation of vitamin E and selenium protection against chronic adriamycin toxicity in rabbits. Cancer Treatment Reports, 64, 315 – 317.

VAN VLEET, J.F., FERRANS, V.J. and WEIRICH, W.E. (1980) Cardiac disease induced by chronic adriamycin administration in dogs and evaluation of vitamin E and selenium as cardioprotectants. American Journal of Pathology, 99, 13 – 24.

VAUPEL, P.W., FRINAK, S. and BICHER, H.I. (1981) Heterogeneous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. Cancer Research, 41, 2008 – 2013.

VON HOFF, D.D., LAYARD, M.W., BASA, P. and et al. (1979) Risk factors for doxorubicin-induced congestive heart failure. Annals of Internal Medicine, 91, 710 – 717.

VON WARTBURG, J.P. and WERMUTH, B. (1980) In Enzymatic Basis of Detoxification, ed. JAKOBY, W. B. Vol. 1, pp. 254. New york : Academic press INC.

VRIGNAUD, P., LONDOS-GAGLIARDI, D. and ROBERT, J. (1986) Cellular pharmacology of doxorubicin in sensitive and resistant rat glioblastoma cells in culture. Oncology, 43, 60 – 66.

WANG, Y.M., MADANAT, F.F., KIMBALL, J.C. and et al. (1980) Effect of vitamin E against adriamycin-induced toxicity in rabbits. Cancer Research, 40, 1022 – 1027.

WARE, C. F. (1985) Protocol for a colorimetric assay to determine cell viability using Bio-Rad EIA microtitration plate reader. Bio-Rad Bulletin, 1203, 1 – 4.

WATSON, I.D., STEWART, M.J. and FARID, Y.Y.Z. (1985) The effect of surfactants on the high-performance liquid chromatography of anthracyclines. Journal of Pharmaceutical and Biomedical Analysis, 3, 555 – 563.

WEENEN, H., VAN MAANEN, J.M.S., DE PLANQUE, M.M., MCVIE, J.G. and PINEDO, H.M. (1984) Metabolism of 4'-modified analogs of doxorubicin. Unique glucuronidation pathway for 4'-epidoxorubicin. European Journal of Cancer and Clinical Oncology, 20, 919 – 926.

WELLNER, V.P., ANDERSON, M.E., PURI, R.N., JENSEN, G.L. and MEISTER, A. (1984) Radioprotective by glutathione ester: transport of glutathionr ester into human lymphoid cells and fibroblasts. Proceedings of the National Academy of Science, 81, 4732 – 4735.

WENDELL, P.L. (1970) Measurement of oxidized glutathione and total glutathione in the perfused rat heart. Biochemical Journal, 117, 661 – 665.

WILLIAMS, C. (1983) All About Cancer, Apractical Guide to Cancer Care. New York: John Wiley and Sons LTD.

WILLIAMSON, J.M., BOETTCHER, B. and MEISTER, A. (1982) Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proceedings of the National Academy of Science, 79, 6246 – 6249.

WINGARD, L.B., TRITTON, T.R. and EGLER, K.A. (1985) Cell surface effects of adriamycin and carminomycin immobilized on cross-linked polyvinyl alcohol. Cancer Research, 45, 3529 – 3536.

WONG, S.H.Y., KNIGHT, J.A., HOPFER, S.M., ZAHARIA, O., LEACH, C.N. and SUNDERMAN, F.W. (1987) Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde–thiobarbituric acid adduct. Clinical Chemistry, 33, 214 – 220.

YAGI, K., NISHIGAKI, I. and OHAMA, H. (1968) Measurement of serum TBA-value. Vitamins , 37, 105 – 112.

YAMAUCHI, N., KURIYAMA, H., WATANABE, N., NEDA, H., MAEDA, M. and NIITSU, Y. (1989) Intracellular hydroxyl radical production induced by

recombinant human tumour necrosis factor and its implication in the killing of tumor cells *in vitro* . Cancer Research, 49, 1671 – 1675.

YESAIR, D.W., THAYER, P.S., McNITT, S. and TEAGUE, K. (1980) Comparative uptake, metabolism and retention of anthracyclines by tumor growing *in vitro* and *in vivo*. European Journal of Cancer, 16, 901 – 907.

YODA, Y., NAKAZAWA, M., ABE, T. and KAWAKAMI, Z. (1986) Prevention of doxorubicin myocardial toxicity in mice by reduced glutathione. Cancer Research, 46, 2551 – 2556.

YU, L.W., LATRIANO, L., DUNCAN, S., HARTWICK, R.A. and WITZ, G. (1986) High-performance liquid chromatography analysis of the thiobarbituric acid adducts of malonaldehyde and *trans,trans* -muconaldehyde.Analytical Biochemistry, 156, 326 – 333.

Appendix to Results

Viability Studies

Viability studies using Trypan blue were carried out using HeLa and A549 cells grown as described in 2.6.1 and 2.6.2 and treated with Doxorubicin at 18 nmol/106 cells (4 μmol/l) for 12 h. The control and doxorubicin-treated flasks were sampled at intervals, the samples stained with Trypan blue and the numbers of viable cells counted. The viability of treated A549 cells at 12 hrs was shown to be 78% (Fig 74) and of HeLa at 5 hrs 98% (Fig 75).

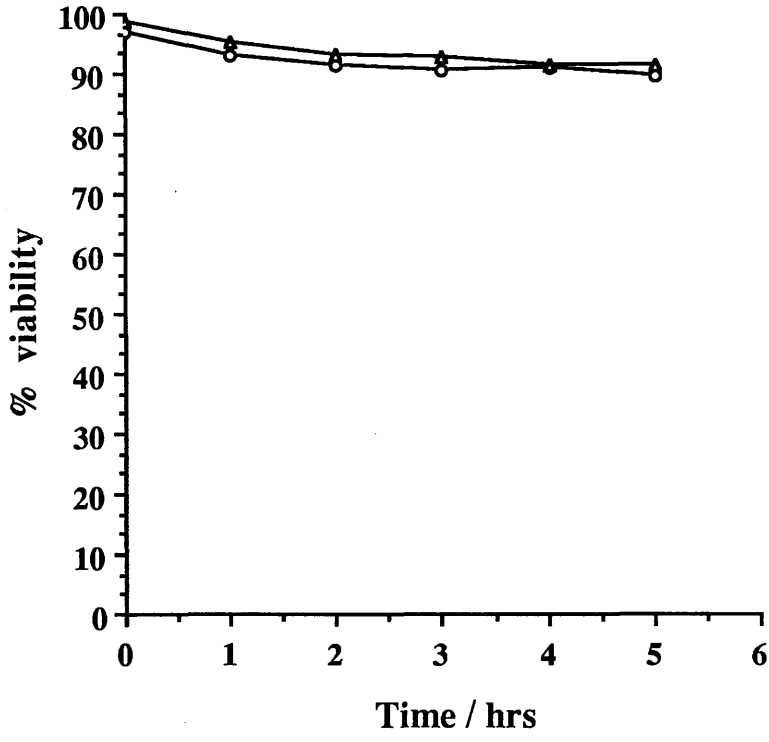


Figure 74 : Viability of Hela cells treated with Doxorubicin over a period of 5 h, Doxorubicin concentration -3.6 nmol /10^6 cells. The two curves are from duplicate experiments.

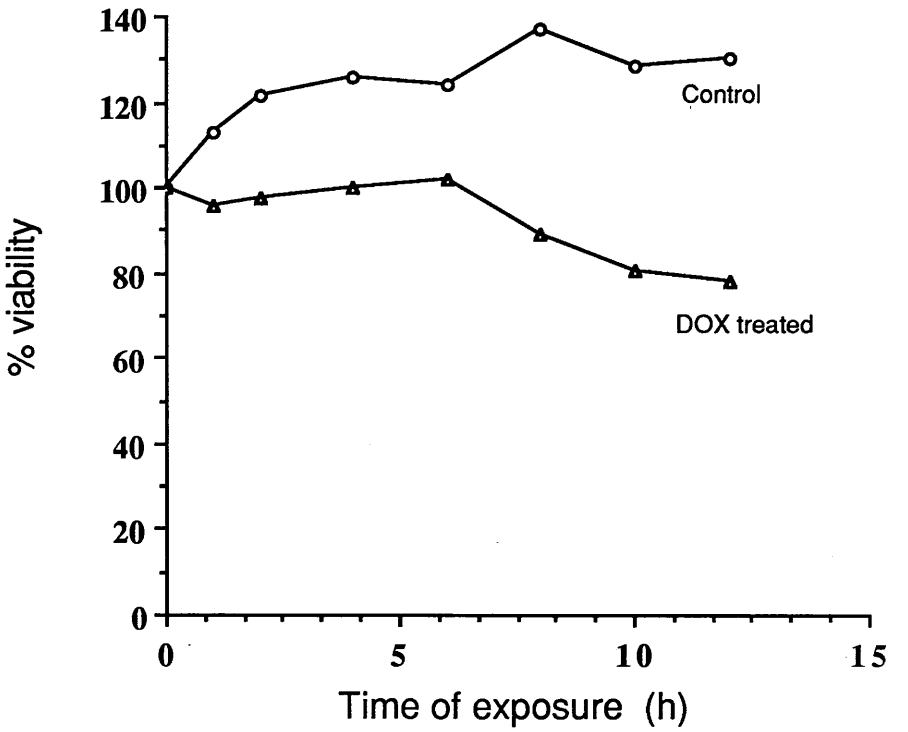


Figure 75 : Viability of A549 cells treated with Doxorubicin over a period of 12 h, Doxorubicin concentration - 18 nmol / 10^6 cells.

THE EFFECT OF DOXORUBICIN ON THE GLUTATHIONE CONTENT AND VIABILITY OF CULTURED HUMAN LUNG CANCER CELL LINES A549 AND GLC_4 210

MUZAHIM AL-KABBAN*, MICHAEL J STEWART, IAN D WATSON AND JOHN REGLINSKI+

Institute of Clinical Biochemistry

Royal Infirmary

Glasgow G4 0SF

* Kufa College of Medicine

Kufa University

Republic of Iraq

+ Department of Pure and Applied Chemistry

Strathclyde University

Glasgow

All proofs and correspondence to Dr M J Stewart at the above address

Key Words: Lung tumours, doxorubicin, glutathione, HPLC

Summary

Glutathione was measured in doxorubicin-sensitive cells from small cell carcinoma of lung (GLC_4 210), and the levels compared with those of cells with acquired resistance and a line of resistant non-small-cell adenocarcinoma A549 (Alveolar type 2). The effect of different doxorubicin concentrations on glutathione were measured by HPLC. The effect of doxorubicin on the viability of the cell lines was studied using thiazole blue dye reduction. An increase in A549 sensitivity to doxorubicin was produced using buthionine-S,R-sulfoximine at a non toxic dose.

Introduction

Measurement of increased intracellular concentrations of glutathione (GSH) in many types of cancer cells have been documented (1), furthermore in certain tumours (eg skin, liver, colon) some carcinogens have been shown to lead to an increase in GSH as well as γ-glutamyl transpeptidase levels . These changes have been suggested as markers for neoplastic change (1,2). Intracellular GSH concentrations may determine the sensitivity of tumour to certain chemotherapeutic agents and irradiation, since GSH can scavenge reactive oxygen intermediates and free radicals (3,4). Thus cells with low GSH levels may be more sensitive and susceptible to chemotherapy and cells with high GSH levels may be protected.

The usefulness of cytotoxic drugs in the treatment of different type of cancer has been limited by the frequent development of drug resistance. Much work has been done in identifying the possible mechanism of this resistance; it is now widely accepted that doxorubicin (DOX) resistance in cancer cells is due to an enhanced outward flux of DOX (5,6). Garman et al (7) showed that DOX resistance is related to changes in the structure of the membrane, both alterations in membrane fluidity and the synthesis of a membrane glycoprotein which transports DOX out of the cells. GSH plays an important role in cellular resistance, and resistance can be reversed by its depletion which may be achieved either by reducing the supply of precursors for synthesis (glutamate, cysteine and glycine) or by using specific inhibitors of synthetic enzymes (8). Buthionine-S,R-sulfoximine (BSO) is a potent and selective agent which acts by inhibition of γ– glutamyl cysteine synthetase. Tumour cells treated with BSO have been

shown to have lower GSH concentrations and increased susceptibility to cytolysis by reactive oxygen intermediates (1,9-13).

The problem with glutathione measurements in cultured cells is the possible oxidation of intracellular GSH during cell disruption and subsequent enzymatic or colorimetric estimations. The HPLC method used in these studies employs direct injection of rapidly disrupted cells in to a non-oxidising environment in the HPLC eluant which is gassed with helium. The method gives results which are comparable with those obtained in intact cells using NMR estimation of reduced glutathione (14).

In this study we measured the GSH level using HPLC in three human lung tumour lines, one sensitive and two resistant to DOX. One of the resistant lines showed inherent resistance, the other acquired resistance on exposure to sublethal concentrations of DOX. The effect of DOX on the viability of these 3 cell lines and the effect of BSO on resistance were studied.

Materials and Methods

Reagents and Chemicals

Doxorubicin, HPLC and tissue culture reagents and chemicals were as reported earlier (14) and from the same sources, except that RPMIX1 1640 culture medium with 20 mmol/l Hepes buffer and 0.9M sodium bicarbonate were obtained from Flow Laboratories (UK). Buthionine-S,R-sulfoximine and MTT dye (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide: thiazolyl blue) were obtained from Sigma Chemical Co. Ltd, Poole, England. Phosphate buffered saline (PBS) was obtained from Gibco (UK) Ltd, Scotland.

Tissue Culture

Three cell lines were used in this study, Alveolar type 2 cells from an adenocarcinoma of lung (A549) resistant to doxorubicin, were obtained from American type culture, Rockville, Maryland, ATCC, CCL 185. A549 cells were routinely grown as a monolayer in culture flasks containing enriched RMP1 1640 medium supplemented with 20 mM Hepes buffer, 1 mM sodium bicarbonate, 10%(v/v) fetal bovine serum (Gibco,UK,Ltd.) and 2 mM L- glutamine (14).

GLC_4 210 cells originating from a small cell carcinoma of lung sensitive to DOX were obtained from the Department of Medical Oncology, University of Gronigen, Holland through the Department of Medical Oncology, University of Glasgow. Resistance was induced in this cell line by growing continuously in DOX-containing RPMI 1640 at a concentration of 900 pmol/l.

GLC_4 210 cells were grown in suspension in the medium as above, with addition of 900 pmol/l DOX to induce resistance.

HPLC

HPLC was carried out as previously reported (14) except that the ratio of methanol/water/40% tetrabutyl ammonium hydroxide was (75:924:1) and the flow rate was 1 ml/min. Since the results obtained by this method have been shown to correlate with those measured in intact cells using ^1H spin echo proton NMR, they are not subject to artefactual changes during cell disruption(14).

Effect of doxorubicin on GSH concentrations

Six F25 flasks (each containing 2 x 10^6 A549 cells in 5 ml culture medium) were prepared. Six Universal tubes were prepared for the GLC_4 210 resistant (R) and sensitive (S) lines, each tube containing between 7 x 10^6 and 8 x 10^6 cells in 5 ml of medium. One flask of A549 and one tube from each of GLC_4 210 sensitive and resistant lines were used as control and the other five were treated with DOX at concentrations from 4.3-69, 1.1-18 and 1.2-20 nmol/10^6 cells respectively. The rest of experimental procedure has been described in detail (14).

Chemosensitivity Assay

The assay depends on the ability of living cells to reduce the tetrazolium dye MTT to the blue coloured formazin (15). For determination of the optimum MTT concentration for the different cell lines, A549 was plated at 24 h prior to the assay at 5 x 10^3 cells/ml (1000 cells per well in 200 ul of culture medium) in 96 well microtitre plates (Flow Laboratories) and incubated at 37°C under 2% CO_2. The other two cell lines were plated out on the day of assay at 3 x 10^4 cells/ml (4,500 cells per well in 150 ul of culture medium) in 96 round-bottomed well plates.

Ten different MTT concentrations (0.5-5 g/l) were prepared in PBS and 50 ul added to each well, one concentration per row of wells after replacing the old medium by fresh in the cases of A549. Plates were wrapped in tinfoil and incubated at 37°C and 2% CO_2 for 4h. MTT-containing medium was removed from the GLC4 210 cell pellet after spinning the plates at 6oo g . MTT formazin crystals were dissolved in 200 ul dimethyl sulphoxide in the case of A549, and 150 ul in the case of GLC_4 210, then Tris-buffer pH 9.5 (25 ul) was added to dissolve the non-dissolved MTT. The MTT absorbance was measured using an ELISA plate reader at an absorbance of 570 nm (Biorad Laboratories, Watford, Herts, England).

The effect of different DOX concentrations on these 3 cell lines was measured by plating the cells as above. Different DOX concentrations were used in fresh medium, one concentration per 3 wells (8.4 nmol/l - 8.6 umol/l). A 549 cells were plated 3 days prior to the Dox-exposure, GLC_4 210 (S) and (R) were subcultured in F75 flasks 3 days prior to the assay and DOX was removed from GLC_4 resistance in this subculture in order not to interfere with the assay.

The cells were left in contact with DOX for 24 h and the medium was then replaced. The medium was changed again once during the next two days. On the last day the medium was again exchanged for fresh and 50 ul of MTT added. The viability was assayed as described above. Chemosensitivity assays were carried out in duplicate for each cell line.

Effect of Buthionine-S,R-sulfoximine pretreatment on A549 resistance to Doxorubicin

Two 96-well plates were plated with A549 as described above. Three days later one plate was treated with BSO-containing medium (2 uM in 200 ul) after removing the old medium, leaving one row as control. At the same time medium in all wells on the other plate was replaced by fresh. Plates were incubated at 37°C and 2% CO_2. Six hours later 50 ul of DOX-containing medium at six different concentrations was added to the wells in six different rows (one concentration per row of wells), to give final concentrations of 1.7- 55.2 nmol/l, leaving one row with BSO only as control in the first plate and medium only controls in both first and second plates. Plates were incubated for 90 min with DOX, then the drug was removed, the medium replaced and the experiment completed as before.

Results

Concentration effect of doxorubicin

The mean basal GSH concentrations in the 3 lung cell lines (measured in duplicate) were 14.6, 1.2 and 0.75 nmol/10^6 cells for A549, GLC_4 210 ((S) to DOX) and GLC_4 210 ((R) to DOX) respectively. There was no significant difference between the duplicated experiments. The effect of DOX on these cells differed qualitatively. The GSH concentration in resistant GLC_4 cells was minimally affected by DOX even at high concentrations, whereas there was a rapid and significant reduction in the sensitive cells. The change in GSH concentration in those cells with resistance induced by DOX fell into an intermediate category (Fig 1).

The observed falls in GSH concentrations in the 3 cell lines were very different. The A549 line has high initial GSH concentrations of the order of 10 times those of the GLC_4 (S) cells, and although GSH fell in both lines in a dose-related manner when treated with DOX, the residual concentration in the resistant cells was still above the basal concentration for the sensitive line, even after treatment with 4 times the concentration of DOX. The GLC_4 cells (R) had basal levels of GSH which were approximately half of those of the sensitive cells but which was unaffected by DOX. These data suggest a different mechanism of resistance in the two cell lines.

Chemosensitivity assay:

The optimum concentrations of MTT used for the chemosensitivity assays (The concentration at which maximum colour development was obtained from untreated cells) , were initially determined as 5 g/l for A549 cells and 3 mg/l for GLC4 ((S) and (R)) (Fig 2).

The measurement of cell kill by different DOX concentrations showed the expected effects of different DOX concentrations with the sensitive cells releasing the dye to a greater extent than the resistant lines ($p<0.01$, Dunnetts test). The LD_{50} of DOX in these 3 cell lines was found to be about 100, 55 nmol/l and 2 umol/l for A549, GLC_4 (S) and GLC_4 (R) respectively (Fig 3 a,b). This indicates that GLC_4 (R) is 20 times more resistant than A549.

Effect of BSO on the prevention of A549 cell resistance:

Six hour pre-treatment of A549 cells with BSO at a non-toxic concentration (2 umol/l) increased the sensitivity of these cells to DOX (Fig 4) when measured using the chemosensitivity assay. Comparison of cells treated with DOX alone, and those pretreated with BSO showed a highly significant increase in sensitivity at all concentrations used ($P<0.01$, t-test). There was no significant difference between control and treated cells at the 3 lowest DOX concentrations, however in the presence of BSO the viability was significantly reduced at all DOX concentrations.

Discussion

The results reported in this study, using a method which is unaffected by artefacts due to cell disruption, indicate that the GSH content in three different lung cancer cells differ from one to another, and is high in inherently resistant cells (A549) compared with initially sensitive lines. These findings are in agreement with Meister et al (3) who showed, using an enzymatic method, that tumours may be found with low, moderate and high intracellular GSH concentrations. Depletion of GSH to that contained in the mitochondrial pool is consistent with viability (12). These data are not at variance with our results as cells grown under the stress of DOX to acquire resistance may deplete the cytoplasmic GSH with only the vital mitochondrial pool remaining. There is evidence that cells will not die until this pool is depleted (12).

A549 DOX resistant cells have a high GSH content which probably contributes to their resistance (4,8,16,17). Certainly the viability of this cell line is only slightly affected by DOX compared with GLC_4 210 (S) which contained moderate levels of GSH that are reduced by DOX. Our results would confirm other findings (11,18), that GSH is protective against cytotoxic agents.

The results of the chemosensitivity assay paralleled the effect on measured GSH concentrations of different DOX concentrations. The LD_{50} of these three cell lines to DOX, differ, in the order GLC_4 210(R) > A549 > GLC_4 410(S). The fact that the cells with acquired resistance show the highest LD_{50} while having a lower intracellular GSH concentration than A 549 cells is due to increased P glycoprotein that results in increased DOX efflux (7). Because of the importance of GSH in the protection of cells against free radical formation caused by certain cytotoxic agents as DOX,

depletion of intracellular GSH in order to increase the sensitivity of resistant cells is a valid method of therapy (9,11, 18, 19). The high level of resistance shown by GLC_4 210(R) cells compared with A 549 agrees with the findings of Shen et al (20) that induced resistant cells are more resistant than wild type tumour cells to certain cytotoxic drugs.

Our results, carried out on a human lung tumour cell line (549), showed that depletion of GSH with BSO 6 h prior to exposure to DOX resulted in decreased resistance.

The results obtained in this study confirm that viability and non-artefactual intracellular GSH depletion, are linked. The application of these methods to the study of known lung tumour lines showing resistance, sensitivity and acquired resistance to DOX further confirms the importance of GSH concentration in resistance to DOX and the potential benefits of agents such as BSO as adjuncts to therapy with DOX.

FIGURE LEGENDS

Figure 1

Effect of DOX treatment on the intracellular GSH content of the three cell lines (each experiment in duplicate).

a) O A 549

Δ GLC_4 210 (S)

X GLC_4 210 (R)

b) Δ GLC_4 210 (S)

X GLC_4 210 (R)

Figure 2

Determination of the optimum concentration of MTT for the measurement of viability in the 3 cell lines.

O A549

X GLC_4 (S)

Δ GLC_4 (R)

Figure 3

Effect of DOX in the viability of cell lines

a) A 549

b) O GLC_4 210 (S)

Δ GLC_4 210 (R)

Bars = standard error

Figure 4

The effect of BSO on the viability of A549 cells treated with DOX

O treated with DOX alone

Δ pretreated with BSO

Bars = standard error

+ - non significant ($p > 0.05$)

* - $p < 0.05$

** $p < 0.01$

Acknowledgements

We thank the Government of Iraq for financial support to M Al-Kabban. Also we would like to thank Dr S Merry and Dr J A Plumb from the Department of Medical Oncology, University of Glasgow for help in chemosensitivity studies.

REFERENCES

1) Meister, A., Anderson M.E. Glutathione. Ann. Rev. Biochem. 1983; 52: 711-760.

2) Murray, G.I. Burke, M.D., Ewen, S.W.B. Glutathione localisation in benign and malignant human breast lesions. Brit. J. Cancer 1987; 55: 605-609.

3) Meister A. Selective modification of glutathione metabolism. Science, 1983; 220: 471-477.

4) Arrick, B.A., Nathan, C.F. Glutathione metabolism as a determinant of therapeutic efficacy. A review. Cancer Research 1984; 44: 4224-4232

5) Louie K.G., Hamilton, T.C., Winker, M.A., et al, Adriamycin accumulation and metabolism in adriamycin-sensitive and resistant human ovarian cancer cell lines. Biochemical Pharmacology 1986; 35: 467-472

6) Twentyman, P.R., Fox, N.E., White, D.J.G. Cyclosporin A and its analogues as modifiers of adriamycin and vincristine in a multi-drug resistant human lung cancer cell line. Brit. J. Cancer 1987; 56: 55-57.

7) Garman, D., Center, M.S. Alterations in cell surface membranes in chinese hamster lung cells resistant to adriamycin. Biochem. and Biophys. Res. Comm. 1982; 105: 157-163.

8) Suzukake, K, Petro, B.J., Vistica D.T., Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. Biochemical Pharmacology 1982; 31: 121-124.

9) Lee F.Y.E, Allalunis-Turner M.J. Siemann, D.W. Depletion of tumour versus normal tissue glutathione by buthionine sulfoximine. Brit. J. Cancer 1987; 56: 33-38.

10) Mitchell, J.B., Russo, A. The role of glutathione in radiation and drug induced cytotoxicity. Brit. J. Cancer 1987; 55: 96-104

11) Jordan, J., d'Arcy Doherty M., Cohen, G.M. Effect of glutathione depletion on the cytotoxicity of agents towards a human colonic tumour cell line. Brit. J. Cancer 1987; 55: 627-631

12) Gaetjens, E.C., Chen, P., Broome, J.D. L1210(A) mouse lymphoma cells depleted of glutathione with L-Buthionine-S-R-sulfoximine proliferate in tissue culture. Biochem. and Biophys. Res. Comm. 1984; 123: 626-632.

13) Crook, T.R., Souhami, R.L., Whyman, G.D., McLean, A.E.M. Glutathione depletion as a determinant of sensitivity of human leukemia cells to cyclophosphamide. Cancer Res. 1986; 46: 5035-5038.

14) Al-Kabban, M., Watson, I.D., Stewart, M.J., Reglinski, J., Smith, E.W., Suckling, C.J. The use of ^{1}H spin echo NMR and HPLC to confirm doxorubicin induced depletion of glutathione in the intact HeLa cell. Brit. J. Cancer 1988; 57: 553-558.

15) Ware, C.F. Protocol for a colorimetric assay to determine cell viability using Bio-Rad EIA microtitration plate reader. Bio-Rad Bulletin 1985; 1203:1-4.

16) Russo, A., Mitchell, J.B. Potentiation and protection of doxorubicin cytoxicity by cellular glutathione modulation. Cancer Treat. Rep. 1985; 69: 1293-1296

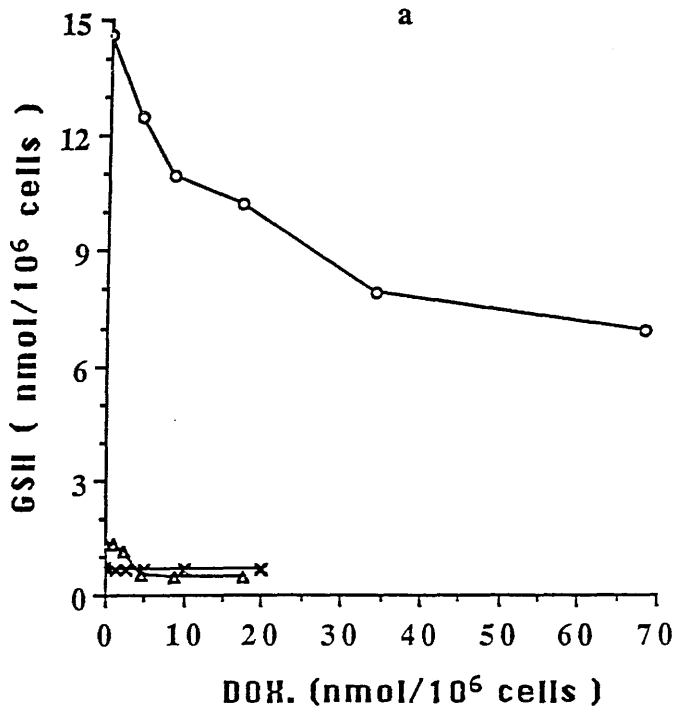
17) Hamilton, T.C., Winker, M.A., Louie, K.G., et al. Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem. Pharmacol. 1985; 34: 2583-2586.

18) Russo, A, DeGraff, W., Friedman, N., Mitchell, J.B. Selective modulation of glutathione levels in human normal versus tumour cells and subsequent differential response to chemotherapy drugs. Cancer Res. 1986; 46: 2845-2848.

19) Arrick, B.A., Nathan, C.F., Griffith, O.W., Cohn, Z.A. Glutathione depletion sensitizes tumour cells to oxidative cytolysis. J. Biol. Chem. 1982; 257: 1231-1237.

20) Shen, D-W, Cardarelli, C., Hwang, J. et al. Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. J Biol. Chem., 1986; 261: 7762-7770

Figure : 1


a
15
12
9
6
3
0
GSH (nmol/10⁶ cells)
0
10
20
30
40
50
60
70
DOX. (nmol/10⁶ cells)

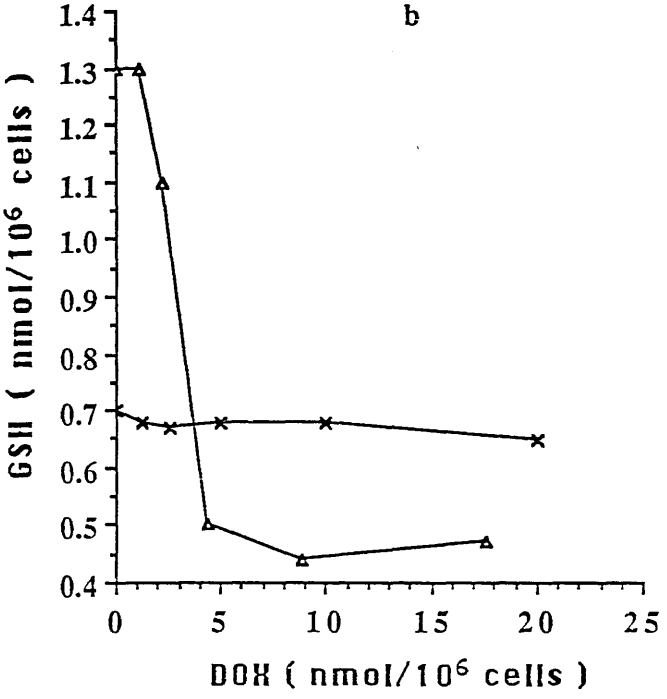

b
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
GSH (nmol/10⁶ cells)
0
5
10
15
20
25
DOX (nmol/10⁶ cells)

Figure : 2

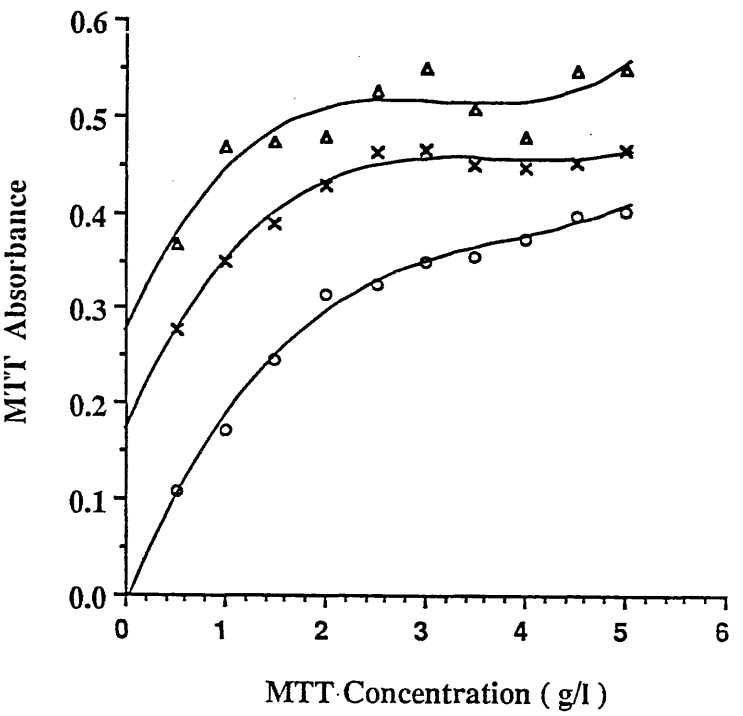
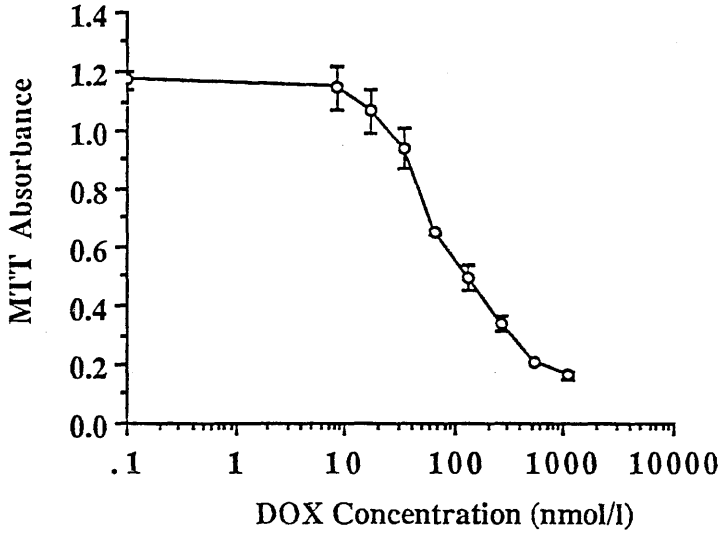

0.6
0.5
0.4
0.3
0.2
0.1
0.0
MTT Absorbance
0
1
2
3
4
5
6
MTT Concentration (g/l)

Figure : 3

a


MTT Absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
10000
DOX Concentration (nmol/l)

b

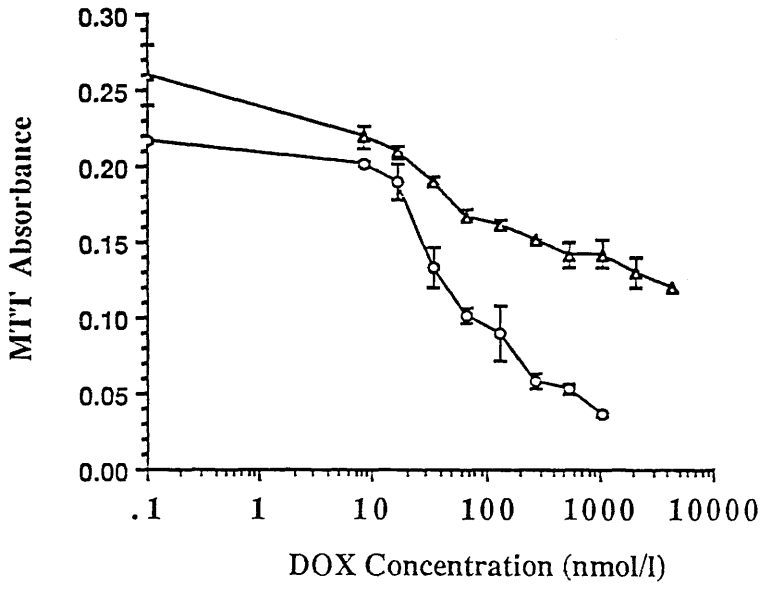
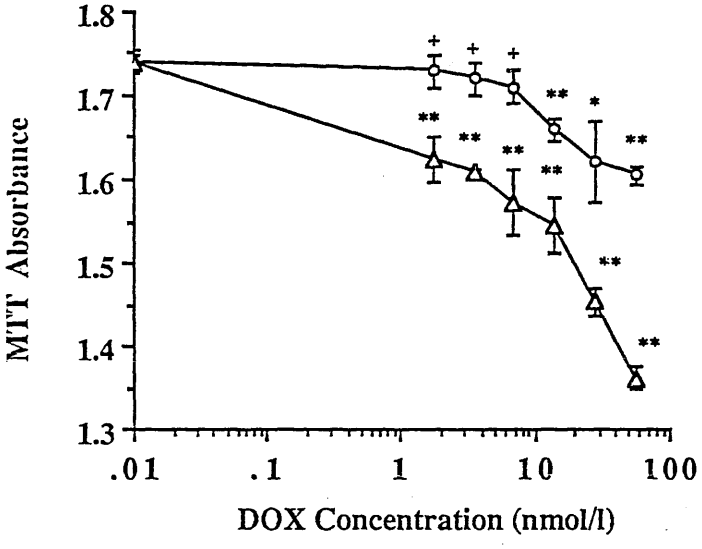

MTT Absorbance
0.30
0.25
0.20
0.15
0.10
0.05
0.00
.1
1
10
100
1000
10000
DOX Concentration (nmol/l)

Figure : 4


1.8
1.7
1.6
1.5
1.4
1.3
MTT Absorbance
.01
.1
1
10
100
DOX Concentration (nmol/l)