# CANCER CHEMOTHERAPY:

# USE OF LOW DENSITY LIPOPROTEINS

# AS TARGETING VEHICLES FOR TREATMENT

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## DECLARATION

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

SHARYN A. HYNDS

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ABBREVIATIONS

The following abbreviations are used in this thesis :-

ACAT : Acyl:Cholesterol Acyl Transferase
ACR : Absolute Catabolic Rate
A-LDL : Adriamycin-LDL Complex
Apo B : Apolipoprotein B
BOP : N-nitrosobis(2-oxopropyl)amine
BSS : Balanced Salt Solution
CBE : Chlorambucil-cholesteryl Ester
CE : Cholesteryl linoleoyl ether
CHD : 1,2-cyclohexanedione
CT : Cellobiose-tyramine
d : density
D-LDL : Daunomycin-LDL Complex
DMH : Dimethylhydrazine
DMPC : Dimyristoyl phosphatidyl choline
DMSO : Dimethyl sulphoxide
DNA : 6'-deoxyribonucleic acid
DNR : Daunorubicin, daunomycin
DOX : Doxorubicin, adriamycin
DPM : Disintegrations per minute
DPPC : Dipalmitoyl phosphatidyl choline
EDTA : Ethylenediaminetetraacetate
FCR : Fractional catabolic rate
FH : Familial hypercholesterolemia
GI : Gastrointestinal
Glu : Glutamate
HDL : High Density Lipoprotein
HEPES : N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HMG Co A Reductase: 3-Hydroxy 3-methylglutaryl coenzyme A reductase
HPLC : High pressure liquid chromatography
$ID_x$ : Dose of drug which inhibits cell growth by x%
IDL : Intermediate density lipoprotein
IV : Intravenous
LCAT : Lecithin:Cholesterol Acyl Transferase
$LD_X$ : Lethal dose which kills x% of those who receive it
LDL : Low density lipoprotein
MTX : Methotrexate
NaIOAc : Sodium iodoacetate
OD : Optical density
p : Probability
PBS : Phosphate buffered saline
R.E. : Reticuloendothelial
$R_f$ : Distance travelled by solute / distance travelled by solvent
RNA : Ribonucleic acid
rpm : Revolutions per minute
SDS : Sodium dodecyl sulphate
SRC : Subrenal capsular
SV 40 : Simian virus 40
TLC : Thin layer chromatography
TCA : Trichloroacetic acid
Tris : Tris(hydroxymethyl)methylamine
WBC : White blood cell

SUMMARY

The feasibility of using low density lipoprotein(LDL) as a selective drug-targeting vehicle is examined in this thesis. A number of animal tumours are seen to express considerable receptor-mediated uptake of the lipoprotein. The organs likely to suffer during chemotherapy using this carrier, viz. liver and adrenals, are afforded some protection by the administration of bile salts and hydrocortisone respectively. Several methods are employed for incorporating adriamycin, chlorambucil and daunomycin into LDL and the drug-LDL complexes so formed show enhanced cytotoxicity *in vitro* and *in vivo*. It is likely that LDL will be a useful targeting vehicle for anticancer agents if the level of drug incorporation can be increased.

## OVERVIEW

Despite recent advances in the design of cytotoxic agents, drug toxicity remains a major problem in the treatment of neoplastic disease. Consequently, there is much interest in methods by which the fraction of the drug dose reaching the tumour may be increased. One such method involves packaging the drugs into macromolecular vehicles, chosen for their ability to seek out tumour cells and deposit their contents only at those sites. In theory, this would markedly increase the efficacy of cytotoxic therapy while minimising any harmful side effects.

Over the years, numerous vehicles have been examined. Drugs have been attached to, for example, DNA[1], proteins [2,3], hormones[4] and monoclonal antibodies[5] and they have been encapsulated in liposomes[6] and liposome-antibody complexes[7]. *In vitro* experiments with liposomes were encouraging[6]. However, when drug-laden liposomes were administered *in vivo*, the cells of the reticulo-endothelial system rapidly removed the complexes from the circulation[8-11]. The work reported in this thesis investigates the potential of a "natural liposome" - Low Density Lipoprotein (LDL) - as a vehicle for cytotoxic drugs.

In 1978 Ho et al observed 3-100 times the normal rate of degradation of low density lipoprotein in human leukemic cells *in vitro* and in 1981 Gal et al[13] reported that a number of human gynaecological cancer cell lines grown in culture exhibited a higher uptake of LDL than did the equivalent non-neoplastic tissue. Other workers[14-23] have observed similar trends in several types of human and animal cancer cells. Recently Vitols et al[24] concluded that mononuclear cells in patients

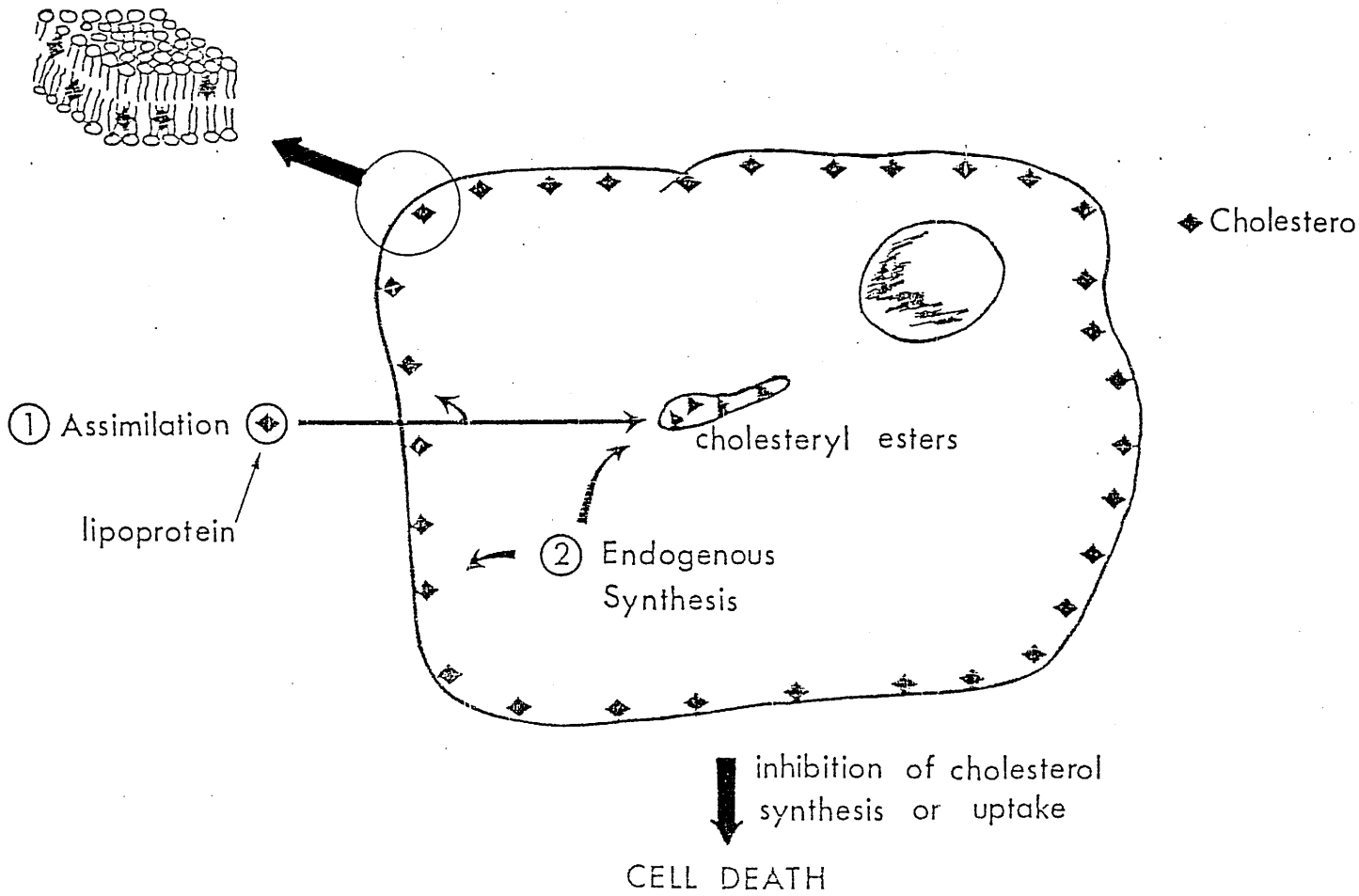
with acute leukemia were capable of metabolising about 35% of the total plasma LDL-cholesterol pool per day.

All cells require cholesterol and they may obtain this in two ways: by endogenous synthesis or by assimilation of cholesterol-containing particles, lipoproteins, from their environment (fig. 1). The most important lipoprotein in this regard is LDL, the major cholesterol-carrying particle in human plasma[26]. LDL consists of an apolar core of cholesteryl esters and triglycerides surrounded by a phospholipid monolayer containing free cholesterol and apoprotein B[27]. Cells may take up this lipoprotein by two distinct pathways, one of which involves specific high-affinity receptors on cell membranes[25]. Following binding to these receptors, located in coated pits[28] on the cell surface, an autoregulated sequence of events occurs which culminates in lysosomal degradation of the lipoprotein and subsequent release of the cholesterol for use in the cell[25].

The LDL particle possesses several characteristics which could be exploited if the particle is used as a drug vehicle:

1. Neoplastic cells readily internalise and degrade LDL by the high-affinity receptor pathway;
2. The LDL core has the capacity to store a substantial amount of drug;
3. Drug sequestration in the core space provides protection from serum enzymes and water; and
4. The entry of drugs into cells via the LDL pathway may circumvent drug resistance mechanisms[23].

Figure 1: CHOLESTEROL METABOLISM



PLAN OF WORK

Until recently the feasibility of using LDL as a vehicle for cytotoxic drug *in vivo* had not been investigated to any great extent. There were no reports of LDL assimilation by solid tumours *in vivo*, although several groups have documented high LDL uptake by tumour cells *in vitro*[12-23]. A brief outline of the plan of work for this project is given below:

(a) The quantitative significance of the LDL-receptor pathway was assessed in several animal models and attempts were made to protect the organs likely to suffer during chemotherapy using LDL as a vehicle.

(b) A number of cytotoxic drugs were chosen for loading into LDL. Several methods of incorporation were employed and the drugs were assayed by HPLC.

(c) The efficacy of the drug-LDL complexes was tested *in vitro* using human cancer cell lines and in mouse models *in vivo*.

# SECTION 1 : INTRODUCTION

## 1.1 LIPOPROTEINS - STRUCTURE AND FUNCTION

Cholesterol is an obligatory constituent of all mammalian cells. It is required for membrane synthesis and is also a precursor in the metabolic pathways of bile acid[29], steroid hormone[30,31] and vitamin D. Since it is insoluble in aqueous solution, cholesterol is carried in complexes with other lipids and protein in plasma[32,33]. These complexes are known as lipoproteins and are composed of apoproteins, cholesterol, cholesteryl esters, phospholipids and triglycerides. All lipoprotein particles share a common structure in which apolar neutral lipids are enveloped in a phospholipid membrane containing free cholesterol and apoprotein B[27,34]. Figure 2 shows a schematic model of a lipoprotein. The lipoproteins are not a single species but a heterogeneous mixture of particles of variable lipid and protein composition[27,34]. Several classes of lipoproteins may be identified by virtue of differences in size, density (fig.3) and other physical properties.

The five major classes of lipoproteins from human plasma, as defined by density, are:-

### 1.1.1. CHYLOMICRONS - d<0.95 Kg/L

These particles are high in triglyceride and are secreted into the lymphatic system by cells of the small intestine during the ingestion of dietary fat [33,35].

### 1.1.2 VERY LOW DENSITY LIPOPROTEINS (VLDL) d 0.95 - 1.006 Kg/L

The liver and small intestine secrete these lipoproteins into the plasma. Like chylomicrons, their main function is to transport triglyceride[33,35].

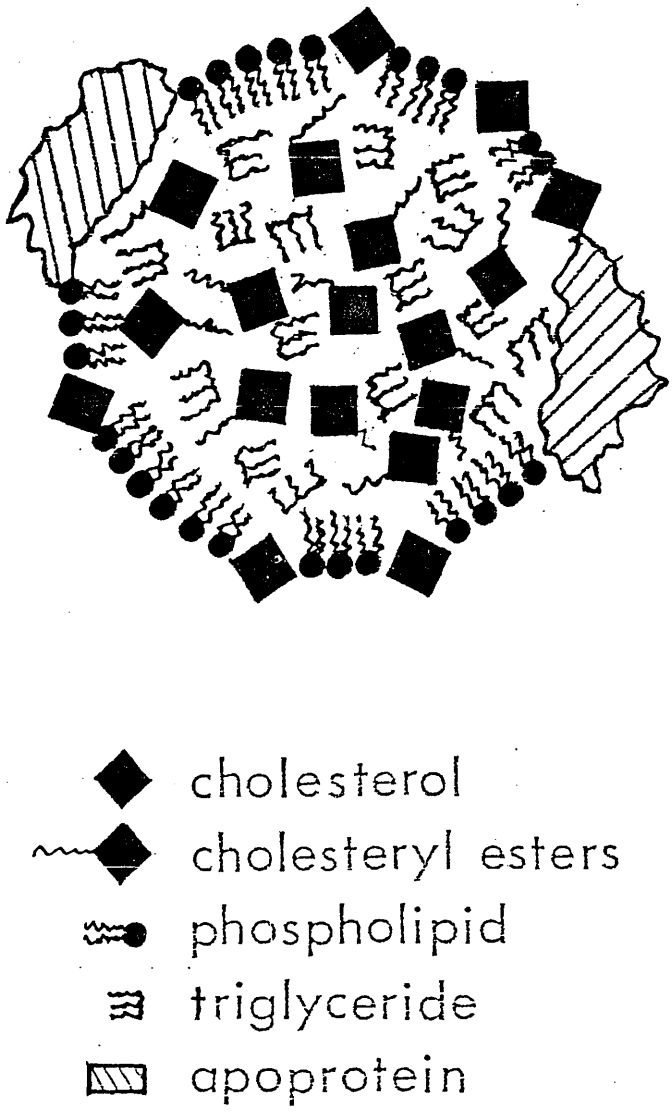
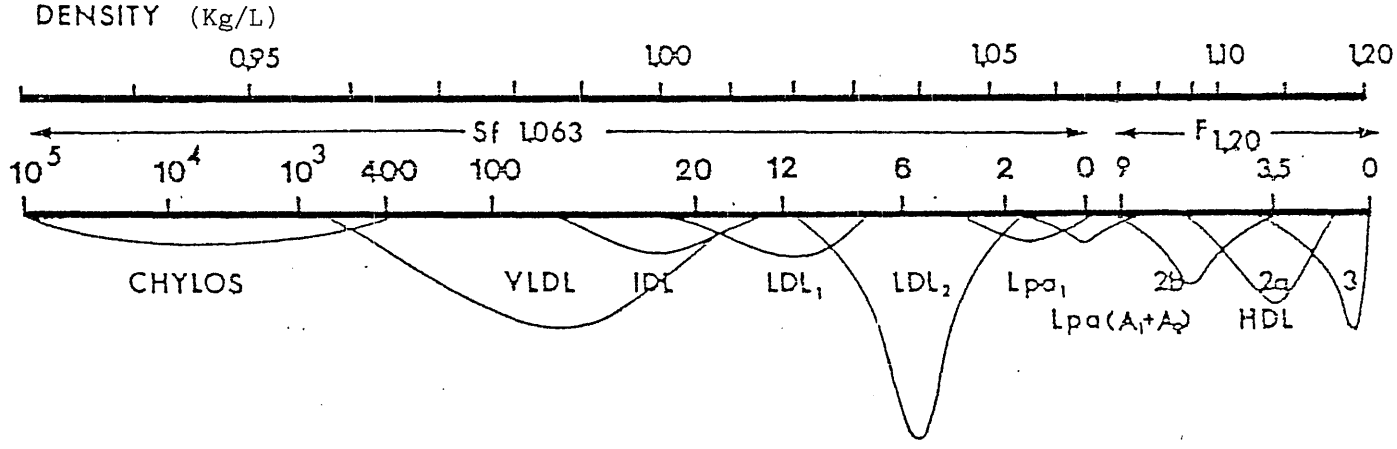



Figure 2: Schematic diagram of lipoprotein structure.

Figure 3: ULTRACENTRIFUGAL ANALYSIS OF LIPOPROTEINS



1.1.3. INTERMEDIATE DENSITY LIPOPROTEINS (IDL) d 1.006 - 1.019 Kg/L

IDL are produced in the plasma as intermediates in the metabolism of VLDL[36]. They may be removed from the circulation by the liver or converted to LDL in the plasma[33,36].

1.1.4 LOW DENSITY LIPOPROTEINS (LDL) d 1.019 - 1.063 Kg/L

These are the major cholesterol-carrying lipoproteins of plasma. Two-thirds of the circulating cholesterol is contained in LDL[26].

1.1.5 HIGH DENSITY LIPOPROTEINS (HDL) d 1.063 - 1.210 Kg/L

HDL are the smallest of the lipoprotein particles and their main lipid component is cholesteryl ester[33,37]. They are involved in the transport of endogenous cholesterol back to the liver from extrahepatic sites.

1.1.6 ELECTROPHORETIC PROPERTIES

Lipoproteins may also be classified according to their electrophoretic mobility relative to the major plasma globulins[35]. Thus chylomicrons remain at the origin, HDL migrate as the $\alpha_1$-globulin fraction and are termed $\alpha_1$-lipoproteins, VLDL, as $\alpha_2$, pre-$\beta$-lipoproteins, and LDL, $\beta$-lipoproteins.

The major properties and compositions of lipoproteins are summarised in table 1.

1.1.7 APOLIPOPROTEINS

Thirteen apolipoproteins have been isolated from human plasma[39] and the functions and primary sequences of many have been determined. They are commonly classified by the system of Alaupovic[39] into structurally and functionally related groups. Their importance in regulating

TABLE 1: PHYSICOCHEMICAL PROPERTIES OF HUMAN PLASMA LIPOPROTEINS[38]

| PROPERTIES | CHYLOMICRONS | VLDL | IDL | LDL | HDL |
|---|---|---|---|---|---|
| DENSITY (Kg/L) | <0.95 | 0.95-1.006 | 1.006-1.019 | 1.019-1.063 | 1.063-1.21 |
| ELECTROPHORETIC MOBILITY | Origin | pre-β | β | β | ≺ |
| $S_f$ * | >400 | 20-400 | 12-20 | 0-12 | - |
| PARTICLE DIAMETER (nm) | 120-1100 | 30-90 | 25-30 | 21-25 | 7-10 |
| COMPOSITION (mean % dry weight) | | | | | |
| MAJOR LIPID CONSTITUENTS | | | | | |
| TRIGLYCERIDE | 88 | 58 | 30 | 9 | 5 |
| PHOSPHOLIPID | 10 | 20 | 22 | 21 | 27 |
| ESTERIFIED CHOLESTEROL | 4 | 15 | 22 | 38 | 14 |
| FREE CHOLESTEROL | 2 | 8 | 8 | 9 | 5 |
| PROTEIN CONSTITUENT | 2 | 8 | 18 | 23 | 48 |
| MAJOR APOLIPOPROTEINS | C,B,A | C,B,E | B,C,E | B | A,C,E |

* Flotation rate in Svedberg units ($10^{-13}$cm/s/dyne/g) in NaCl solution of density 1.063 g/mL at 26°C

lipoprotein metabolism arises from their ability to interact with key enzymes and cell membrane receptors. The cardinal properties of apoproteins are shown in table 2.

## 1.2 LIPOPROTEIN METABOLISM

Two classes of lipids with distinct physical properties are carried by the lipoprotein transport system: the apolar lipids (cholesteryl esters and triglycerides); and the amphipathic lipids (cholesterol and phospholipids). These are organised into pseudomicellar structures in which the apolar lipids are sequestered from the bulk aqueous phase in the hydrophobic interior of lipoprotein particles (fig.2) Prior to their use in the cell the apolar lipids must be metabolised to yield free cholesterol and fatty acids[41]. The majority of triglycerides are removed by adipose tissue or muscle where their fatty acids are stored or oxidised to provide energy. Cholesteryl esters are transported to all cells of the body where they yield sterol for inclusion in membrane structures and as precursors of steroid hormones [30,31] and bile acids[29].

Cholesterol present in the circulation may originate from dietary intake or from endogenous synthesis in the liver and extrahepatic cells[42,43]. The two "types" of cholesterol are initially transported in different pathways - the "exogenous" and "endogenous" routes.

### 1.2.1. EXOGENOUS LIPID TRANSPORT

Dietary lipids are absorbed by cells of the small intestine and secreted into the lymphatic system as large triglyceride-rich chylomicrons[33,35]. The nascent chylomicrons possess apo B and

TABLE 2: HUMAN PLASMA APOLIPOPROTEINS[40]

| APOLIPOPROTEIN | DENSITY CLASS | MOLECULAR WEIGHT | PROPOSED FUNCTION |
|---|---|---|---|
| AI | HDL | 28,000 | Cofactor for LCAT |
| AII | HDL | 17,000 | Cofactor for Hepatic Lipase |
| AIV | CHYLOMICRONS | 45,000 | ? |
| B-100 | VLDL, IDL, LDL | 550,000 | Structural protein in VLDL, IDL & LDL. Receptor interaction in LDL pathway. |
| B-48 | CHYLOMICRONS, REMNANTS | 210,000 | Structural protein of chylomicrons |
| CI | VLDL, HDL | 6,500 | ? |
| CII | VLDL, HDL | 10,000 | Cofactor for Lipoprotein Lipase |
| CIII | VLDL, HDL | 10,000 | ? |
| D | $HDL_3$ | 20,000 | ? Cholesteryl ester exchange protein interaction |
| $E_2$-$E_4$ | VLDL, LDL, HDL | 35,000 | Receptor interaction in chylomicron remnant pathway |
| F | HDL | 30,000 | ? |
| G | VHDL | 75,000 | ? |
| H | CHYLOMICRONS & VLDL | 50,000 | ? |

apo AI and AII on their surface and obtain further apoproteins (apo C and apo E) from HDL upon entering the bloodstream. In the course of their metabolism 90% of the chylomicron apo C returns to HDL[44] while apo B, an essential structural component of chylomicrons, remains with the particle throughout its lifetime in plasma[45]. Chylomicrons are too large to pass through the endothelial barrier[46] and enter interstitial fluid. Their triglyceride is hydrolysed in the plasma compartment, yielding free fatty acids immediately adjacent to the cells which use them. This is accomplished by the action of lipoprotein lipase (E.C. 3.1.1.34) an enzyme which is attached to the luminal surface of capillary beds in muscle and adipose tissue. The lipase is activated by apo CII[47] on the chylomicron surface and the products of the reaction pass into adjacent adipocytes or muscle cells. Having lost much of its core, the chylomicron now has an excess of surface phospholipid which is shed and assimilated by HDL. The triglyceride-depleted particle, known as chylomicron remnant, is composed mainly of cholesteryl esters, with apo B and apo E as the major apoproteins. This lipoprotein is removed rapidly from the circulation by a receptor mechanism which recognises apo E on the particle surface[48,49]. Following binding the remnants are subject to endocytosis and transferred to lysosomes where they are degraded. The average half-life of chylomicrons in the circulation is only a few minutes[50,51] and consequently, in normal individuals, they are abundant only in post-prandial plasma. In this way dietary cholesterol finds its way to the liver, the major organ involved in regulating cholesterol homeostasis. Dietary cholesterol in excess of immediate metabolic requirements is secreted in bile, both as free cholesterol and in the oxidised form of bile acids[29,32,50,51]. Much of the former material is reabsorbed in the gut, transferred to chylomicrons and eventually returned to the liver, thus completing an enterohepatic

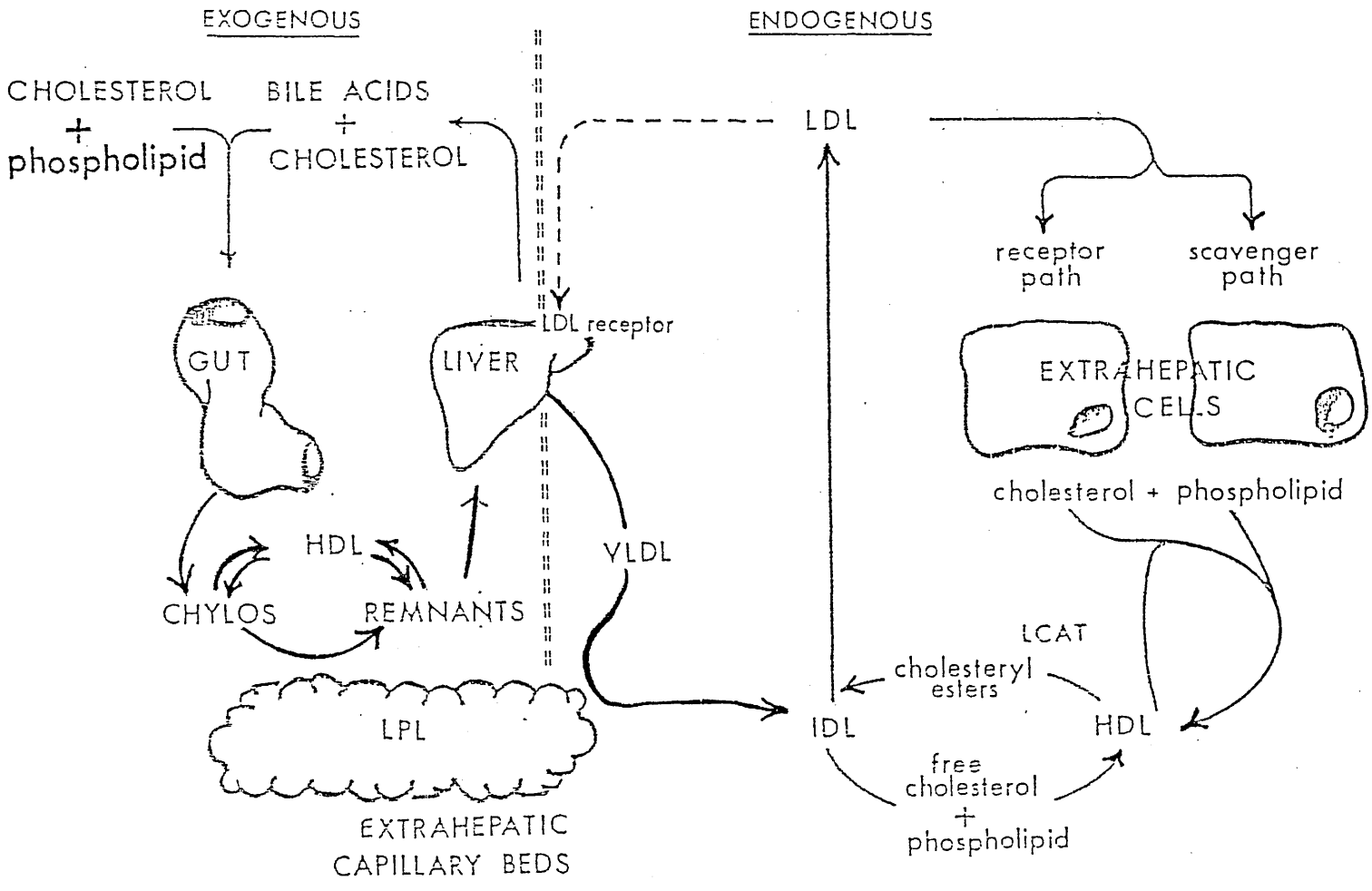
circulation, (fig. 4). Each time the cycle is completed a portion of the cholesterol is lost from the gut. Typically, humans acquire about 1 gram of cholesterol each day, 800 mg from endogenous synthesis and the remainder from dietary sources[51]. This is balanced by the faecal loss of neutral and acidic steroids.

### 1.2.2. ENDOGENOUS LIPID TRANSPORT

Fatty acids and carbohydrates are converted to triglyceride in the liver and this lipid is incorporated into lipoproteins prior to its secretion into the circulation. Similarly, cholesterol and cholesteryl esters are packaged in lipoproteins for delivery to extrahepatic cells. As noted above, some of the cholesterol is obtained from the diet and the remainder is produced endogenously[27,52] from acetate under the regulation of 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A Reductase (HMG CoA Reductase, E.C. 1.1.1.34) the rate-limiting enzyme in cholesterol biosynthesis[53].

VLDL are the major lipoproteins synthesised in the liver and when released into the bloodstream they, like chylomicrons, bind to lipoprotein lipase and, under its action, lose much of their tryglyceride. Apo CII is again required for activation of the enzyme[47] and is mainly acquired by transfer from plasma HDL[44]. The apo B of VLDL remains an integral component of the particle throughout the delipidation cascade to IDL and LDL[45]. The apo B of chylomicrons (termed apo B-48)[54] synthesised in the small intestine is known to be different from the VLDL apo B (apo B-100)[54] of hepatic origin. VLDL catabolism is much slower than that of chylomicrons, (half-life 1-3 hours[36]) due possibly to their lower efficiency of binding to lipoprotein lipase[51]. Again excess surface phospholipid and cholesterol is transferred to HDL. The particles produced by the action of lipoprotein lipase are the triglyceride-deficient and much smaller intermediate density

Figure 4: CHOLESTEROL TRANSPORT IN LIPOPROTEINS



lipoproteins (IDL)[36,55] which, like VLDL have Apo B as the major apoprotein, with trace amounts of apo C and apo E[45]. At this stage, there is some removal of the IDL from the plasma by the liver[56]. The remainder is metabolised to LDL, which are smaller particles, rich in cholesteryl ester, and containing apo B as the only apoprotein. The site of this second stage of conversion is uncertain but it is thought to occur in the liver. The acquisition of too much cholesterol by extrahepatic cells necessitates its export into the plasma. This is thought to be achieved by transfer of the sterol from the cell surface to HDL[57] and within the lipoprotein it is esterified by the action of Lecithin : Cholesterol Acyl Transferase (LCAT). The cholesteryl esters so formed are eventually transferred to VLDL, IDL and LDL via the agency of lipid transfer proteins[57,58]. Several genetic disorders result in elevation of one or more of the lipoprotein fractions. Such defects contribute to about 20% of all myocardial infarctions occurring in those under the age of 60[59].

### 1.2.3. THE LDL RECEPTOR PATHWAY

In man, LDL carries more than two-thirds of the total plasma cholesterol[26]. Plasma LDL has a half-life of the order of 2-3 days and delivers cholesterol to all cells of the body[60]. Cells may obtain the cholesterol they require by endogenous synthesis of the sterol from acetate units or from LDL. When available, LDL is usually the preferred source. The lipoprotein is assimilated by high-affinity and low-affinity routes. On binding to specific high-affinity receptors in coated pits on the cell surface, LDL is rapidly taken into the cell by endocytosis[61] (fig.5). The endocytic vesicle so formed migrates to the interior of the cell and fuses with lysosomes, exposing LDL to hydrolytic enzymes which release cholesterol, fatty acids, monoglycerides and amino acids. Cholesterol taken up in this way

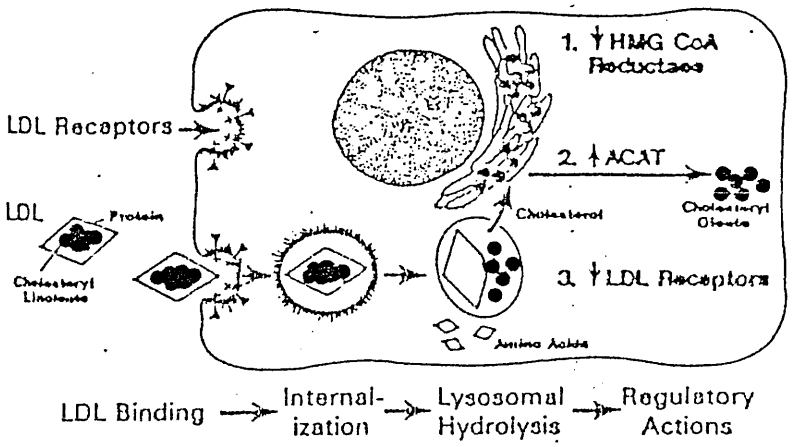



Figure 5: The LDL receptor pathway in cultured human fibroblasts[25].

(ACAT - Acyl : Cholesterol Acyl Transferase
HMG CoA Reductase - 3-Hydroxy 3-Methyl-Glutaryl Coenzyme A Reductase)

regulates several intracellular events which collectively mediate cholesterol homeostasis within the cell, i.e. the activity of HMG CoA Reductase, the rate-limiting enzyme in cholesterol biosynthesis, is reduced[62,63] and Acyl : Cholesterol Acyl Transferase (ACAT) activity is increased[64] so that LDL cholesterol may be esterified for storage as cytoplasmic lipid droplets. A further consequence of receptor-dependent LDL uptake by the cell is the suppression of receptor synthesis, which prevents over-accumulation of cholesterol[65]. Thus, by the use of these three mechanisms, the intracellular and extracellular sterol concentrations may be constantly balanced to maintain a stable cholesterol environment within the cell. About half of LDL cholesterol assimilation is accomplished via the receptor pathway in man. Low-affinity routes, e.g. bulk absorptive endocytosis, account for removal of the remainder of the LDL from plasma. One such route is thought to involve the reticulo-endothelial system[66,67] and when plasma LDL-cholesterol levels are high, macrophages ingest LDL and can become "foam cells" which are characteristic components of atherosclerotic plaques[68].

The exact proportion of cellular sterol which comes from receptor-mediated LDL uptake in each type of cell is uncertain but it is thought that de novo synthesis is sufficient for cells with a very slow rate of cholesterol turnover[25]. However, cells which are heavy users of cholesterol, e.g. adrenals[30,31] and liver[29,50], preferentially obtain cholesterol from the LDL receptor pathway.

### 1.2.4. THE LDL RECEPTOR

LDL receptors from bovine adrenal cortex were purified by Schneider et al[69] in 1982 and have been observed in a number of human and animal cells (table 3). The receptor molecule was found to be an acidic glycoprotein, with an apparent molecular weight of 164,000 on SDS

TABLE 3: CULTURED CELLS WHICH OBTAIN CHOLESTEROL VIA THE LDL RECEPTOR PATHWAY*

| | |
|---|---|
| HUMAN | Fibroblasts |
| | Fibroblasts transformed by SV40¥ |
| | Smooth muscle cells |
| | Endothelial cells |
| | Lymphoblasts |
| | Burkitt lymphoma cells |
| | Acute myelogenous leukemia cells[12] |
| | HeLa cells |
| | Epidermoid cervical carcinoma(EC-50)[13] |
| | Endometrial adenocarcinoma(AC-258)[13] |
| | Glioma cells[20] |
| | Hep-G2 Hepatoma cells[14] |
| | Non-small-cell lung carcinoma (L-DAN)# |
| MOUSE & RAT | Adrenal cells(Y-1 clone) |
| | Teratocarcinoma cells |
| | L cells |
| | L1210 leukemia cells |
| | P388 leukemia cells[144] |
| HAMSTER | Chinese hamster ovary cells |
| | Fibroblasts |
| DOG & SWINE | Fibroblasts |
| | Smooth muscle cells |
| COW | Adrenocortical cells |
| | Endothelial cells |

* From reference 28 unless otherwise stated

¥ Simian Virus 40

\# This thesis, Section 2.4.8

polyacrylamide gel electrophoresis. The protein chain is composed of 839 amino acids and may be divided into 5 domains, each with a particular function[70]. The $NH_2$-terminal 322 amino acids constitute the first domain. This section contains many cysteine residues whose sulphydryl groups are bound in disulphide links, thus conferring rigidity and stability on this part of the receptor. Overall the first domain is negatively charged and probably provides the site for interaction with the positive arginine and lysine residues known to be crucial in the binding of apo B and apo E[71]. Second and third domains are 350 and 48 amino acids long respectively. The third domain is immediately adjacent to the cell and probably serves to extend the receptor so that binding sites are clear of the membrane. The fourth domain spans the plasma membrane. This region of the receptor has 22 amino acids, all of which are apolar. The carboxy terminal end of the receptor forms the fifth domain which projects into the cytoplasm. It is thought that this sequence binds to clathrin or a clathrin-associated protein, enabling the receptor to be incorporated into coated pits[28]. The carbohydrate chains are attached, two through N-linkages to asparagine and 18 via O-linkages to serine and threonine[72]. The exact location of these chains on the protein backbone is unknown. The LDL receptor is synthesised as a lower molecular weight precursor in the rough endoplasmic reticulum and converted to the mature receptor in the Golgi complex by addition and modification of the carbohydrate side chains[73]. It is inserted into specialised areas of the plasma membrane, which allows it to function as an effective transport mediator. These areas are called "coated pits"[74]. In 1976 Anderson[74], in collaboration with Goldstein and Brown, showed that, in cultured human fibroblasts, LDL receptors were found almost exclusively in these regions of the plasma membrane. The characteristic feature of these indented segments of the plasma membrane is a cytoplasmic surface lined

predominantly with the protein clathrin of molecular weight 180,000[75]. Continually forming and pinching off, coated pits are transient in nature and their lifespan is less than five minutes[76]. Their rate of formation and internalisation is not affected by the presence of LDL or LDL receptors[28] and each pit contains receptors for many different macromolecules[28].

Unlike LDL, the receptor molecule is not degraded on fusion with lysosomes within the cell. Instead, it returns to the cell surface and into the coated pits[28]. This was demonstrated by blocking the synthesis of new receptors with cycloheximide and observing that there was no reduction in the rate of binding or internalisation of LDL by human fibroblasts over several hours[65]. The coated pit, enclosing the LDL-receptor complex, forms an endocytic vesicle which breaks away from the plasma membrane. The endosome, containing the LDL, which has lost its clathrin coat and its complement of receptors[28] is carried through the plasma to fuse with a primary lysosome[74] where its contents are exposed to hydrolytic enzymes. Here apo B is enzymically degraded to its component amino acids and cholesteryl esters are hydrolysed to yield fatty acids and unesterified cholesterol[26].

### 1.2.5 SPECIFICITY OF LDL RECEPTORS IN VITRO

The receptor binds LDL by interaction with apo B on the particle's surface[25]. This binding is dependent on a limited number of functionally significant arginine and lysine residues and it can be abolished by modification of these particular residues on the lipoprotein[79]. Studies with human fibroblasts by Mahley et al[78,79] showed that, in addition to apo B, the LDL receptor can bind apo E-containing lipoproteins, viz. chylomicrons and IDL. This property was discovered in experiments where dogs and swine were fed a high cholesterol diet which caused the appearance of an HDL species, $HDL_c$,

which is high in cholesteryl ester and contains apo E as the only apoprotein[78]. $^{125}$I-apo E-HDL$_c$, when placed in the culture medium of human fibroblasts, was taken up by LDL receptors with 10-25 times the affinity of LDL[80]. It was also observed that the receptors became saturated by HDL at 25% of the number of LDL particles required for saturation. Pitas et al[80] deduced that the enhanced affinity of HDL$_c$ was due to the fact that four apo E molecules in each HDL particle bound to a receptor on the cell surface.

### 1.2.6 HEPATIC LIPOPROTEIN RECEPTORS

The liver is known to remove chylomicron remnants, VLDL, IDL, and LDL from the plasma by receptor-mediated mechanisms[81]. Although as noted above, chylomicron remnants may be recognised by the LDL receptor, most are removed from the plasma by interaction with a receptor which is distinct from the former. Apo E binds to this receptor and initiates endocytosis and degradation of the lipoprotein of which it is part. Cholesterol so obtained by the cell suppresses HMG CoA Reductase[82] as in the LDL receptor pathway. In dogs $^{125}$I- apo E-HDL$_c$ is rapidly cleared by the liver via the "chylomicron receptor pathway". Clearance is retarded by preventing receptor binding by modifying the lysine residues of apo E. Liver uptake of HDL$_c$ is saturable and subject to competition from chylomicron remnants[48]. The observations that LDL does not compete for chylomicron remnant uptake, and LDL receptor-deficient animals and humans have normal chylomicron metabolism, indicate that the two receptors are distinct. This is further illustrated in familial dys-$\beta$-hyperlipoproteinemia, where a form of apo E is synthesised which is not recognised by receptors, and remnants and IDL persist in the plasma, but LDL is metabolised as normal[84].

A summary of the properties of the LDL receptor is given in table 4.

TABLE 4: PROPERTIES OF THE LDL RECEPTOR[#]

| | |
|---|---|
| LOCATION: | Concentrated in regions of cell surface membrane called clathrin coated pits |
| BINDING CAPACITY: | Saturation at 50μg of LDL protein per mL |
| BINDING AFFINITY: | $K_m$ – 2μg of LDL protein/mL at 4°C<br>10–15μg of LDL protein/mL at 37°C |
| BINDING SPECIFICITY: | Affinity for LDL more than 200-fold higher than for HDL. Affinity for canine $HDL_c$ 23-fold higher than for LDL[61]. |
| NUMBER OF BINDING SITES PER CELL: | 7,500–15,000 at 4°C, 1,500–70,000 at 37°C |
| MOLECULAR WEIGHT: | 164,000[69] |
| RATE AT WHICH BOUND LDL IS INTERNALISED INTO THE CELL: | $t_{½}$ – 3 minutes at 37°C |
| RATE OF TURNOVER OF RECEPTORS: | $t_{½}$ – approx. 20 hours |
| BINDING CHARACTERISTICS: | Requires divalent cations<br>Abolished by chemical modification of lysine and arginine residues of apoB & apoE[37] |
| BINDING pH OPTIMUM: | 7.5 |
| ENZYME TOLERANCE: | Sensitive to proteases.<br>Resistant to glycosidases. |
| METABOLIC REGULATION: | Receptor synthesis repressed by LDL, cholesterol or oxygenated sterols. |

# Data from reference 25 except where otherwise indicated

### 1.2.7 THE LDL RECEPTOR PATHWAY IN VIVO

Results of *in vitro* experiments examining the regulation of LDL metabolism within the cell cannot be quantitively extrapolated to apply to the *in vivo* system. In order to calculate the uptakes of LDL by the receptor pathway in various cells *in vivo* it would be necessary to know the exact LDL concentration at the surfaces of extravascular cells and the extent of receptor expression in each cell type. Also, the LDL concentration in human interstitial fluid[85] is known to be high enough for suppression of cultured human fibroblast LDL receptors. Other methods have therefore been devised for the study of receptor mechanisms *in vivo*.

Since the lipids of LDL are constantly exchanging[86], their labelling yields data of limited value in following the kinetics of the intact LDL particle. Instead, the apoprotein moiety of the LDL is usually trace-labelled since it remains with the particle throughout its lifespan in the plasma, and the amount of apo B per particle is constant[39,87]. Thus apo B metabolism reflects the metabolism of the whole LDL particle. In typical experiments donors are injected with their own LDL which has been labelled with $^{125}I$ [88]. Blood samples are withdrawn at intervals and the clearance rate of LDL determined from serial measurements of plasma radioactivity. Iodotyrosine, generated in the degradation of LDL is rapidly filtered from the circulation by the kidneys. When results are expressed as percentages of initial radioactivity, a decay curve for LDL catabolism may be constructed (fig.6). This allows calculation of the fractional catabolic rate (FCR) of apo B[88]. The decay curve for human $^{125}I$-LDL was found to be biexponential[89] and this phenomenon may be explained with the use of a simple two-compartment model (fig.7), with intra- and

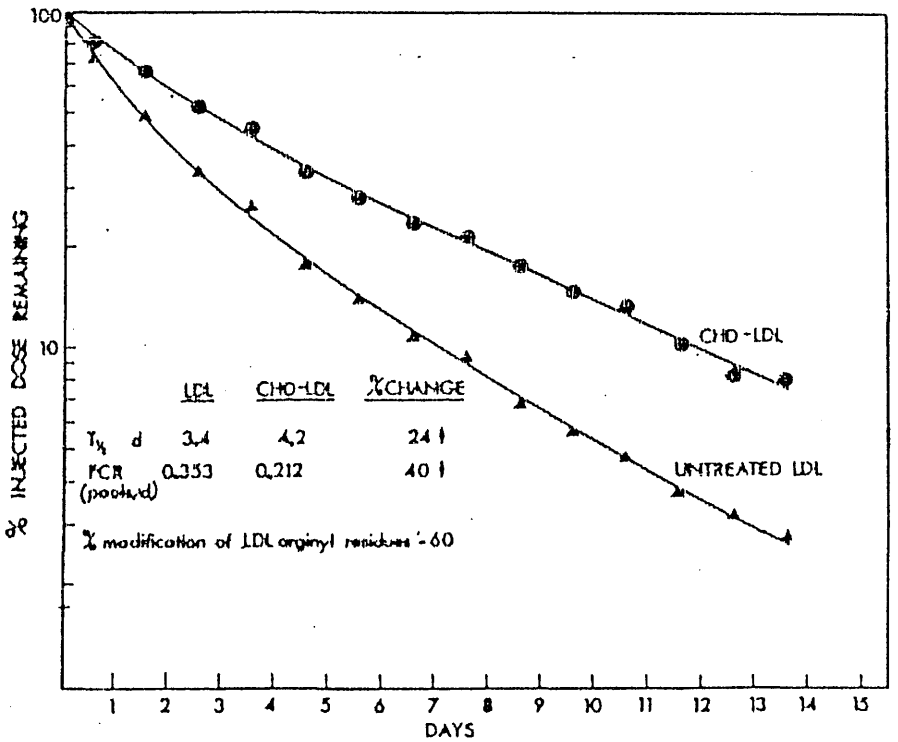



Figure 6: Plasma decays of Native- and 1,2-cyclohexanedione(CHD)-modified- LDL in man[93].

(FCR : Fractional Catabolic Rate)

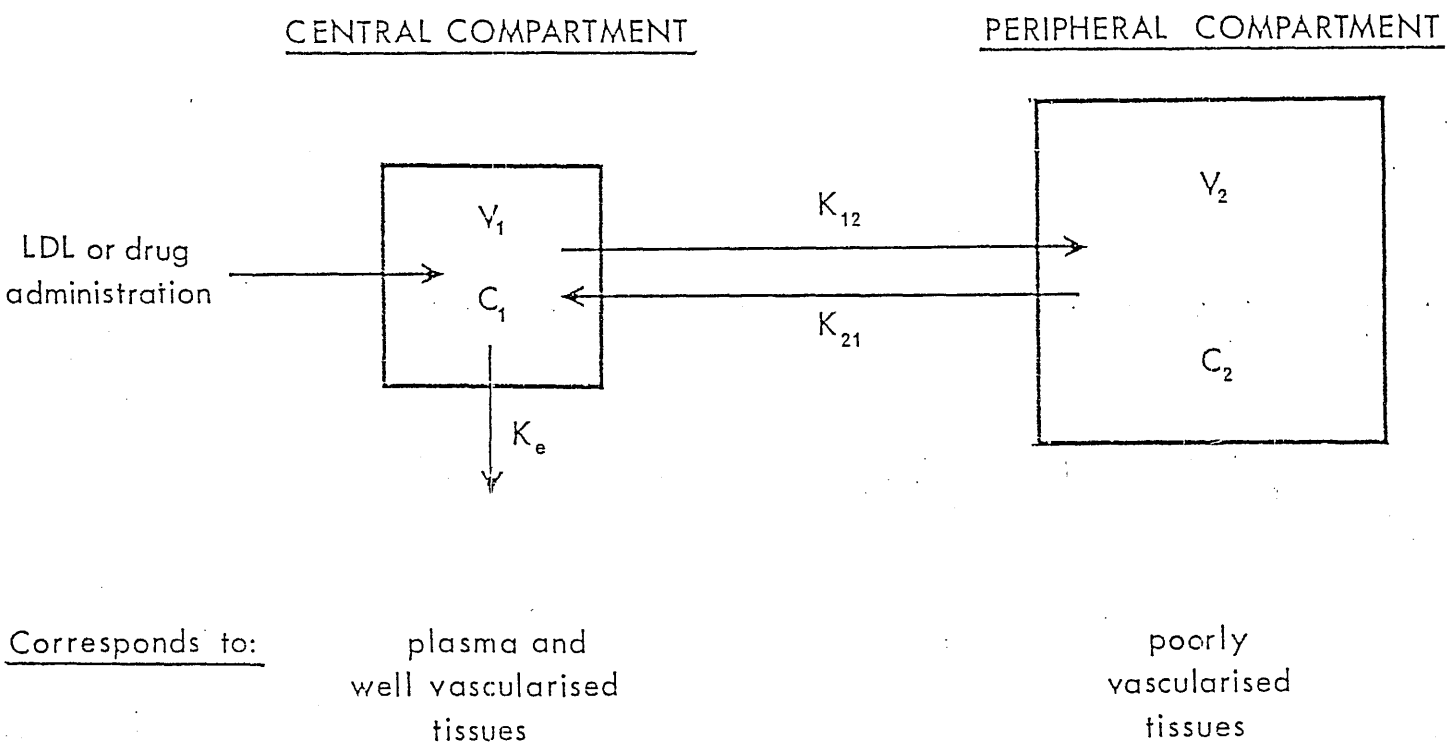



Figure 7: Diagram of the two-compartment model. $C_1$ and $C_2$ represent drug concentrations in, and $V_1$ and $V_2$ are the apparent volumes of, the central and peripheral compartments, respectively. $K_{12}$ and $K_{21}$ are first order rate constants of drug transfer between the compartments. $K_e$ is the first order rate constant for drug elimination from the central compartment.

extravascular pools. The first compartment contains approximately two-thirds of the total LDL pool. This encompasses LDL in the intravascular pool and in rapidly exchanging tissue spaces. The remaining LDL is located in slowly exchanging extravascular spaces. After 5-6 days the two pools have equilibrated and consequently the decay curve becomes monoexponential[89]. The FCR and apo-LDL concentration together are used to calculate the absolute catabolic rate (ACR), commonly expressed as mg Apo-LDL/day/Kg body weight. In the steady state the ACR is equivalent to the rate of synthesis of apo B[89]. Although this method allows investigation of total LDL kinetics in vivo, it reveals no information about the extent of the contribution of the receptor pathway to LDL catabolism.

As mentioned previously, the lysine and arginine residues on the binding site of apo B may be chemically modified so that the binding of the apoprotein to its receptors is abolished[90,91]. Consequently, LDL modified in this way cannot gain entry to cells by the receptor pathway. It may enter only by non-receptor-dependent mechanisms and therefore modified lipoprotein is cleared much more slowly from the plasma[92], (fig.6). The clearance of LDL by the receptor pathway may then be expressed as the difference between total LDL removed (native LDL) and the removal by receptor-independent methods (modified LDL). Using this approach, it has been shown that the LDL pathway makes a significant contribution to cholesterol metabolism in man[93,94], and several animal species[91,92,95]. Reports of the proportion of LDL cleared by receptor vary according to the method employed to modify the receptor-recognition site of apo B. Values are in the range 30-60% of the total LDL catabolised.

Studies conducted using 1,2-cyclohexanedione (CHD) - modified LDL appear to underestimate receptor activity[91,96,97]. The partial reversal of CHD-modification has been shown to occur spontaneously at

pH 7.4 *in vitro*[91,98], but this seems not to affect significantly the results obtained *in vivo*[99]. Reductive methylation of the lysine residues of apo B yields a stable modified LDL[100]. This complex is, however, rapidly cleared from the plasma in man and dogs, with a half-life of only a few minutes[100] suggesting that it is recognised as a foreign particle. In some species (rats, rabbits, monkeys), however, methylation is successful in retarding the removal of LDL from plasma and gives a reasonable estimate of LDL clearance by non-receptor routes[91,92,101]. More recently, LDL whose lysine residues have been glycosylated has been used as a tracer. Estimates of receptor-LDL uptake by this method indicate that more than 50% of plasma LDL is metabolised via the receptor pathway[94].

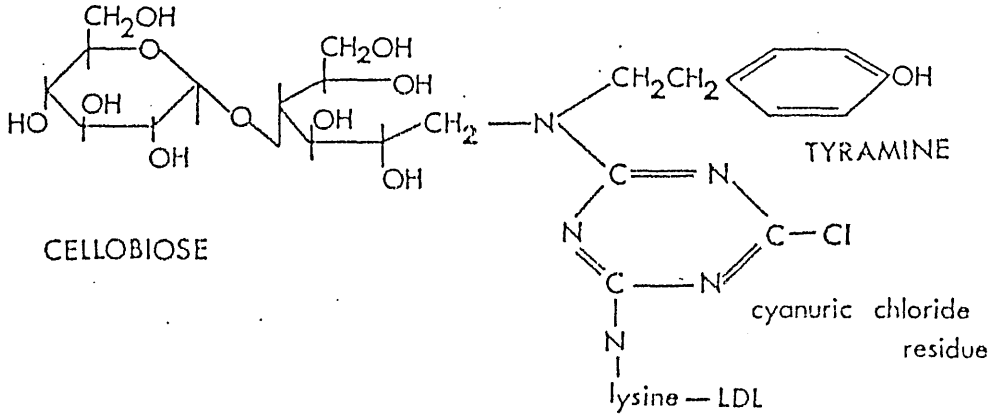
The above methods have also been used to assess the effects of genetic defects, drugs, and hormones on LDL catabolism[93,94]. For example, cholestyramine[102] and thyroxine[103] have thus been shown to stimulate the LDL receptor pathway in man and animals and thereby increase LDL catabolism.

### 1.2.8 SITES OF LDL CATABOLISM IN VIVO

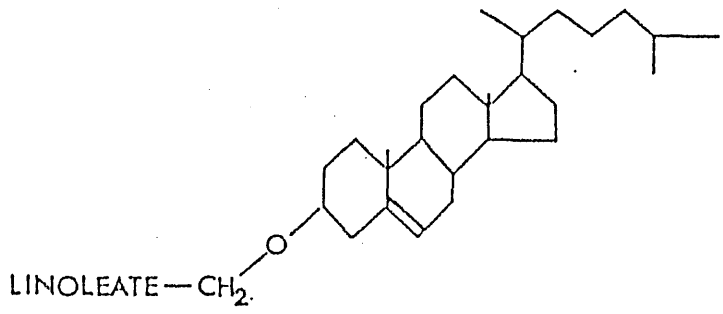
Different types of cells have different requirements for cholesterol, therefore, the degree of expression of cellular LDL receptors is variable. Several methods have been developed to assess tissue receptor activity *in vivo*. Assays on isolated cell membranes[30] have shown that the receptors are present on most tissues (table 3), although the majority have relatively low activity[61]. Those tissues which actively utilise cholesterol, such as liver, adrenal glands and ovaries are all high in receptors[61]. Adrenals have a rapid throughput of cholesterol which they use as a precursor for steroid hormones[30,31]. Similarly, ovaries require cholesterol as a substrate for hormone synthesis, while the liver utilises LDL cholesterol in the synthesis

of bile acids and lipoproteins.

A second method of determining tissue receptor status is to use non-degradable markers of LDL catabolism (fig.8). The radioiodinated LDL commonly used in kinetic studies is degraded by the cell, releasing iodotyrosine which is rapidly excreted by the kidneys. Thus the tissue uptake values obtained using this tracer reflect only the catabolism occurring immediately prior to assessment. Non-degradable tracers have the advantage of remaining inside the cells they have entered and give a cumulative assessment of LDL catabolism over the time-course of the experiment. Pittman et al have used $^{14}$C-sucrose[104] and $^{125}$I-cellobiose-tyramine[105] covalently bound to LDL as cumulative probes of the metabolic fate of the lipoprotein. Also, Stein et al[106] have studied the metabolism of LDL whose core has been loaded with non-degradable $^{125}$I-cholesterol linoleyl ether. Regardless of the tracer used, such experiments have indicated that the adrenal glands are the most active tissues in LDL receptor-mediated uptake per gram weight but, due to its size, the liver makes the largest contribution to receptor catabolism of LDL, removing over 50% of plasma LDL by this process[99,104,106,107].




CELLOBIOSE TYRAMINE-LDL




CHOLESTERYL LINOLEOYL ETHER

Figure 8: Non-degradable markers of LDL catabolism.

## 1.3 LDL AND CANCER

### 1.3.1. HYPOCHOLESTEROLEMIA IN CANCER

Hypocholesterolemia has been observed in many cases of cancer[108,109]. In 1930 Muller reported hypocholesterolemia as a frequent finding in cases of leukemia[110]. Since then several epidemiological studies have been conducted[111]. Of these 15 provided evidence that men in the lowest quintile of plasma cholesterol concentration were at increased risk of cancer and 12 showed no such relationship. Hence hypocholesterolemia, although common in cancer victims, cannot be used as a predictive factor. Until recently, there was considerable controversy as to whether the low plasma cholesterol observed in cancer patients is a cause of the neoplasm or an effect of its presence. Bases et al[112] showed that plasma cholesterol levels returned to normal in leukemic patients receiving successful chemotherapy, so supporting the latter argument. Further studies conducted in 1980[113] and 1982[114], indicated that most subjects with low plasma cholesterol who died of cancer, did so within 1-2 years of detection of the hypocholesterolemia and the concensus view now is that hypocholesterolemia in these patients was probably a consequence of cancer present, but undetected, at the time of blood sampling.

### 1.3.2. PLASMA LDL CHOLESTEROL

Ginsberg et al[115] examined the cholesterol content of lipoprotein fractions in groups of hypocholesterolemic-leukemic patients and normal subjects. The lowering of plasma cholesterol in the former was mainly due to a decrease in LDL cholesterol. The plasma concentration and metabolism of VLDL were found to be normal but the catabolic rate of LDL was significantly increased. They postulated that the increased mass of actively proliferating neoplastic cells had

elevated cholesterol requirements and so metabolised large quantities of LDL. This agrees with the findings of Gal et al[16] that cancer cells in culture exhibit high LDL receptor uptake and endogenous cholesterol synthesis when in a logarithmic growth phase. Gilbert et al[116] later showed that plasma cholesterol levels in a patient with chronic myelocytic leukemic fluctuated inversely with disease activity. This is further evidence that hypocholesterolemia is an effect of cancer rather than a cause.

### 1.3.3. LDL RECEPTOR ACTIVITY IN NEOPLASTIC CELLS IN VITRO

Ho et al in 1978[12] showed the rate of receptor-mediated degradation of LDL was 3-100 times higher than normal in mononuclear cells from patients with acute myelocytic leukemia,than in those from normal subjects. In these cells there was also a higher rate of endogenous synthesis of cholesterol. Despite the increased cholesterol availability in these cells, the cholesterol:protein ratio was 50% lower than normal. This implies a rapid turnover of cholesterol, which may be attributable to a rapid efflux of the sterol from the cells or, more probably, to a high rate of utilisation of cholesterol for cell growth and replication. The LDL receptor pathway in four human gynaecological cell lines was observed by Gal et al[16] to be subject to all the regulatory mechanisms observed in the pathway in human fibroblasts[65]. Furthermore, the rate of receptor-mediated degradation of LDL was found to vary according to the cell density in the cultures, with cells in log phase exhibiting the highest rate of LDL degradation. These workers later reported fifty times the LDL receptor activity in cultured neoplastic gynaecological tissue as compared to the equivalent non-neoplastic tissue cultures[13]. The cancer cells also had elevated HMG CoA Reductase activity and overall the neoplastic cells were metabolising much larger quantities of

cholesterol than their non-neoplastic counterparts. When the same cell lines were propagated in mice, the tumours had 15-30 times more binding capacity for LDL than any of the vital organs. Tumour cells may therefore exhibit a much higher uptake of LDL than "normal" tissues and LDL, on the basis of these observations may therefore constitute a good vehicle for the delivery of cytotoxic drugs or radioisotopes selectively to cancer cells. The classic LDL receptor pathway has also been observed in human hepatoma cells[14,117] and Leydig cells[118] in vitro, and LDL has been shown to provide cholesterol for steroid hormone synthesis in the latter.

More recently, Vitols et al[119] have correlated leukocyte LDL receptor activity and plasma cholesterol levels in leukemic patients. Mononuclear cells were isolated from the blood of healthy donors and from patients with leukemia. Elevated receptor-mediated degradation of LDL was observed in cells from leukemic patients and there was an inverse relationship between plasma cholesterol and receptor activity. On analysis of the chromosomes of leukemic cells[120], those taken from patients with significantly higher LDL receptor activity were found to have an extra chromosome 8. On the basis of this finding Lindquist et al[120] postulated that chromosome 8 may include genes of importance in the expression of the LDL receptor pathway. Further studies[24] revealed that leukemic cells in the bloodstream are capable of the catabolism of 35% of the plasma cholesterol pool per day. They conclude that the increased assimilation of LDL by leukemic cells may be the cause of hypocholesterolemia in acute leukemia.

## 1.4 CURRENT APPROACHES AND PROBLEMS IN CANCER CHEMOTHERAPY

The factors which influence drug efficacy in the treatment of diseases other than cancer apply equally in the management of cytotoxic therapy. As with any type of drug, absorption, binding, distribution, metabolism and excretion all affect the overall success or failure of treatment but, in addition, other properties of cytotoxic agents are important. For most anticancer agents the difference between an ineffective dose and a lethally toxic dose is very small, that is, these drugs have a very narrow therapeutic range[121](fig.9). Many of the drugs commonly used are relatively unstable and are effective at very low concentrations. Some agents work only on cells at a particular stage of the cell cycle[122]. Major organ dysfunction is not uncommon with cancer and may complicate chemotherapy since the absorption, excretion, etc. of the drug may be consequently modified, leading to increased toxic effects unless the dosage is changed accordingly. One way of improving cancer chemotherapy is to look for highly specific agents, but to-date there are no drugs which are completely specific for malignant cells. This is due to the lack of a qualitative difference between "normal" and "cancer" cells. For specificity, cytotoxic drugs rely on quantitative differences in distribution[123], the presence of target biochemical reactions within neoplastic cells[124], and differences in cell kinetics[125]. In general, tumours possess a large proportion of actively dividing cells and it is these cells which are most sensitive to cytotoxic therapy. Other rapidly dividing cells in the body, including bone marrow[126,127] and gastrointestinal tract epithelium[128] usually suffer the toxic effects of chemotherapy. A second method of obtaining better results with the anti-cancer compounds has been to take advantage of their dose response curve (fig.9) which has been shown in animal models[121] to be very steep. The number of tumour cells which

## DOSE RESPONSE CURVE

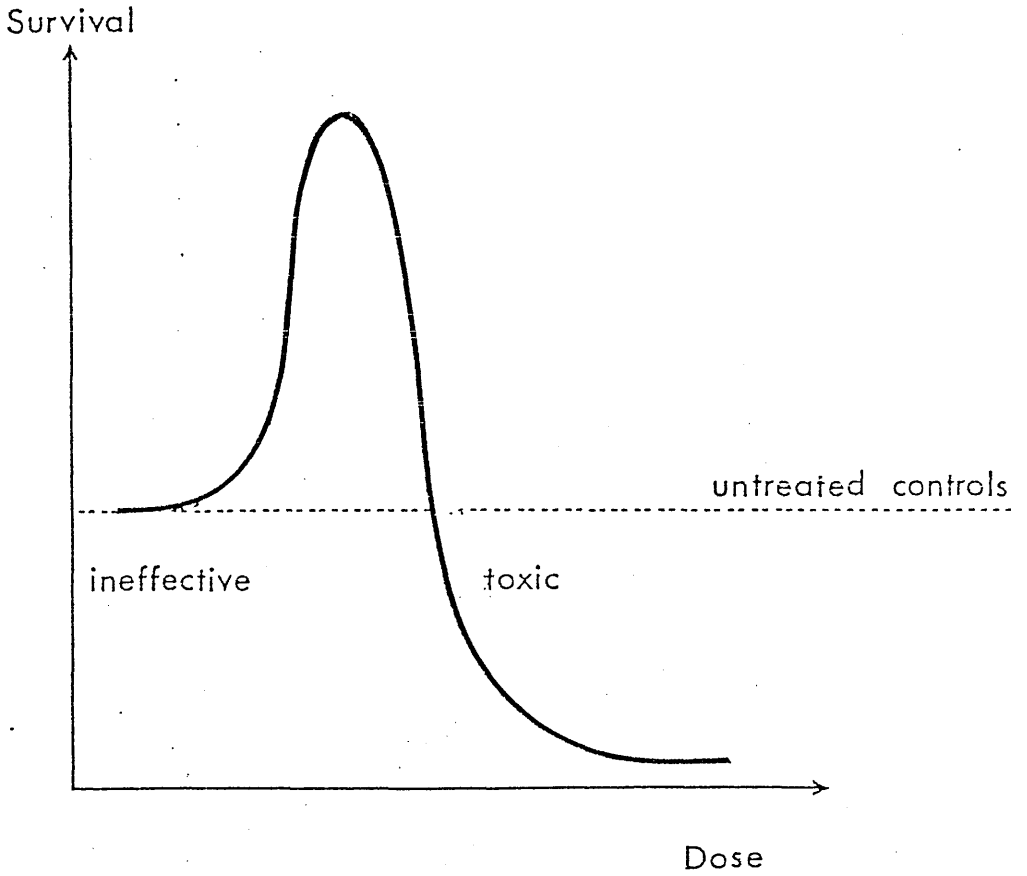



Figure 9: Dose response curve for cytotoxic drugs

survive may vary by several orders of magnitude between toxic and non-toxic doses. It is on this basis that high-dose chemotherapy is justifiable, despite the often inevitable myelosuppression and mucositis.

### 1.4.1 PHARMACOKINETICS

In order for any cytotoxic agent to be therapeutically effective, it must penetrate the tumour cell cytoplasmic membrane. Once inside, it must be present in sufficient concentrations and must be exposed to tumour for an adequate length of time. Physical and chemical properties of the drug determine tissue distribution and dictate its metabolism and excretion. These factors contribute to the concentration-time profile of the drug within body fluids, different tissues, cells and subcellular compartments.

The observed kinetics of many antineoplastic agents can be explained by a simple two compartment model (fig.7). This model assumes that the drug is distributed unevenly between a central compartment, of small volume, and a peripheral compartment of larger volume. Drug enters the central compartment and is rapidly distributed among the body fluids and tissues which it comprises. Although the two compartments are "theoretical spaces" and do not correspond directly to specific anatomical sites, the central compartment can be said to consist of serum and the extracellular fluid of well-vascularised tissues. Drugs in the serum and tissues within this compartment equilibrate rapidly. The peripheral compartment, in contrast, equilibrates very slowly with serum. This compartment is thought to consist of the more poorly-perfused tissues e.g. muscle, skin, fat and some tumours.

Drug may enter or leave the system only by the central compartment. The apparent volume of drug distribution for each compartment is dependent on the extent of blood flow to each tissue, the ability of

the drug to penetrate the tissues and the drug's affinity for them. The peripheral compartment serves as a reservoir for the central compartment. As drug is excreted from the central compartment, it is replaced by drug from the peripheral compartment.

### 1.4.2. DRUG ADMINISTRATION

There are several routes by which antineoplastic agents may be administered. The method by which a particular drug is given depends on its stability, solubility and sclerosant characteristics. Some drugs are unsuitable for oral administration because they are destroyed by enzymes or low pH in the gastrointestinal tract, or precipitate at acidic pH. Others cannot be given intravenously because they are insoluble in aqueous solvents.

Oral administration is convenient and suitable for many drugs, e.g. chlorambucil. However, gastrointestinal (GI) function is frequently abnormal in cancer patients as a result of pre-existing GI complaints, the presence of abdominal tumour or side effects of treatment. Even in the absence of GI complications there is great variability in absorption of drugs between patients.

Intramuscular, intraperitoneal, intrathecal and subcutaneous routes are less commonly used. Anthracyclines, for instance, cannot be given by these routes since the high local concentration of drug generated may cause bleeding and tissue necrosis around the injection site[129]. Intrathecal injection is useful in cases where drug would not otherwise penetrate the blood-brain barrier in sufficient concentrations.

Intravenous drug administration is the most common of parenteral methods. Drug is injected directly into the central compartment and a stable high drug concentration may be maintained over a defined time by continuous infusion. Drawbacks to this approach are the possibility of thrombosis or infection and drug extravasation may cause necrosis at the

injection site. Intra-arterial injection may be given to induce a high local drug concentration at the tumour. However, this requires surgery and a sizeable tumour artery must be located. This procedure, like IV administration, carries the risks of infection and thrombosis. Also, the administration of drugs like cyclophosphamide by this method is of no added benefit, since the drug requires enzymic activation in liver microsomes[130,131].

### 1.4.3. DISTRIBUTION, METABOLISM AND EXCRETION

The distribution of cytotoxic drugs in different tissues of the body depends on the chemical structure and physical properties of the drug in question. Polar drugs have difficulty in crossing lipid membranes and hence are largely excluded from the central nervous system. The concentration of drug in a particular organ is dependent on the size of the organ, the rate of blood flow through it, and the lipid solubility and polarity of the drug. Certain sites are relatively impermeable to polar drugs, e.g. central nervous system, testicles, large tumours. There may be other pharmacological "sanctuary sites" in which the penetration of particular drugs is limited. Response rates to chemotherapy in metastic breast cancer are higher in some metastases than others[132]. This may be a result of biochemical and kinetic differences between sites, but it is likely that drug distribution is important. A "third space" has been described where drugs may equilibrate very slowly with the central compartment. This third compartment probably includes pleural or peritoneal effusions. The rate of drug elimination from patients with these "extra" reservoirs may be so greatly prolonged as to produce increased toxic side effects.

Many cytotoxic drugs are extensively metabolised. Some require enzymic activation, e.g. cyclophosphamide becomes a potent alkylating agent only after metabolism by microsomal enzymes in the liver[130,131].

Nucleic acid antimetabolites are activated in several sites in the body, including tumour cells[133]. It is important that the sites of activation function properly. Organ dysfunction[123,124] may result in a prolonged half-life of the ineffective form of drug, with a failure to attain sufficiently high levels of the metabolised active agent. Conversely, in the case of drugs destroyed on metabolism or inefficiently excreted, toxic levels of drug may persist in the body for dangerously long periods.

Some drugs are excreted as the active drug, therefore impairment of the excretion of these drugs leads to increased toxicity and doses must be reduced accordingly. Anthracyclines are excreted in bile[135] and consequently any blockage of the biliary tract, e.g. gallstones, reduces or prevents excretion and anthracycline concentrations may rise above the safe therapeutic limit. Similarly, drugs excreted via the kidney are more likely to reach toxic levels and may even produce nephrotoxicity if administered to patients with kidney dysfunction[136].

### 1.4.4. CELL KINETICS

Growing tumours may be composed of both proliferating and non-proliferating cells, and within the former subpopulation, the cells may be at various phases of the cell cycle[122] (fig.10.) Thus in the optimisation of drug schedules account must be taken of the kinetics of tumour growth, since certain agents may be markedly more effective at particular stages of the cell cycle. As an alternative to classification by their mode of action, anticancer drugs are commonly classified according to the stage of the cell cycle during which they are most toxic. Thus three classes of cytoxic agent are defined:-

1) cell cycle non-specific

2) cell cycle and phase-specific

3) cell cycle non-phase-specific

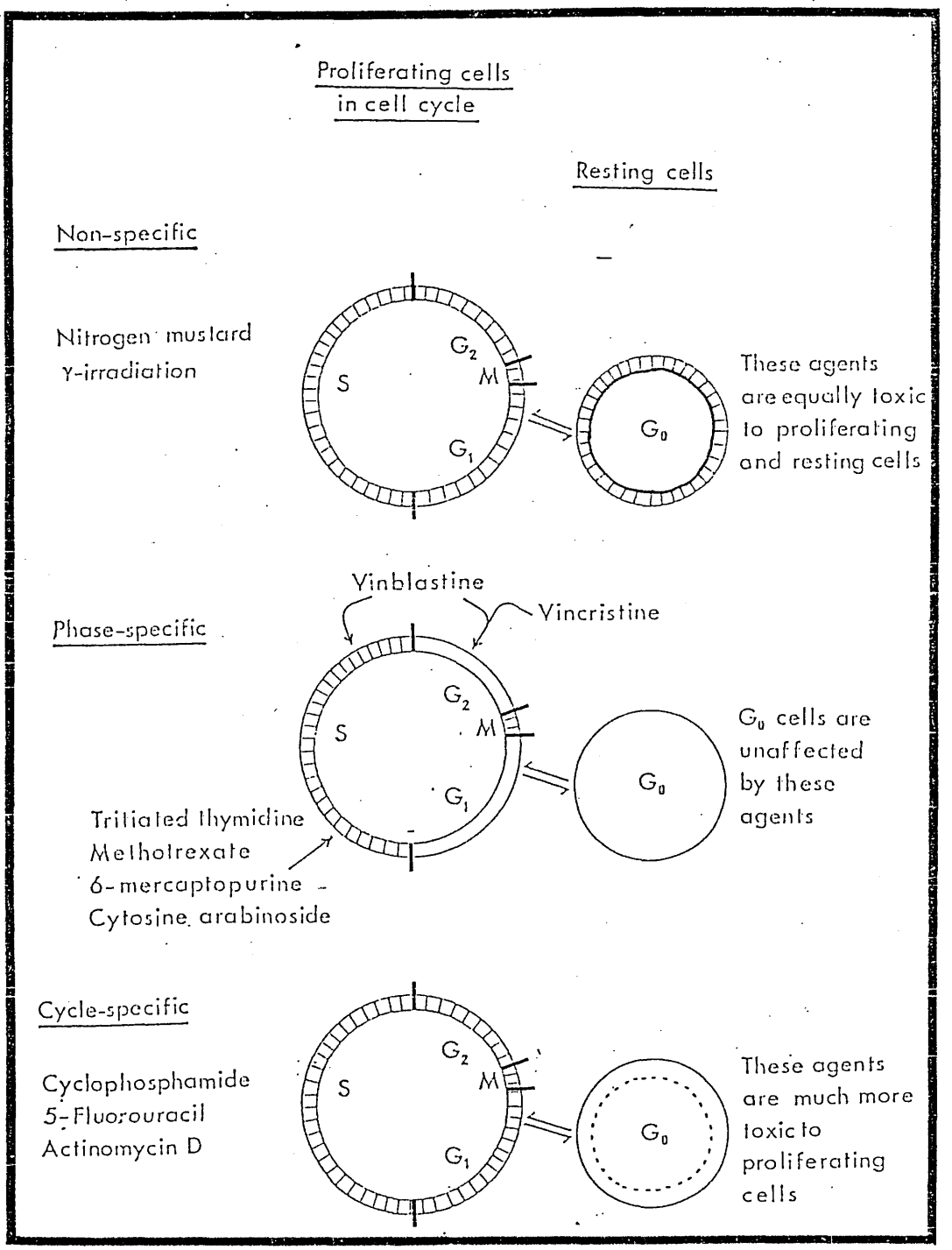



Figure 10: Cell phase and cycle specificity of cytotoxic drugs

*(Adapted from "Basic Principles of Cancer Chemotherapy", Calman, K.C. et al 1980 Pub. McMillan, LONDON.)*

The first of these classes includes γ-irradiation and nitrogen mustard, and these agents are toxic to both dividing and non-dividing cells. Methotrexate, vinblastine and cytosine-arabinoside belong to the second category. Drugs of this type are ineffective against cells which are not actively proliferating. The third type of drug e.g. cyclophosphamide, nitrosoureas, actinomycin D, is much more toxic to proliferating than non-proliferating cells. Since most tumours are composed of cells in various stages of the cycle, drugs from each of the above categories are commonly given in combination in order to maximise the proportion of tumour cells under attack during treatment. Consequently combination chemotherapy is usually more effective than single-agent therapy.

### 1.4.5. DRUG RESISTANCE

Drug resistance is often a complicating factor in the treatment of cancer. The resistance may occur in normal tissues as well as tumour and may be intrinsic (e.g. bleomycin and cytosine arabinoside are not effective against bone marrow and bowel cancer respectively) or acquired. Resistance may be conferred by a combination of pharmacological, biochemical and kinetic factors[137]. One beneficial aspect of acquired resistance is that normal cells may develop an increased tolerance of chemotherapy with repeated drug treatments. Acquired resistance[138] is often due to induction of drug metabolism[133] and changed kinetics, in both normal tissues and tumour. Another mechanism of resistance in tumours is believed to be a result of mutation, whether induced by drugs or irradiation therapy. In general, normal tissues are less susceptible to mutation since they contain a relatively smaller proportion of dividing cells than tumours. The frequency of mutation increases with tumour size and is variable between different tumour types.

### 1.4.6 DRUG TARGETING

Thus there are a number of problems which beset the clinical oncologist in his efforts to rid the patient of cancer cells and retain normal ones. There are two obvious ways of improving the benefit/risk ratio of cytotoxic drug therapy:-

(1) Improved Drug Design - drugs or prodrugs should be designed so that they have a high affinity for the site at which their action is required, or are only converted to their active form at that site. This should markedly increase the efficacy of therapy while minimising any harmful side effects. However, this approach is limited by the strength of the target/agent relationship which is generally far from ideal.

(2) The Use of Targeting Vehicles - As long ago as 1906, Ehrlich[139] proposed that molecules with an affinity for certain tissues could be used as carriers for therapeutic agents. By encapsulating drug within a vehicle, the distribution, uptake and overall efficacy of the drug may be beneficially altered so that tumour cells are damaged substantially more than normal cells. The ideal vehicle would deliver drugs only to their site of action and, once there, they would be passively released or internalised upon recognition of their target.

In addition to drug selectivity, numerous other factors, e.g. distribution, kinetics may act to reduce the intrinsic activity of a highly selective drug. Thus it is preferable to target drugs within some sort of carrier system. Drug vehicles should be non-toxic, biodegradable and of size and shape to allow the incorporation of many drug molecules, without evoking a response from the host's immune system. They should have a high affinity for their target site, while ignoring irrelevant areas. There must be a mechanism by which the drugs enter the cells along with, or after release from, their carrier. Several types of drug carrier have been investigated. These fall into three main categories: drug-antibody complexes; drug-macromolecule complexes;

and drug microspheres.

### 1.4.7. DRUG-ANTIBODY COMPLEXES

Drug-antibody complexes often have improved toxicity against malignant cells[5,140,141,142]. Binding of the immunoglobulin to its antigen on the cell surface is thought to initiate endocytosis[141], followed by lysosomal degradation of the antibody-drug complex and release of the free drug inside the cell. Isolation and purification of target-specific antibodies, however, remains difficult and it is also possible that the antigenic specificity of the tumour may change during its progression. Furthermore, drugs may distort the antigen-binding site, and render the complex susceptible to removal by the reticulo-endothelial system. The drug-antibody interaction may be subject to competition from endogenous circulating antibodies against target cells and the host may even develop an allergic reaction to the drug-antibody complex.

### 1.4.8. DRUG-MACROMOLECULE COMPLEXES

Phagocytes and some malignant cells are able to actively endocytose macromolecules. Among the many macromolecular carriers which have been studied are DNA, albumin, liposomes and LDL. Deprez-de Campeneere and co-workers[1] have studied daunorubicin (DNR) and doxorubicin (DOX) linked covalently to DNA. In mice with leukemia, both complexes were more effective than the native drugs and the overall toxicity of DOX-DNA was significantly less than that of doxorubicin. Cornu et al[143] have shown drug-DNA complexes to be effective on certain tumours in man. Proteins such as albumin have also been used as carriers. β-amantin coupled to albumin[144] was selective in damaging kidney proximal tubules and liver sinusoidal cells *in vivo*. Daunorubicin covalently bonded to albumin[3] was found to be more effective than the

free drug, both in vitro and in vivo. The major drawback of drug-protein carriers is that liver and kidneys will suffer during chemotherapy since they constitute the main sites of protein endocytosis.

A great deal of work has been done with drug-liposome complexes, where the drugs are carried entrapped in lipid vesicles[6]. Although liposomes provide a protective environment for the drug, they are leaky and small drug molecules may easily diffuse out into the plasma. This has been overcome to some extent by incorporating drug-macromolecule complexes e.g. DNA, poly-L-Glu[145] into the liposomes. In early work, the size of the lipsomes used (μm) precluded their entry to small capillaries and induced their rapid clearance from the plasma by cells of the R.E. system[8-11]. Recently, much smaller, more stable particles have been synthesised, but the liver, spleen and lungs remain the major targets for liposomes in vivo[8-11]. Inclusion of monoclonal antibodies in the liposome surface has increased the specificity of the carrier and, consequently, the efficacy of the drug[146]. In the case of methotrexate in liposomes conjugated with antibody to L929 murine fibroblasts[7], a 10-fold increase in drug efficacy in vitro was observed. Another promising macromolecular drug carrier is LDL. LDL is, in effect, a "natural liposome" and as such has many of the advantages of liposomes, with the added benefit that its removal from the circulation by the R.E. system is likely to present less of a problem. The potential of LDL as a drug vehicle is discussed fully later (1.4.10).

### 1.4.9. MICROSPHERES

These are relatively large (0.2-300 μm) microspherical colloids of polymers such as albumin[147]. They are employed in the controlled localised release of drugs. Their target is dictated largely by their

size. Very small microspheres are cleared from the circulation by macrophages of the R.E. system and are ultimately delivered to Kuppfer cells in the liver. Particles 7-12 μm become trapped in the lungs. Liver and spleen filter out particles 2-12 μm. Particles larger than 12 μm stick in the first capillary bed they encounter. Thus large microspheres will become entrapped in tumour vasculature if they are injected into the arterial supply of the tumour[148]. A high local concentration of drug is therefore achieved with reduced effects on non-target tissues. Microspheres 1-2 μm may be coated with magnetic substances[149] and directed to the required sites by external application of two-dimensional magnetic fields. Senyei et al[150] demonstrated that magnetic carriers of doxorubicin can achieve high levels of drug in the tumour with no drug in any other organ.

### 1.4.10 STRUCTURAL AND FUNCTIONAL ADVANTAGES OF LDL AS A VEHICLE FOR CYTOTOXIC DRUGS

Low density lipoproteins are particles composed of an apolar core of cholesteryl esters and triglycerides surrounded by a phospholipid monolayer containing free cholesterol and apoprotein B[27,34]. The proportions of each of the lipids is shown in table 1. The apolar lipid core contains approximately 1500 cholesteryl ester molecules and provides an excellent site for sequestration of lipophilic molecules. Since the studies of Rudman et al[151], it has been observed that numerous lipophilic agents partition into the lipoprotein fractions *in vivo*. Gal et al[13] indicated that LDL might prove to be a suitable vehicle for targeting of cytotoxic agents. Membrane fractions from human tumour cells (EC50), after propagation in mice, were found to have 15-30 times the affinity for LDL expressed by vital organs of the mouse. Studies in our own laboratory[152] and by other workers[153,154] indicate too that mouse tumours *in vivo* have a high affinity for LDL,

which they obtain by the high-affinity receptor pathway. Cell experiments have shown that the LDL pathway in neoplastic cells[13,14] has all the characteristics of the classic LDL receptor pathway described by Goldstein and Brown in cultured human fibroblasts[61]. On binding to its receptors on the cell membrane, LDL is rapidly endocytosed and degraded in the lysosomes liberating its charge to the inside of the cell (fig.5). LDL has the potential to act like the "ideal" drug vehicle in many respects[155], providing adequate amounts of drug for therapeutic activity can be incorporated into the lipoprotein.

### 1.4.11 IN VITRO STUDIES WITH DRUG-LDL COMPLEXES

Recently numerous groups have reported successful arrest of fibroblast or tumour cell growth in vitro using antineoplastic agents carried in LDL.

Using "reconstituted" LDL[156], Mosley et al[18] delivered the photosensitising agent, pyrene, via the LDL receptor pathway. Subsequent exposure to U.V. light led to cell death, since pyrene activated in this way produces oxygen radicals. Human A431 epidermal carcinoma cells, SV40-transformed human fibroblasts and chinese hamster ovary cells were among those killed using this method. Fibroblasts from a patient with familial hypercholesterolemia (FH) which had no LDL receptors, were unable to take up LDL by the receptor pathway. Consequently, pyrene accumulation in these cells was very low and they were not killed after exposure to U.V. light. In later experiments[19], chinese hamster ovary cells pre-incubated with pyrene were exposed to LDL containing photoprotective azo dyes, scavengers of singlet oxygen. After U.V. irradiation, cells exhibiting high LDL receptor activity were undamaged by the pyrene and it was established that the photoprotective dyes were delivered to the cells by a receptor-dependent process.

In 1983, Rudling et al[20] showed that cellular accumulation of LDL containing the anthracycline Aclacinomycin A was dependent on LDL receptor activity in cultured human glioma cells. LDL accumulation was found to be highest when the cells were proliferating rapidly. The receptor-dependent uptake of drug-LDL was subject to competition from native LDL but not HDL. Chloroquine, an inhibitor of LDL degradation caused increased LDL receptor uptake. The drug-LDL complex inhibited growth in glioma cells but not in FH fibroblasts.

Firestone et al[15] synthesised a number of lipophilic derivatives of cytotoxic agents with a view to reconstituting them in LDL. Two of these derivatives, both steroid nitrogen mustard carbamates, reconstituted well with LDL and were able to arrest the growth of chinese hamster ovary cells and SV40-transformed human fibroblasts at low drug concentrations. Using one of these compounds, (N-[[[2-[3β-(oleoyoxy)-androst-5-en-17β-yl] propyl] oxy] carbonyl]-N,N-bis (2-chloroethyl)-amine), the growth of 98% of the cells was arrested at 10 μg/mL of the drug in LDL. However, only 50% of these cells were actually killed. Increasing the drug concentration gave no enhancement in cytotoxicity. There were two possible reasons for this: 1) saturation of the LDL pathway; and 2) inefficient hydrolysis of the drug-steroid bond in the lysosomes. A second derivative (N-[[[4-[3β-(oleoyloxy)androst-5-en-17β-yl] pentyl] oxy] carbonyl]-N,N-bis-(2-chloroethyl)amine) was synthesised, in which the steric congestion around the drug-steroid link was reduced, so facilitating the rapid enzymic liberation of free drug within the cell. This derivative, when reconstituted with LDL was able to kill 100% of the cells at concentrations of 5 μg/mL and above.

Lipophilic derivatives of adriamycin were used by Vitols et al[21,22] for reconstition in LDL. In cultured human fibroblasts, accumulation of (N-(N-retinoyl)-L-leucyl doxorubicin-14-linoleate)-LDL[21] was measured

and found to be equivalent to the theoretical uptake implied by the observed rates of LDL assimilation and degradation. As in the aforementioned studies, FH fibroblasts showed little or no uptake of the drug-LDL complex. Drug-LDL complexes whose receptor-binding ability had been abolished by methylation were not assimilated by the cells. The drug-LDL complex was found to inhibit growth of normal fibroblasts. In human white blood cells from normal and acute myelocytic leukemia patients, the uptake of AD-32-LDL[22] was many times higher than the amount which could have been assimilated by the receptor pathway alone. Therefore, in this system, the authors concluded that non-specific mechanisms were responsible for the accumulation of a large proportion of the drug. This was analgous to the results of Remsem and Shireman[157] who observed that the carcinogen benzo(a)pyrene, when carried within LDL, was able to enter cultured fibroblasts in significant amounts even in the absence of LDL receptors, apparently via a rapid distribution of the compound between lipoprotein and cell membrane.

Daunomycin was incorporated into LDL by Iwanik et al[23] using a simple incubation procedure. After fluorescence quenching with DNA and KI, they concluded that the daunomycin had partitioned into two domains of the LDL particle viz. the core and the surface. An interesting finding was that the complex was toxic to both daunomycin-sensitive and daunomycin-resistant P388 leukemic cell sublines. In both cases more drug entered the cells when daunomycin-LDL was administered than when free daunomycin was used. Subfractionation of subcellular organelles showed the drug to be located mostly (86%) in insoluble nuclear material when native daunomycin was used. After exposure to the drug-LDL complex, some of the drug was found in the plasma membrane and nuclear membrane fractions, but the majority (65%) was in the mitochondrial-microsomal-lysosomal membrane fraction and insoluble

nuclear pellet. The daunomycin distribution in organelles correlated well with the distribution of $^{125}I$-labelled native- and daunomycin-LDL. In subsequent work[158], the same group showed the LDL-drug complex to be more cytotoxic than the native drug at shorter exposure times. Uptake of daunomycin-LDL was subject to competition from LDL but not HDL. On analysis of the DNA content of treated cells it was apparent that cells exposed to daunomycin-LDL had accumulated in the $G_2$-M phase of the cell cycle, whereas those given daunomycin were evenly distributed in each phase, thus illustrating the enhanced cytotoxic effects of the daunomycin-LDL delivery system.

Halbert et al[17] linked methotrexate (MTX) covalently to the apo B moiety of LDL. The complex was thirty times less active against murine leukemia in vitro than native drug. It is likely that the drug binding to apo B caused distortion of the protein molecule, so preventing its recognition by the LDL receptor, although it has been reported[159] that other covalent methotrexate-macromolecule complexes are less active than MTX when tested under similar conditions. Another possible explanation for this decreased cytotoxicity is that methotrexate is not being efficiently hydrolysed from LDL and insufficient concentrations of free MTX are being released inside the cell.

### 1.4.12 LDL AS A CYTOTOXIC DRUG VEHICLE IN VIVO

From the results of the many cell experiments with drug-LDL complexes, it is apparent that LDL has potential as a drug vehicle. It carries drug in appreciable quantities primarily to cells which express specific LDL receptors. On binding to the receptors, the drug, inside LDL, is internalised by endocytosis and transferred to the lysosomes. Here the complex is degraded releasing the drug, free to apply its cytotoxic action within the cell. LDL is an endogenous particle and as

a carrier, it is not susceptible to the possible resistance mechanisms and allergic responses that some other macromolecular drug vehicles evoke.

For LDL to act as a drug-vehicle *in vivo* it is crucial that:

1) the structure of the particle is not changed in such a way that it is rendered "foreign";
2) the conformation of apo B is maintained to allow efficient binding to LDL receptors.

Some of the above studies[15,18,19,21,22,157] used LDL which had been freeze-dried and reconstituted with drug by Kreiger's method[160]. This procedure was originally developed for replacing cholesteryl esters in the core of LDL with their labelled counterparts[156]. The particles produced act like native LDL *in vitro* but, when injected *in vivo*, they are recognised as foreign and cleared rapidly from the circulation by cells of the R.E. system.

Masquelier et al[161] found that when AD-32-LDL was injected into mice, 98% of the complex was removed from the plasma within 90 minutes. By comparison only 40% of injected native LDL was removed. High relative uptakes of AD-32-LDL were found in liver, kidneys and spleen, indicating that removal of the complex was mediated largely by the R.E. system. In an effort to reduce the rate of clearance of the complex, the method of drug incorporation was modified. Lyophilisation of LDL with 25% sucrose instead of starch, followed by reconstitution with AD-32 as before, produced a particle which acted like native LDL *in vivo*. The possibility of using a complex in which AD-32 was covalently linked to lysine residues on apo B was examined. As reported by Mahley[82], modification of more than 15% of the lysine residues greatly inhibits apo B-receptor interaction. Consequently, degradation of the drug-LDL

particle decreased with increasing conjugation of drug to protein. The authors concluded that drug could be incorporated into LDL, producing particles which acted like LDL *in vivo*.

The use of LDL as a drug vehicle is, of course, not without its drawbacks. Drug-LDL has the potential to harm any of the cells in the body, since they all utilise LDL to some extent. Organs which, under normal circumstances, use large quantities of LDL, namely adrenal glands and liver, are likely to suffer along with tumour during chemotherapy, unless steps are taken to protect them. Drug toxicity will be limited by the rate of the receptor mechanism and it is therefore possible that the receptor pathway could become saturated before toxic drug levels are reached within the target cells. Drug uptake via LDL would also be subject to dietary influence, since cellular drug accumulation would obviously be lowered when receptor expression had been downregulated in response to dietary intake of cholesterol, and vice versa. Another potential drawback is the current lack of techniques for making synthetic LDL which acts like the endogenous particle *in vivo*. Preparation of native human LDL requires fresh, unfrozen plasma, which would ideally come from the patient who is to receive chemotherapy. However, this may not always be practical, and unless a substantial proportion of the 1500 cholesteryl esters in each LDL particle is replaced by drug, the quantity of LDL required to administer a therapeutic drug dose may be too large in terms of both the volume of plasma required and the induced rise in the level of plasma cholesterol following re-injection of the drug-laden LDL.

## 1.5 CHLORAMBUCIL, ADRIAMYCIN AND DAUNOMYCIN

Three drugs, namely chlorambucil, daunomycin and adriamycin - (fig.11), were incorporated into LDL in the course of this project and the following section outlines the modes of action, toxicity and pharmacokinetics of each of them.

### 1.5.1. CHLORAMBUCIL

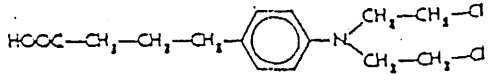
Sulphur mustard poisoning in World War I was observed to cause a combination of gastrointestinal tract ulceration, lymphoid tissue dissolution and bone marrow aplasia. The toxic effects of nitrogen analogues of sulphur mustard on lymphoid tissue were studied by Goodman, Gilman and Dougherty in 1942 and their observations on transplanted lymphosarcoma in mice[162] prompted them to undertake clinical trials with the nitrogen mustards, the first of the antineoplastic chemotherapeutic agents. Since then many nitrogen mustard derivatives have been synthesised, although few have had any more cytotoxic activity than the original compound.

All nitrogen mustards are considered chemically unstable. However, modification of the side chain increases the stability of some analogues. For example, chlorambucil (fig.11), the phenylbutyric acid derivative of mechlorethamine (fig.11), is stable enough to be prepared in tablet form and administered orally, while cyclophosphamide (fig.11), is stable and inactive prior to activation by the cytochrome P450 system in the liver[130,163]. In contrast, mechlorethamine is so hygroscopic that it is prepared as dry crystals and reconstituted with water only, immediately prior to injection.

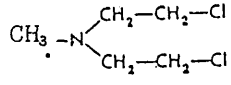
### 1.5.2. CHEMICAL ACTION OF CHLORAMBUCIL

The nitrogen mustards act by virtue of their ability to form carbonium ions which subsequently participate in electrophilic attack on

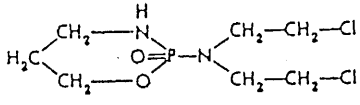
ALKYLATING AGENTS



CHLORAMBUCIL

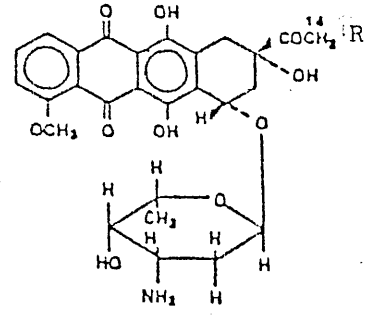


MECHLORETHAMINE



CYCLOPHOSPHAMIDE

ANTHRACYCLINES



R = H , DAUNOMYCIN

R = OH ,ADRIAMYCIN

Figure 11: Chemical structures of some alkylating agents and anthracyclines

biological molecules[164] (fig.12). They readily form covalent bonds with amino, sulphydryl, hydroxy, carboxy and imidazole groups. Many cell sites are susceptible to this method of alkylation, including DNA, RNA and enzymes. Therefore, cell function and viability may be greatly affected by nitrogen mustards. The alkylation of DNA, however, is thought to be the main mechanism of the cytotoxic action of these agents. By altering DNA, alkylating agents disrupt basic cellular process. They interfere with mitosis and cell division in rapidly proliferating tissues.

Nitrogen mustards exert their effects on cells at all stages of the cell cycle but toxicity is usually expressed in the S-phase with blockage at the $G_2$ (pre-mitotic) phase[165]. However, synchronised cells in culture are generally more sensitive in late G, or S-phases than $G_2$, mitosis or early $G_1$. This is possibly because polynucleotides are more susceptible to alkylation in the unpaired state, during DNA replication (S-phase), than in the $\alpha$-helical conformation. Cells accumulated in the $G_2$ phase continue to synthesise cellular materials, without dividing. Lethal toxicity may be the result of damage to many cellular sites, for example, glycolysis, respiration, protein synthesis and nucleic acid synthesis are all processes which may be affected. Non-proliferating cells may later differentiate, without further mitosis, to adult cells. Slowly proliferating cells are more likely to recover from alkylation since the effects of alkylated DNA may not be of great importance prior to mitosis. Slowly dividing cells therefore have a much longer time in which to attempt DNA repair and so overcome the effects of the alkylating agents.

Interaction of bifunctional agents like chlorambucil with DNA tends to have toxic effects, whereas monofunctional agents cause only mutations or carcinogenesis[164]. Bifunctional molecules have the ability to cross-link two DNA chains. This is potentially more harmful than

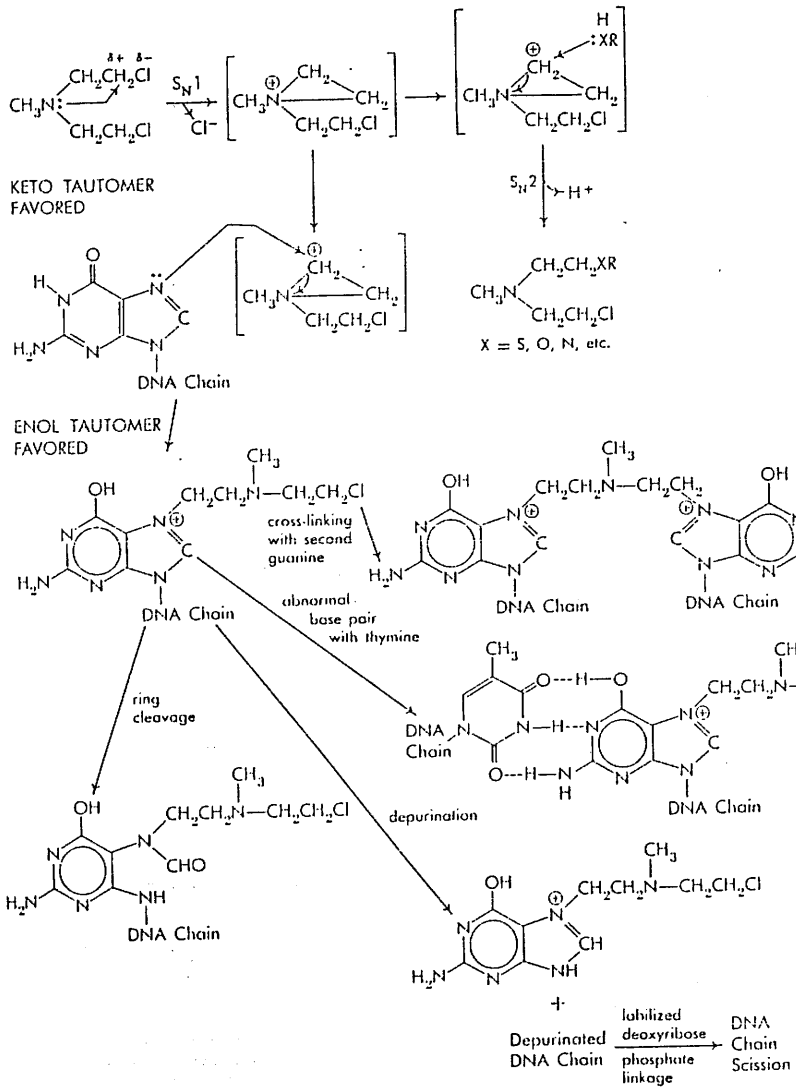



Figure 12: The mechanism of action of alkylating agents

mono-alkylation, where the modified DNA may be compatible with the continued life of the cell and may be successfully passed to the next generation. DNA repair may recover the monoalkylated DNA, whereas highly cross-linked DNA is much more difficult to free from the damaging alkyl groups. High-dose chemotherapy circumvents the DNA repair system to some extent by causing formation of extensively cross-linked DNA, whereas DNA damage during low-dose therapy may be easily corrected.

Due to their properties of lipophilicity, acid dissociation, and stability in aqueous solutions, alkylating agents are able to cross cell membranes. This is crucial to their activity, as are the two (2-chloroethyl) groups. Several different groups have been used as the third substituent on the nitrogen in an attempt to increase drug selectivity and decrease overall toxicity, with varying degrees of success[164]. A degree of specificity may be obtained because of the inherent difference in proliferation rates between normal and neoplastic tissues. Since normal tissues proliferate more slowly, the effects of alkylating agents are not immediately apparent and these cells may repair their DNA successfully before division occurs.

### 1.5.3. CHLORAMBUCIL - THERAPEUTIC USES AND TOXICITY

Chlorambucil is commonly used in the treatment of leukemia, Hodgkin's Disease and malignant lymphomas. Administration of the drug is usually oral, in doses of 0.1-0.2 mg/Kg daily. Absorption from the gastrointestinal tract is variable but is generally complete within 2 hours. There is little information on the metabolism, distribution and elimination of chlorambucil. Toxic effects of chlorambucil are most apparent in bone marrow, gastro-intestinal tract, skin and occasionally in the liver[166].

## 1.5.4. RESISTANCE TO ALKYLATING AGENTS

There is little information available on the possible mechanisms of resistance to alkylating agents but it has been widely observed that acquired resistance to one alkylating agent confers cross-resistance to all others[137]. The acquisition of resistance has generally been observed to be slow, and is thought to be a result of several mutations, not just a single one as is the case with antimetabolites. In resistant cells, there is a decrease in the permeability of the cell membrane to the drug. Production of nucleophiles is increased to compete with DNA for alkylation e.g. administration of cysteine[137] reduces the antineoplastic efficacy of alkylating agents and several resistant animal tumour cells have increased free thiol concentrations as compared to sensitive cells. It is likely too that resistance is potentiated by an increase in the activity of the DNA repair system.

## 1.5.5. PHARMACOKINETICS OF CHLORAMBUCIL

Alberts et al[167] observed in man that chlorambucil was rapidly absorbed from the gastrointestinal tract and cleared from plasma with a terminal phase half-life of 92 minutes. On administration of chlorambucil at 0.6 mg/Kg, the peak drug concentration was found to be 1.1 μm/mL plasma. The major metabolite of chlorambucil, phenylacetic acid mustard, was seen in plasma within fifteen minutes of drug administration. Comparison of gastrointestinal and IV routes of administration[168] of chlorambucil showed peak plasma concentrations to be higher after IV injection. Peak plasma concentrations were observed 40-70 minutes after gastrointestinal administration. Thereafter, decay curves for the drug were identical in both cases. Two metabolites were detected, one of which was the β-oxidation product of chlorambucil 2[4-N,N,-bis-(2-chloroethyl)-aminophenyl]-acetic acid (phenyl acetic acid mustard),

a compound known to have alkylating action in animals.

## 1.5.6. ANTHRACYCLINES

These are relatively new antineoplastic agents. The anthracyclines (fig.11) are antibiotics and were first isolated in the early 1960's from fermentation products of the fungus Streptomyces peucetius, var. caesius. Daunomycin was simultaneously discovered by groups in France[169] and Italy[170] and as a result it is known by several different names (daunorubicin, rubidomycin and daunomycin). Adriamycin was later isolated in Italy[171] and differs from daunomycin in only one hydroxy substituent. Structures for both drugs are shown in figure 11. The anthracycline molecule consists of a tetracyclic ring structure joined to the sugar daunosamine by a glycosidic linkage (fig.11). Although their structures are almost identical, the two drugs are effective in the therapy of quite different neoplasms. Daunomycin is used mainly in the treatment of leukemia and lymphomas, whereas adriamycin is effective against a much wider spectrum of tumours.

## 1.5.7. CYTOTOXIC ACTION OF ANTHRACYCLINES

The mechanism of action of anthracyclines is not fully understood. X-ray diffraction studies and DNA model building[171a] suggest that anthracyclines bind tightly to DNA. It is widely accepted that these drugs intercalate the strands of DNA[172]. The $\alpha$-helix of the DNA molecule is thus disrupted and no longer forms an effective template for RNA synthesis[173]. Adriamycin has a great affinity for myocardial DNA and this is thought to be a cause of the cardiotoxic properties of this drug[174]. Both drugs may also have cytoplasmic routes of action. Adriamycin is thought to cause intracellular damage as a result of free-radical formation[130] and cell membrane behaviour may also be affected. As expected, maximal cytotoxic effects are expressed, after

exposure to these drugs, during the S-phase of the cell cycle, although some cytotoxicity is observed at other stages[129,175].

## 1.5.8. METABOLISM OF ANTHRACYCLINES

The reason for the observed differences in the spectra of cancers against which these agents are active is largely unexplained, but differences in their metabolism have been observed. Both complexes are extensively metabolised[176,177]. The major metabolites are adriamycin-ol and daunomycin-ol which are the results of enzymic reduction of the C-13 ketone[176] and both express antitumour activity. In acute myelocytic leukemia, the activity of "daunomycin reductase" has been directly correlated with patients' response to daunomycin therapy. Two other metabolites of daunomycin[176] and five of adriamycin[177], all aglycone fragments of the drugs, have been observed. Major sites of anthracycline absorption are heart, lungs, kidneys, liver and spleen[123,178,179]. Anthracyclines do not appear to cross the blood-brain barrier and, when administered by intrathecal injection[180], were found to be toxic to cells of the central nervous system. The liver is the primary site of anthracycline metabolism and excretion, with 60% of adriamycin and 20% of daunomycin[178] removed here and subsequently excreted in bile[179]. These drugs are also excreted in urine, 25% and 6% of adriamycin[179] and daunomycin respectively are removed from the body by this route[178].

## 1.5.9. THERAPEUTIC USES AND TOXICITY OF ANTHRACYCLINES

Both adriamycin and daunomycin are effective in the treatment of acute leukemias and malignant lymphomas. Daunomycin is also effective in the treatment of solid tumours in children but, unlike adriamycin, it is of limited usefulness against solid tumours in adults. Cardiotoxicity[174] is a major dose-limiting factor in the administration of anthracyclines,

and consequently the cumulative dose of these drugs is generally kept below 550 mg/$m^2$. Dose regimens for both drugs vary depending on the tumour type and the extent of its progression. Daunomycin is usually given at a dose of 30-60 mg/$m^2$ daily for 3 days or once weekly[166]. The recommended dose for adriamycin is 60-75 mg/$m^2$, administered as a single IV infusion and repeated after 21 days[166]. Toxic effects of anthracyclines include stomatitis, alopecia, gastrointestinal disturbances and cardiomyopathy. Myelosuppression is frequently the main dose-limiting factor.

### 1.5.10 RESISTANCE TO ANTHRACYCLINES

In common with numerous other antineoplastic agents, acquired resistance to anthracyclines is frequently observed and the reasons for it are poorly understood. As in the case of alkylating agents, resistance to one anthracycline confers complete resistance to the other anthracyclines[138,164]. Cross-resistance has also been reported between anthracyclines and other natural products viz. dactinomycin and the vinca alkaloids[181,182]. It is likely that this type of cross-resistance is the result of modifications in cellular permeability. Cellular drug accumulation has been found to be lower in several resistant sublines when compared to the sensitive sublines[183,184]. It has not been established whether this is a consequence of increased drug efflux or decreased influx, although a number of authors have shown the former to be more important in several tumour models in vitro, and in vivo[138].

### 1.5.11 PHARMACOKINETICS OF ANTHRACYCLINES

Anthracyclines are administered intravenously and so are immediately distributed in the central compartment. The plasma decay curve for daunomycin is biphasic with half-lives of 45 minutes for the initial

rapid distribution phase, and 55 hours for the terminal phase[178]. Adriamycin removal is triphasic, reflecting the participation of a larger number of metabolites in adriamycin pharmacokinetics. Half-lives of the drug in the three clearance phases are 12 minutes, 3.3 hours and 29.6 hours, as measured by Bachur et al[179].

# SECTION 2 : MATERIALS, METHODS AND RESULTS

## 2.1 MATERIALS

Suppliers of the materials used in the following experimental sections are detailed below. All other reagents and solvents were of Analar grade.

Adriamycin and daunomycin were supplied as gifts from Farmitalia Carlo Erba, Milan, Italy, and May & Baker Ltd., Dagenham, U.K., respectively. Chlorambucil was donated by The Wellcome Foundation, Crewe, U.K., or purchased from Sigma Chemical Co. Ltd., Poole, Dorset.

Radioisotopes ( $^{125}I$, $^{131}I$, [7-$^{3}H$]-cholesterol, and [4,5-$^{3}H$]-leucine) were obtained from the Radiochemical Centre, Amersham, Bucks.

| MATERIAL | SUPPLIER |
|---|---|
| Bio-Rex 70 Cation Exchanger | Bio-Rad Laboratories, Watford, Herts |
| Brij-35 | Sigma Chemical Co., Poole, U.K. |
| Cellobiose | Sigma |
| Cholesterol | British Drug Houses (BDH) Poole, U.K. |
| Cholesterol Oleate | Sigma |
| Cholesterol Tosylate | " |
| Cholic Acid | BDH |
| Cyanuric Chloride | Sigma |
| 1,2-Cyclohexanedione | Fluorochem Ltd., Glossop, Herts. |
| Dimethyl Hydrazine | Sigma |
| Dimyristoyl Phosphatidyl Choline | " |
| Dipalmitoyl " " | " |
| $Na_2EDTA$ | BDH |

| MATERIAL | SUPPLIER |
|---|---|
| Dulbecco's Modified Eagle's Medium | Gibco Biocult Ltd., Paisley, U.K. |
| Folin-Ciocalteu Reagent | BDH |
| Glycine | " |
| Ham's F10 Medium | Gibco |
| Human Serum Albumin | Sigma |
| Hydrogen Peroxide Solution(40% w/v) | BDH |
| Hydrocortisone sodium succinate for injection | Organon Laboratories, Newhouse, Scotland |
| Iodine Monochloride | Sigma |
| Lecithin | " |
| Microtitre Cell Culture Plates | Corning Medical Halstead, U.K. |
| Millipore Filters | Millipore Corpn., Bedford, Mass. |
| N-Nitrosobis(2-oxopropyl)amine | Ash Stevens Inc., Detroit, USA. |
| PD10 Columns | Pharmacia(GB)Ltd, London |
| QAE-A50 Anion Exchanger | " |
| Scintillation Liquid (Dimilume 30) | Packard Instruments |
| Silica | Sigma |
| Silica Gel60$_{F254}$ TLC Plates | BDH |
| Sodium Dodecyl Sulphate | BDH |
| " Iodoacetate | Sigma |
| " Potassium Tartrate | BDH |
| " Taurocholate | Sigma |
| Spectrapor Dialysis Tubing | Spectrum Medical Industries Los Angeles, USA. |
| Tissue Culture Flasks | Flow Labs., Irvine, Scotland |
| Tris(hydroxymethyl)methylamine | Sigma |
| Triton X-100 | Sigma |
| Trypsin | Gibco |

## EQUIPMENT

| | |
|---|---|
| Beckman L5-65 Centrifuge and rotors | Beckman Instruments Ltd., Glenrothes, Scotland |
| Isco $UA_5$ U.V. Absorbance Monitor | MSE Scientific Instruments Crawley, W. Sussex |
| Amicon B15 Concentrating Cells and Membranes | Amicon Corpn. Lexington, Mass., U.S.A. |
| Packard Scintillation Counters | Packard Instruments Illinois, U.S.A. |
| Iridectomy Scalpel & Trocar | MacCarthy's Surgical Instruments Dagenham, U.K. |

## ANIMALS & DIETS

NMRI and CFLP mice were obtained from inbred colonies at the Departments of Medical Oncology, Glasgow University, and Pathology, G.R.I., respectively. NIC B6D2F1 mice were purchased from Bantin and Kingman Ltd., Hull, U.K.

Rats, Sprague-Dawley and Wistar were purchased from Strathclyde University and Lions Lab., Ringwood, Hants., respectively.

New Zealand White rabbits were purchased from Leslie Moore Ltd., Bradford, U.K. and fed diet SG1 (Oxoid Ltd., Basingstoke, U.K.). Standard small animal diet was obtained from Special Diet Services, U.K, Cholesterol and bile salt-enriched diets were prepared as described in Section 2.1.13.

## 2.1.1. PREPARATION OF LDL

LDL was separated from human plasma by rate-zonal ultracentrifugation by the method of Patsch et al[185]. A diagram of the apparatus used in this procedure is shown in figure 13. Using a Beckman L5-65 ultracentrifuge and Ti-14 zonal rotor, the LDL from 40-60 ml of human plasma was separated from other plasma constituents by flotation through a linear sodium bromide gradient, density range 1.0-1.3 Kg/L, at 45,000 rpm and 10°C for 110 minutes. Eluant from the rotor was collected in fractions of 14 mL and the optical density at 280 nm monitored using an Isco UA5 Ultraviolet Absorbance Monitor. A typical elution profile is shown in figure 14. The peak fractions containing LDL were pooled and concentrated by pressure filtration in an Amicon ultraconcentration cell using an XM100 Å filter, under nitrogen pressure at 25 p.s.i. Excess sodium bromide was removed by washing with working buffer (0.15M NaCl, 0.01% EDTA, pH 8.1) and reconcentrating, several times, or by exhaustive dialysis against working buffer.

## 2.1.2. PREPARATION OF HDL AND d>1.21 Kg/L PLASMA FRACTION

HDL was prepared from human plasma by sequential ultracentrifugation at 1.063 Kg/L and 1.21 Kg/L[186]. Human plasma was made up to density 1.063 Kg/L by the addition of 0.0834 g/mL of solid potassium bromide, overlayed with solution of the same density and centrifuged at 45,000 rpm and 10°C for 18 hours in a Beckman Ti60 rotor and L5-65 ultracentrifuge. Buoyant lipoproteins of density less than 1.063 Kg/L were aspirated. The solution containing HDL and plasma proteins at the bottom of the centrifuge tubes was made up to density 1.21 Kg/L by the addition of 0.236 g/mL of solid potassium bromide, overlayered with 1.21 Kg/L density solution and centrifuged overnight as above.

# Lipoprotein Separations on the Zonal Centrifuge

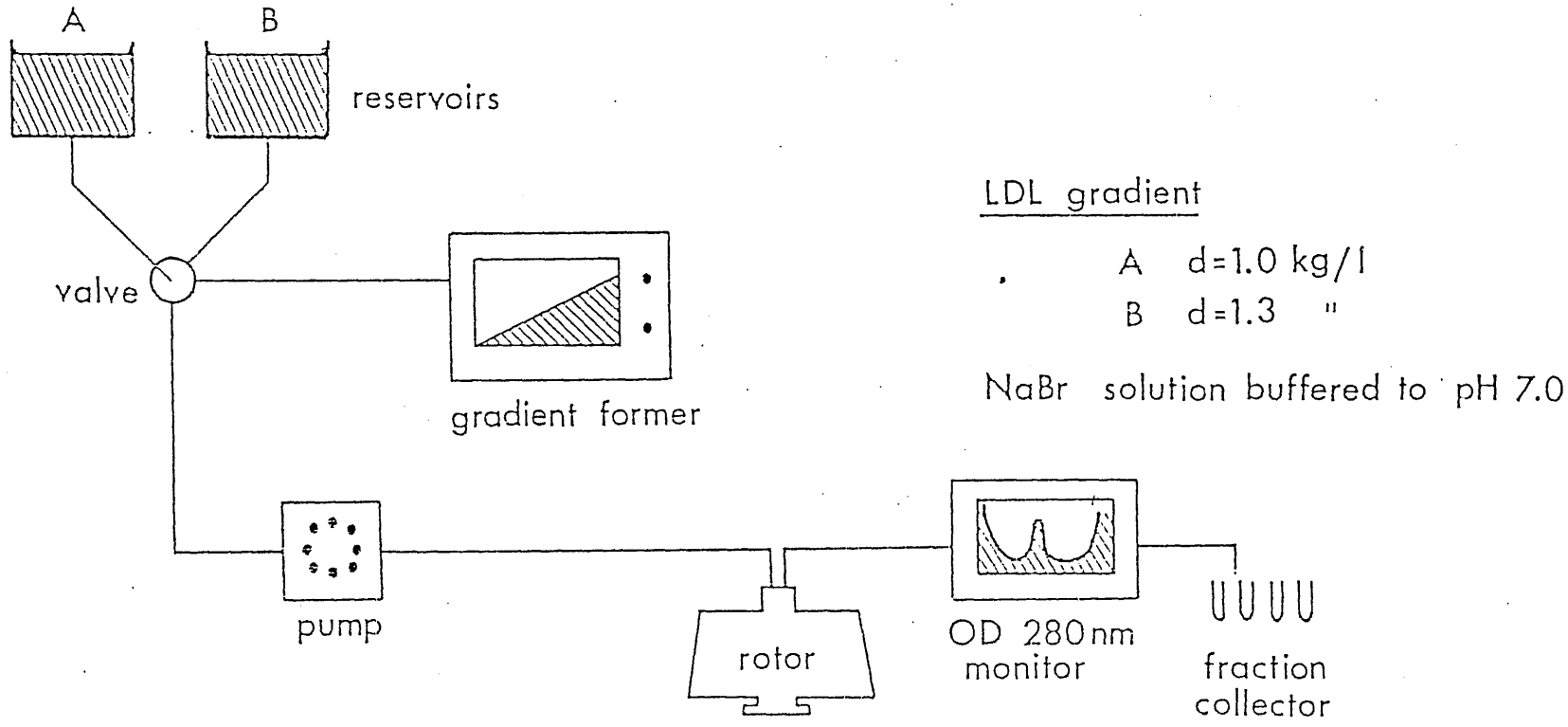



Figure 13: Zonal preparation of LDL as described by Patsch et al[185]

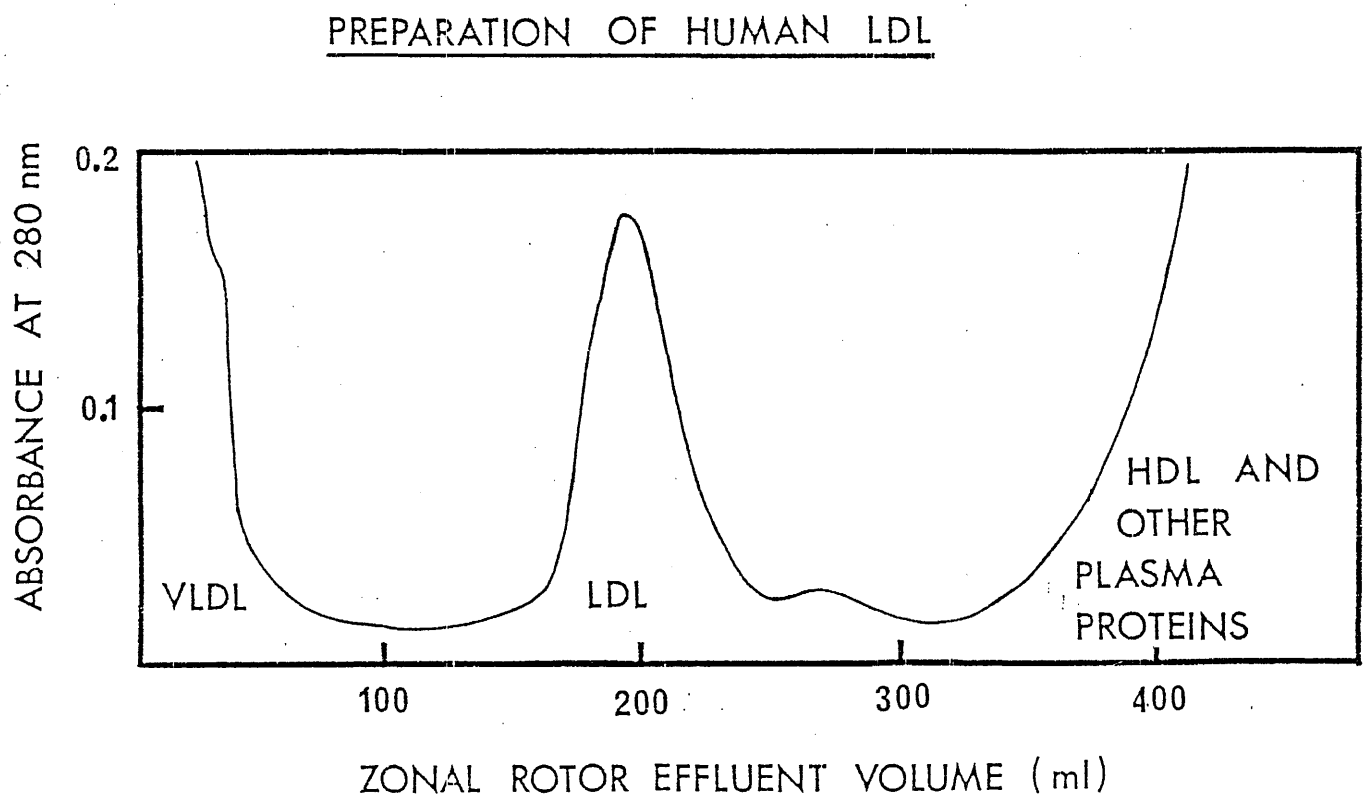



Figure 14: Elution profile of human plasma after zonal centrifugation to prepare LDL

HDL (d 1.063 - 1.21 Kg/L) was aspirated from the tops of the tubes and desalted, either by dialysis against working buffer or gel matrix filtration through Pharmacia PD10 columns with working buffer (0.15 M NaCl, 0.01% EDTA, pH 8.1) as eluant.

The remaining fraction at the bottom of the tube was dialysed against distilled water for 24 hours at 4°C, prior to further dialysis against working buffer (0.15M NaCl, 0.01% EDTA, pH 8.1) with several changes of solution.

### 2.1.3. RADIOIODINATION OF LDL

The protein moiety of LDL was labelled using a modification[93] of the iodine monochloride method of McFarlane[186]. This involves the substitution of iodine at the meta-position on the phenyl ring of tyrosine residues of the apoprotein. Since this reaction is only 40% efficient with LDL, a 2.5-fold excess of ICl is required in order to label each mole of apoprotein, taken as 64000 daltons, with one mole of iodine. Apoprotein concentration was determined by the method of Lowry et al[188], Section 2.1.9. or estimated by measuring absorbance at 280 nm on a Beckman Du-7 Spectrophotometer and relating 1 optical density unit to a concentration of 1 mg/mL. (It should be noted here that, since this method was first described, the molecular weight of apoprotein B has been reported to be 500,000 Daltons. Thus the estimation of apo B concentration here was purely empirical and overestimates the amount of protein present by a factor of 7.8. Consequently each mole of apoprotein is labelled with 7.8 moles of iodine). To each mL of LDL solution (protein 2-5 mg/ml) prepared as described in Section 2.1.1. was added 250 µl of 1M glycine buffer, pH 10.

After addition of the required amount of $^{125}I$ or $^{131}I$, usually 1-2 mCi in 10-20 μL, the appropriate volume of 25 nM ICl was added, giving a final ICl concentration of 125-315 nM. The reaction is complete in a few seconds and glycine and unreacted iodide were removed by gel filtration through a Pharmacia PD10 column, using working buffer (0.15 M NaCl, 0.01% EDTA, pH 8.1) as eluant.

## 2.1.4. SYNTHESIS AND IODINATION OF CHOLESTERYL LINOLEOYL ETHER

Cholesteryl Linoleoyl ether (CE), figure 8, was synthesised as described by Halperin and Gatt[189]. The cholesteryl ether was labelled by mixing 15 mg of the ether, in 250 μL of chloroform, with 250μL methanol, 2 mCi of $^{125}I$ or $^{131}I$ and 250 μL of 25nM ICl. This mixture was washed several times with distilled water to remove unreacted iodide. The labelled ether was further purified by thin-layer chromatography on silica gel $60_{F254}$ plates. These were developed in heptane: ethyl acetate (9:1) and the cholesteryl ether band (which migrated near the solvent front leaving iodide at the origin) was scraped off into a plastic bag. The radioiodinated cholesteryl ether (I-CE) was extracted into chloroform (3 x 5 mL) and transferred to a pre-weighed glass tube before evaporation of the solvent under nitrogen at 40°C. The yield of I-CE was usually ca. 10 mg.

## 2.1.5. INCORPORATION OF LABELLED CHOLESTERYL LINOLEOYL ETHER INTO LDL

This procedure (figure 15) has been adapted from Stoffel's method[190] for loading HDL with labelled cholesteryl esters and fatty acids. In brief, the I-CE was co-sonicated with cholic acid and lipids to generate micelles which, when mixed with HDL undergo exchange of lipids. The ester-containing HDL was purified and mixed with LDL and the $d > 1.21$ Kg/L fraction from human plasma which was a source of cholesteryl ester transfer protein[191].

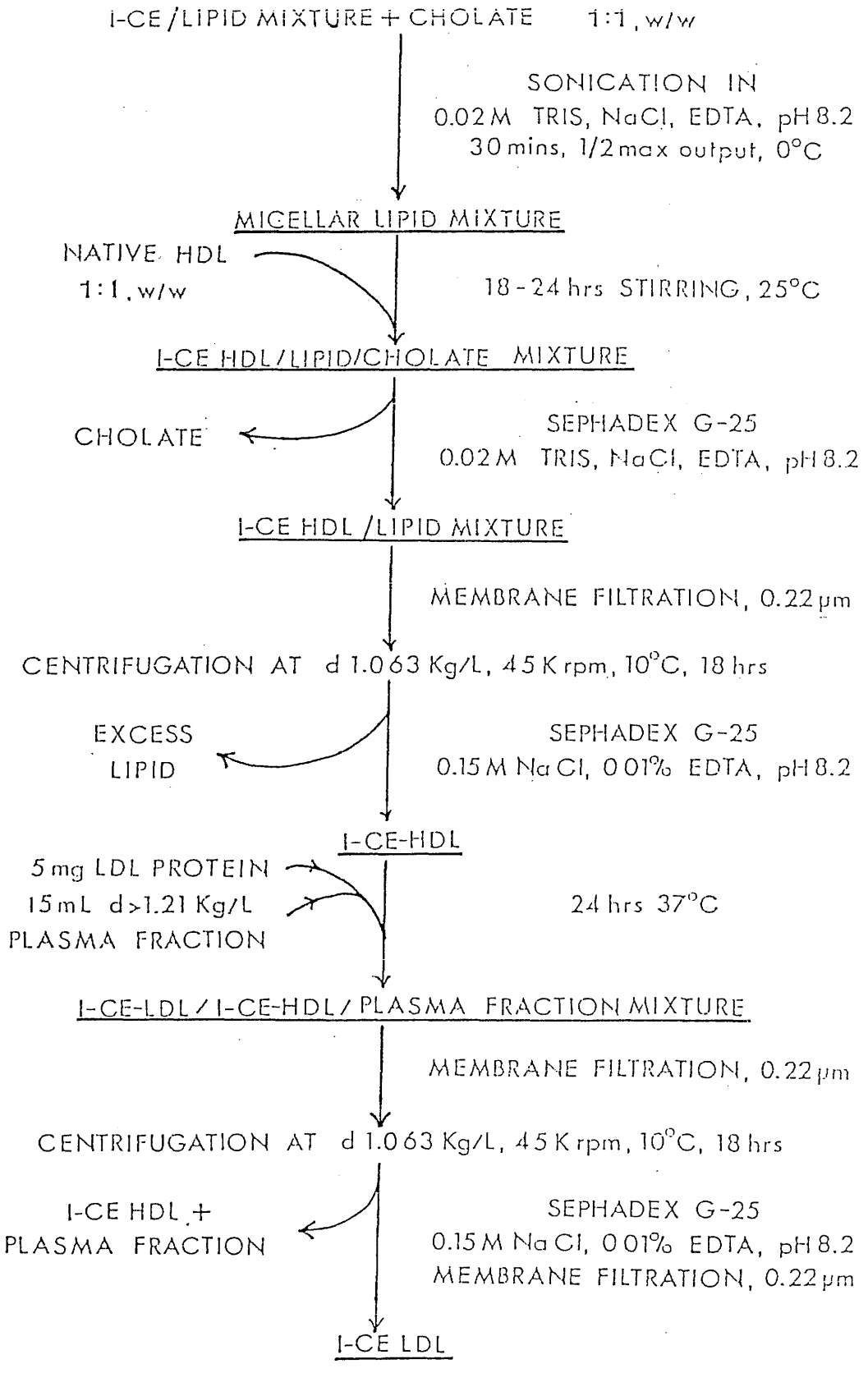



Figure 15: Protocol for the preparation of radioiodinated cholesteryl ether-LDL (I-CE-LDL) by the modified Stoffel method, Section 2.3.2.

The labelled LDL was purified by centrifugation, and desalted by gel-matrix chromatography.

A) PREPARATION OF CHOLATE MICELLES[190]

Phospholipid, cholesterol and I-CE, in chloroform, were mixed in molar ratios of 80 : 24 : 53 and dried into the surface of a conical glass tube under a stream of nitrogen. This required 10.9 mg lecithin (13.6 μmol), 1.6 mg cholesterol (4.1 μmol ) and 10 mg I-cholesteryl ether (9.0 μmol). Cholic acid was dissolved in tris buffer (0.02 M tris(hydroxymethyl) methylamine, 0.02 M NaCl, 0.02 M EDTA, pH 8.2) to a final concentration of 4.6 mg/mL. Equivalent weights of cholic acid and lipids (i.e. 5 mL cholate solution to above quantities of lipids) were mixed and sonicated at 0°C for 30 minutes.

B) LOADING OF HDL WITH CHOLESTERYL ETHER

Cholate micelles and HDL were mixed 1 : 1 (weight/weight) and stirred overnight at room temperature. After 18-24 hours cholate micelles were removed by filtration of the mixture through 0.22 μm millipore filters. The HDL solution was made up to density 1.063 Kg/L by the addition of 0.0834 g/mL of KBr, overlayered with d1.063 Kg/L density solution and centrifuged at 45,000 rpm for 18 hours in a Beckman Ti60 rotor and L5-65 centrifuge. Fragments of HDL and lipid micelles and density solution were aspirated from the top of the centrifuge tube, leaving HDL labelled with I-CE at the bottom. The I-CE-HDL was desalted by filtration through a Pharmacia PD10 column, eluting with working buffer (0.15M NaCl, 0.01% EDTA, pH 8.1).

C) TRANSFER OF CHOLESTERYL ETHER FROM HDL TO LDL

By utilising the cholesteryl ester transfer protein found in human plasma[57,58,191] the I-CE was transferred from HDL to LDL. Fifteen mL of d > 1.21 Kg/L bottom fraction was incubated with I-CE-HDL and 5 mg LDL (protein) for 24 hours at 37°C.

D) PURIFICATION OF LABELLED LDL

The above mixture, after incubation, was made up to density 1.063 Kg/L and overlayered with d1.063 solution prior to centrifugation at 45000 rpm and 10°C in a Beckman Ti60 rotor. Labelled LDL was removed by aspiration from the tops of the centrifuge tubes and desalted on a Pharmacia PD10 column using working buffer or phosphate - buffered saline, (PBS; 0.15M phosphate; 0.15 M NaCl pH 7.4) as eluant.

## 2.1.6. TRANSFER OF CHOLESTERYL ETHER FROM LDL TO OTHER LIPOPROTEINS

Man and many animals possess a cholesterol ester transfer protein[57,58,191] which facilitates the exchange of cholesteryl esters between the lipoprotein classes. If I-CE-LDL is to be used as a tracer of LDL catabolism, it is essential that the animal whose metabolism is being studied does not have high cholesteryl ester transfer activity. It was therefore necessary to establish which of the animal models used had suitably low transfer activity.

$^{125}$I-cholesteryl ether HDL was prepared as described above (2.1.5.) One mL of mouse, hamster, rat, rabbit and human plasma were each incubated with 10 µL of $^{125}$I-CE-HDL (approx. 20,000 cpm) at 37°C for 24 hours.

The LDL from each sample was separated by centrifugation at 1.063 Kg/L in a Beckman Ti40.3 rotor at 39K rpm and 4°C overnight. On completion of the spin, 1 mL fractions were removed from each tube and their $^{125}I$-activity measured using a Packard Twin-Channel Autogamma Liquid Scintillation Counter. The percentage of the total activity found in each fraction is shown in figure 16. It was concluded that hamster and rabbit plasma have high transfer activity, like human plasma, whereas mouse and rat have much lower activity. The use of I-CE-LDL as a probe of LDL catabolism is therefore likely to be confounded by transfer of the cholesteryl ether between lipoproteins in the former two species.

## 2.1.7. CELLOBIOSE-TYRAMINE-LDL:SYNTHESIS AND LABELLING

Cellobiose is a disaccharide (4-O-β-D-Glucopyranosyl-D-⊀-Glucopyranose) which has a reducing aldehydic group available on the second ring. Tyramine is a primary amine which will react rapidly with aldehydes to form a Schiff's base. This linkage can then be reduced to make a stable bond to LDL-lysine residues via cyanuric chloride (figure 8 ). Cellobiose-tyramine (CT) was synthesised in a manner similar to that described by Pittman et al[105]. 1 mmol each of cellobiose and tyramine and 5 mmol of sodium cyanoborohydride were suspended in 10 mL of distilled water and dissolved by heating to 50-60°C in a stoppered glass tube. The mixture was allowed to react at 37°C for 24 hours. Unreacted tyramine was removed by chromatography using a 1.6 x 100 cm column of Sephadex G15 in 0.1 M ammonium acetate. The reaction mixture was applied to the column and eluted with ammonium acetate buffer at a flow rate of 20 mL/hr. Fractions were collected and assayed for tyramine, by measuring absorbance at 280 nm in a Beckman DU-7 spectrophotometer, and sugar by the anthrone reaction. Two peaks were obtained, the first contained 75% of the

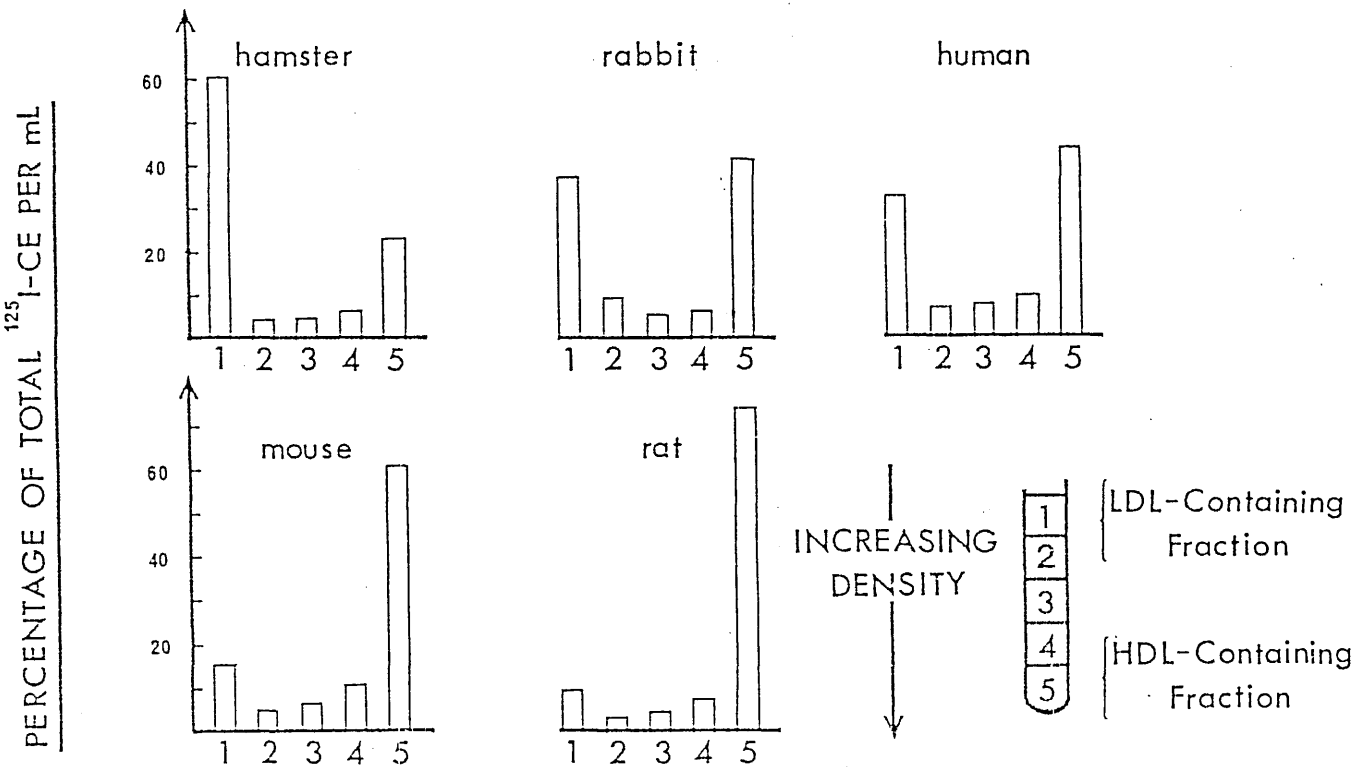



Figure 16: Percentages of total $^{125}$I-CE found in fractions of animal and human plasma following incubation of the labelled lipoprotein with the plasma and centrifugal separation of HDL from LDL as described in Section 2.1.5 The presence of radioactivity in fraction 1 indicates cholesteryl ester transfer activity.

applied $OD_{280}$ and all of the sugar, the second contained the remainder of the $OD_{280}$, that is, non-conjugated tyramine. Excess celloboise was removed by ion exchange chromatography using Bio-Rex 70 Cation ($Na^+$ form) Exchanger, 200-400 mesh. The resin was washed in 50 volumes of 0.12N HCl, three times, followed by three washes with 50 volumes of water. The column was packed and eluted with water until the eluate was of pH 5-6. Peak I from the sephadex column was applied in three aliquots, each interspersed with 5 mL water. The unretained peak was eluted with water and was found to contain sugar. The water elution was continued until there was no detectable absorbance at 280 nm. The retained, positively charged conjugate was eluted using 3.5% ammonia solution. Ammonia and water were removed by overnight lyophilisation leaving pure cellobiose-tyramine.

To 250 nmol of CT in 50 µL of distilled water was added 10 µL of 0.3M $KH_2PO_4$, pH 4.5, 10 µL of Na $^{125}I$ or Na $^{131}I$ solution (1m Ci) and 10 µL of ICl (250 nmol). The mixture was allowed to stand for 15 minutes before the addition of 500 µL of distilled water. Unreacted iodide was removed on a 0.9 x 6 cm column of QAE-A50 (Anion Exchanger) in distilled water. The I-CT was eluted in 3-4 mL of water and lyophilised overnight.

Linkage of CT to LDL : A dried aliquot of the freshly made I-CT was dissolved in 5 x 10 µL of distilled water and 10 µL of the solution mixed with 20 µL of cyanuric chloride (2.5 mg/mL in acetone), 15 µL of 0.008 M NaOH and 10 µL of distilled water. This was allowed to stand for 15 seconds before the rapid addition of 5 mg LDL protein in 1 mL of working buffer. After 2-3 hours at room temperature, the I-CT-labelled LDL was purified on a Pharmacia PD10 column, eluting with working buffer.

## 2.1.8. CHEMICAL MODIFICATION OF LDL

In order to prevent binding of LDL to its receptors, the arginine residues of apo B were modified by 1,2- cyclohexanedione (CHD), as described by Mahley et al[90], (figure 17). 0.15M CHD was made up in 0.2 M sodium borate buffer pH 8.1. Two volumes of CHD solution were mixed with each volume of the LDL solution (protein concentration 2-5 mg/mL), and incubated at 35°C for two hours. Sodium borate and unreacted CHD were subsequently removed by gel filtration through Pharmacia PD10 columns, eluted with working buffer. This process modifies at least 55% of the arginine residues in the apoprotein B[90], so preventing CHD-LDL from binding to LDL receptors.

Reversal of modification *in vitro* is possible by incubating the CHD-LDL with a strong nucleophile such as hydroxylamine[90,98]. For this reason tris buffers should not be used in the preparation of CHD-LDL.

Reaction of the arginine molecules with CHD removes the positive charge characteristic of arginine residues. As a result, the modified LDL has substantially more anodic mobility than native LDL on agarose gel electrophoresis.

## 2.1.9. PROTEIN DETERMINATION

The apoprotein content of LDL was assayed by the method of Lowry et al[188] using human serum albumin as standard. Reagents were prepared as follows:

Solution A - 2% $NaHCO_3$ in 0.1N NaOH;

Solution B - 2% NaK Tartrate in deionised water;

Solution C - 1% $CuSO_4$ in deionised water.

Biuret reagent - to 100 mL of solution A was added 1 mL of solution B and 1 mL of solution C.

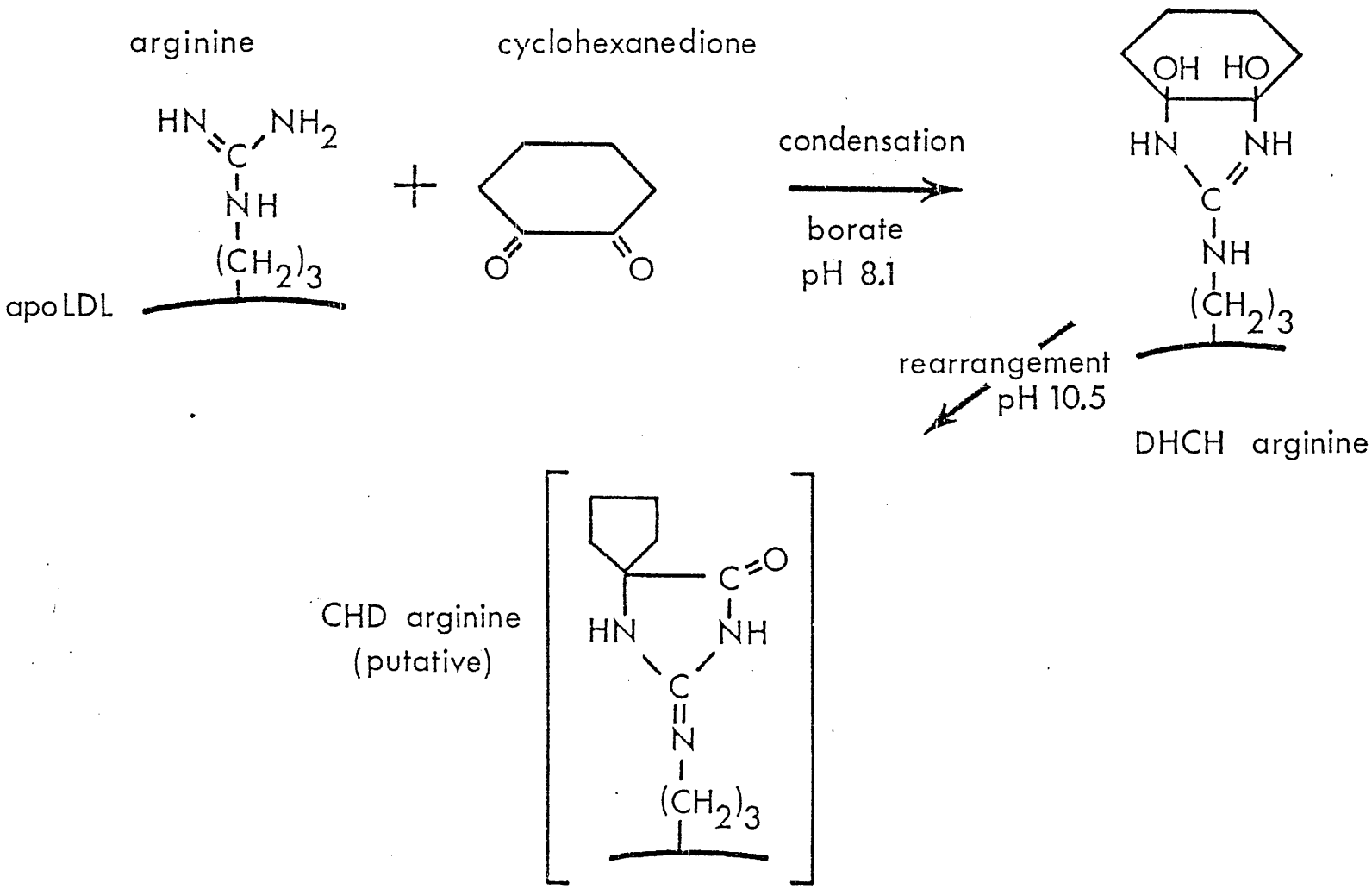



Figure 17: Chemical modification of LDL with 1,2-cyclohexanedione(CHD), DHCH - dihydroxycyclohexyl

Folin-Ciocalteu reagent - the stock reagent was diluted 1 : 1 with distilled water.

Standards

Human serum albumin 1 mg/mL was diluted with deionised water to give 25-100 µg protein / 400 µL. Bovine serum albumin, 0.3 mg/ mL, was diluted 1 : 4 and used as a quality control.

Method

To each 400 µL sample, control and standard was added 2 mL of Biuret reagent. Following vortexing and a 10-minute stand, 200 µL of Folin-Ciocalteu reagent was added to each tube during further vortexing. Colour was allowed to develop for 30 minutes and the optical density at 750 nm was measured using a Beckman DU7 spectrophotometer. A standard curve of $OD_{750}$ vs. protein concentration was constructed and unknowns were read from the curve.

## 2.1.10. STERILISATION OF SAMPLES FOR INJECTION

All solutions used for injection into animals were sterilised by filtration through sterile millipore filters, pore size 0.22 µm.

## 2.1.11. COLLECTION OF BLOOD SAMPLES

Blood removed from a peripheral vein was placed in 10 mL glass tubes containing 0.1% $K_2$ EDTA, unless serum was required, in which case 10 mL glass tubes containing no anticoagulant were used.

## 2.1.12. INDUCTION OF TUMOURS

A) NMRI/MAC13 - This was carried out at the Department of Oncology at Glasgow University with the cooperation of Dr J Welsh. Solid MAC-13 tumour was passaged in two-month old NMRI mice from an inbred colony at the above department by subcutaneously injecting each animal with approx $1mm^3$ of finely minced tumour. After 10 days the tumour had grown to approximately 1.5 g in weight ($1.49 \pm 1.13$g, n=14). At this stage the animals were used in the study, or tumour was passaged through another group of mice.

B) CFLP/PAROTID ADENOMA - Parotid adenomata were induced in these mice by inoculating them subcutaneously in the neck with A2LP polyoma virus within 24 hours of birth, as described by Lamey et al[192]. Following a latent period of 10-12 weeks the tumours became visible and therafter the animals were ready for use in ca. 3 weeks. At this stage the tumours weighed approximately 0.5 g ($0.43 \pm 0.35$ g, n = 8).

C) SPRAGUE - DAWLEY RATS/ COLONIC TUMOURS - Rats weighing 300g were injected for 16 weeks with dimethyl hydrazine in 0.05% EDTA solution, at a dose of 15 mg/Kg. Controls were injected with EDTA solution only[193]. Two to three weeks after the last injection, tumours were approximately 1g in size ($0.92 \pm 0.87$, n = 25).

D) WISTAR RATS/WALKER256 Tumour - Walker256 tumour was maintained in serial subcutaneous passage, in a colony of inbred Wistar rats, by Dr K Fearon at the Department of Oncology, G.U. Tumour was cut into fragments of 100 mg and placed under the skin in the flanks of rats weighing 200-250g. After 10 days, the tumours had grown to approximately

8g ($8.01 \pm 3.75$, n=8). The rats were used at this stage. This type of tumour was used for implantation in the subrenal capsular assay, described later, Section 2.3.14 .

E) HAMSTERS/PANCREATIC TUMOURS - Pancreatic tumours were induced in syrian hamsters by repeated weekly injections of N-nitrosobis (2-oxopropyl) amine in normal saline at a dose of 5 mg/Kg over 23 weeks as described by Pour et al[194].

## 2.1.13. ANIMALS AND DIETS

Unless otherwise stated, rats, mice and hamsters were maintained on standard small animal diet and allowed free access to drinking water (containing 1% potassium iodide for 1 day before and during LDL turnover studies, to inhibit thyroidal uptake of radioiodide).

### Sodium Taurocholate - Enriched Diet

Standard small animal diet was spray-coated with a saturated solution of sodium taurocholate in ethanol. The ethanol was allowed to evaporate and the preparation had a final sodium taurocholate composition of 1% (W/W).

### High Cholesterol Diet

Cholesterol was heated in coconut oil (1g in 10) and used to coat standard diet pellets. The final cholesterol content of the diet was 1% (W/W).

## 2.1.14. MEASUREMENT OF LDL RECEPTOR ACTIVITY IN VIVO

LDL was prepared by rate-zonal ultracentrifugation of human plasma, as previously described (2.1.1.). Aliquots of the concentrated and desalted LDL were labelled, one with $^{125}I$ and the other with $^{131}I$ (2.1.3.). One aliquot, usually the $^{131}I$-labelled, was then chemically modified with cyclohexanedione. (2.1.8.) Tumour and control animals were each injected with 0.1 mL of solution containing 5 μCi of each LDL tracer via a tail vein in the mice, and by jugular vein in the rats. After 18 hours, the animals were sacrificed and exsanguinated. The organs were removed, rinsed in saline, blotted, weighed and $^{125}I$-LDL and $^{131}I$-CHD-LDL radioactivity measured using a Packard Twin-channel Autogamma Liquid Scintillation Counter. Results were expressed as counts per minute/gram of tissue per mL of plasma for each isotope.

This approach relies on the observation that CHD-modified LDL is cleared more slowly than native LDL from the plasma (figure 6) since it is unable to interact with LDL receptors and cannot therefore be catabolised via the LDL receptor pathway[90]. CHD-modified LDL is consequently cleared only by receptor-independent mechanisms. Whereas native LDL is subject to catabolism via receptor-dependent and receptor-independent routes. Thus LDL receptor activity may be expressed as the difference between native- and CHD-LDL clearances (figure 18), if it is assumed that the distribution of modified LDL is identical to that of native LDL in the plasma and extravascular spaces.

Tissue radioactivity due to uptake of native LDL is a composite of receptor-bound, internalised and degraded LDL, and a non-specific component of LDL trapped in interstitial fluid. Since no

## LDL RECEPTOR ACTIVITY

NATIVE-LDL uptake = uptake by RECEPTOR-DEPENDENT and RECEPTOR-INDEPENDENT routes ------ 1

CHD-LDL uptake = uptake by RECEPTOR-INDEPENDENT routes only ------ 2

thus,

1 - 2 = RECEPTOR ACTIVITY

Figure 18: Calculation of LDL receptor activity using native and 1,2-cyclohexanedione (CHD)-modified LDL

difference is assumed between the distribution of native and chemically modified lipoproteins in interstitial fluid, calculated CHD-LDL uptake contains this same non-specific component. The calculated receptor-mediated LDL uptake into each tissue may then be attributed to receptor-bound, internalised and degraded LDL. The low-molecular weight LDL degradation products after extraction of tissue homogenates with trichloroacetic acid were found to correspond to less than 10% of the total radioactivity in all tissues except kidney. Ninety percent of the observed LDL uptake was then a result of the presence of high molecular weight, presumably intact lipoprotein. In the kidney 35% of the total radioactivity consisted of degradation products, reflecting the role of this organ in the excretion of iodotyrosine. Radioactivity assimilated via the receptor pathway generally accounted for 25-40% of the total tissue activity. The major breakdown products of LDL labelled in this way are rapidly excreted from the cell. Thus LDL receptor activity measured using I-LDL as a tracer reflects only the LDL metabolism occuring immediately prior to assessment in any particular tissue. In order to measure cumulative LDL metabolism, it is necessary to employ a tracer which is not excreted so rapidly from the cells which it enters. To this end, several cumulative probes of LDL catabolism have been investigated. Stein et al[106] have used radioiodinated cholesteryl ether and, more recently, Pittman et al[105] have studied radioiodinated cellobiose-tyramine.

## 2.2 RESULTS I

### 2.2.1. MEASUREMENT OF RELATIVE LDL RECEPTOR ACTIVITY IN ANIMAL TUMOUR MODELS USING NATIVE AND 1,2-CYCLOHEXANEDIONE-MODIFIED LDL.

Eighteen hours after injection of native and CHD-modified LDL into control and tumour-bearing animals, the radioactivity in their tissues and plasma was measured and used to calculate relative receptor-mediated LDL uptakes (2.1.14). The values obtained for each tumour type are shown in tables 5,7-9 and figures 19,21-23.

A) MAC-13 TUMOUR IN NMRI MICE

All tissues examined showed some degree of receptor-mediated LDL uptake (figure 19). The liver is clearly the most active in receptor expression per gram of tissue (table 5), and per organ (figure 19). Apparently high receptor activity is found in the kidney (table 5) although this is partly a result of this organ's role in the excretion of the degradation products of iodinated LDL, namely iodotyrosine and free iodide - note the high TCA-soluble activity in figure 19. In animals with tumours, receptor-mediated hepatic assimilation of the lipoprotein fell ($p<0.05$, Wilcoxon Rank Test) while uptake into other tissues remained the same as in the control situation. The tumour was moderately active in taking up LDL by the receptor pathway, and when account was taken of its mass (figure 19) it was found to be second only to liver in activity. The presence of MAC-13 tumour in these mice did not perceptibly change their total plasma cholesterol value (table 6), nor did it change the whole body clearance of native or CHD-modified LDL (figure 20).

## Receptor mediated organ uptake of LDL in NMRI/MAC 13 mice

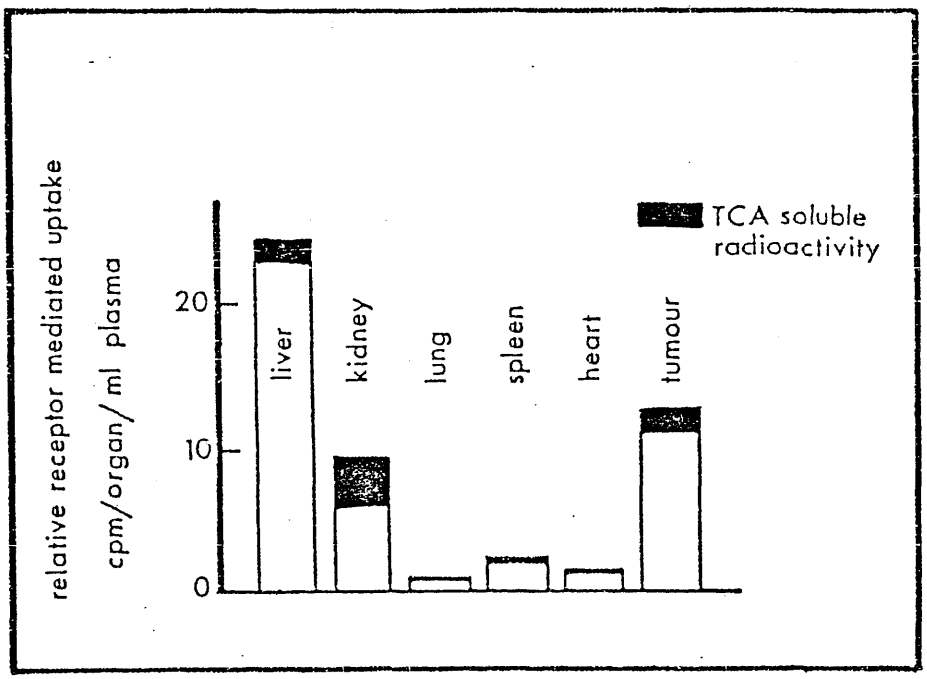



Figure 19: Assimilation of LDL via the receptor pathway, in NMRI mouse tissues and MAC-13 tumours,. measured as described in Section 2.1.14. The low molecular weight degradation products of the lipoprotein were extracted from tissue homogenates using trichloroaceticacid(TCA).

| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/GRAM TISSUE/mL PLASMA) | |
|---|---|---|
| | CONTROL GROUP (n=11) | TUMOUR GROUP (n=15) |
| Liver | 30±11 | 21±9 ** |
| Spleen | 14±11 | 15±7 |
| Kidney | 23±12 | 26±5 |
| Heart | 13±5 | 19±6 |
| Lung | 14±9 | ND * |
| Muscle | 6±3 | 11±3 |
| Tumour | - | 16±8 |

Table 5 : Receptor-mediated LDL uptake into the tissues of NMRI/MAC 13 mice[152].

* ND : Not determined.

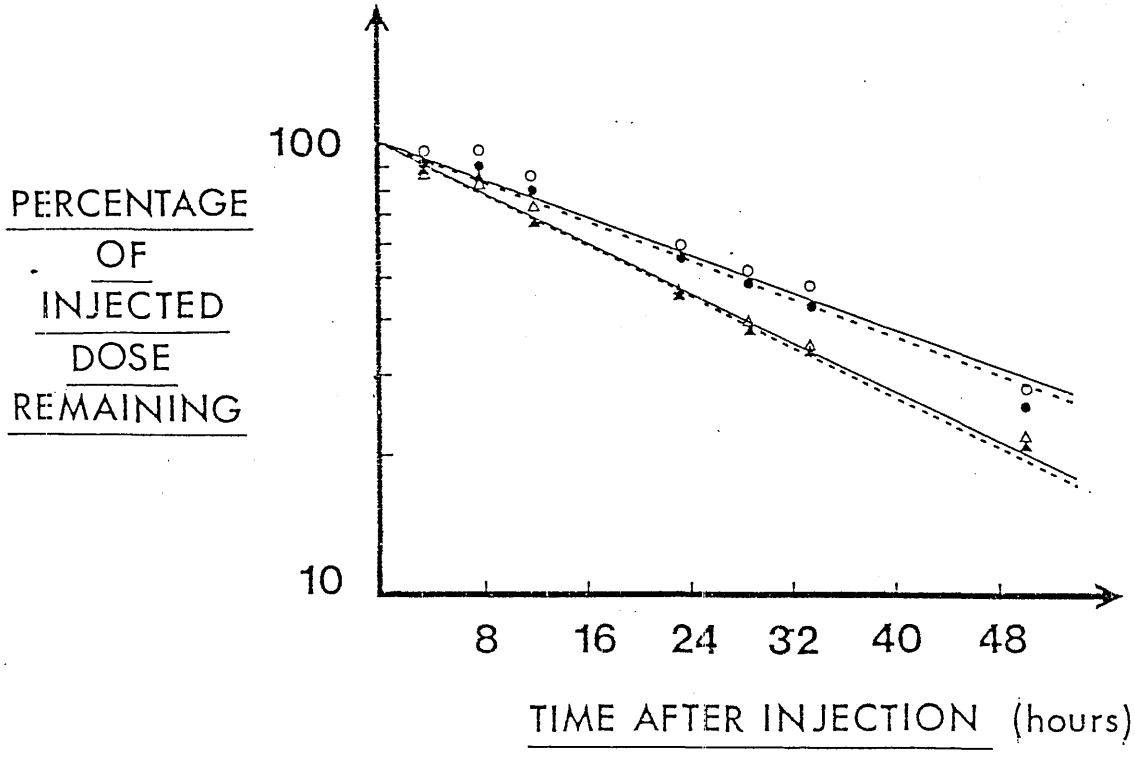
** Wilcoxon Rank Test, $p<0.05$

| GROUP | PLASMA CHOLESTEROL (mmol/L) |
|---|---|
| Control* (N=11) | 2.91±1.07 |
| Control, cholesterol fed (n=12) | 4.62±0.64 |
| Tumour* (n=15) | 2.72±0.81 |
| Tumour, cholesterol fed (n=15) | 4.44±0.79 |

* Tissue uptakes of LDL for these groups are shown in Table 13.

Table 6 : Effects of cholesterol feeding on plasma cholesterol concentrations in control and MAC-13 tumour inoculated NMRI mice[152].

# DECAY OF NATIVE- AND CHD- LDL IN CONTROL AND TUMOUR-BEARING NMRI MICE




$^{131}$I-CHD-LDL
$^{125}$I-NAT-LDL
CONTROLS n=10

$^{131}$I-CHD-LDL
$^{125}$I-NAT-LDL
MAC-13 TUMOURS n=10

| FCR $hr^{-1}$ | CONTROL | TUMOUR |
|---|---|---|
| CHD | 0.024 | 0.025 |
| NAT | 0.032 | 0.033 |
| %RM | 25 | 24 |

Figure 20: Plasma decays of $^{125}$I-native- and $^{131}$I-CHD- LDL in control and MAC-13 tumour-inoculated NMRI mice

B) POLYOMA VIRUS-INDUCED ADENOMATA IN CFLP MICE

The pattern of receptor-mediated LDL assimilation observed in NMRI mice was repeated in this model. Uptakes of LDL per gram of tissue are shown in table 7 . Again the liver, the most active organ in the control group, was suppressed by the presence of tumour ($p<0.05$, Wilcoxon Rank Test). In these animals the opportunity was taken to measure adrenal LDL uptake. As expected, it was very high per gram of tissue (table 7), but because of its size, made a negligible contribution to overall LDL catabolism (figure21). The tumour was also very active on a per gram basis and was matched only by hepatic uptake when its mass was taken into account (figure 21).

C) DIMETHYLHYDRAZINE-INDUCED COLONIC TUMOURS IN RATS

As in the mouse models, liver was the most active normal organ in receptor-mediated uptake of LDL, whether uptake was expressed per gram (table8) or per organ (figure 22). The presence of this type of tumour in the rats did not suppress hepatic LDL uptake (figure 22), however, there was a significant ($p<0.05$, Wilcoxon Rank Test) reduction in receptor-mediated assimilation of LDL by the ovaries of animals with tumours. Once again, both adrenals and ovaries had high LDL receptor activity per gram (table 8) although their contribution to overall LDL catabolism is minimal (figure 22). Tumour made a large contribution to receptor-mediated LDL catabolism with higher uptake of LDL by this pathway than any other organ.

| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/GRAM TISSUE/mL PLASMA) | |
|---|---|---|
| | CONTROL GROUP (n=9) | TUMOUR GROUP (n=9) |
| Liver | 60±14 | 27±9 * |
| Spleen | 22±18 | 22±7 |
| Kidney | 36±12 | 28±10 |
| Heart | 31±5 | 23±7 |
| Lung | 36±16 | 24±7 |
| Muscle | 8±2 | 7±15 |
| Adrenal | 51±36 | 36±24 |
| Tumour | - | 31±9 |

Table 7 : Receptor mediated LDL uptake into the tissues of CFLP/viral adenoma mice[152].

* Wilcoxon Rank Test, $p<0.05$

## Receptor mediated organ uptake of LDL in CFLP/adenoma mice

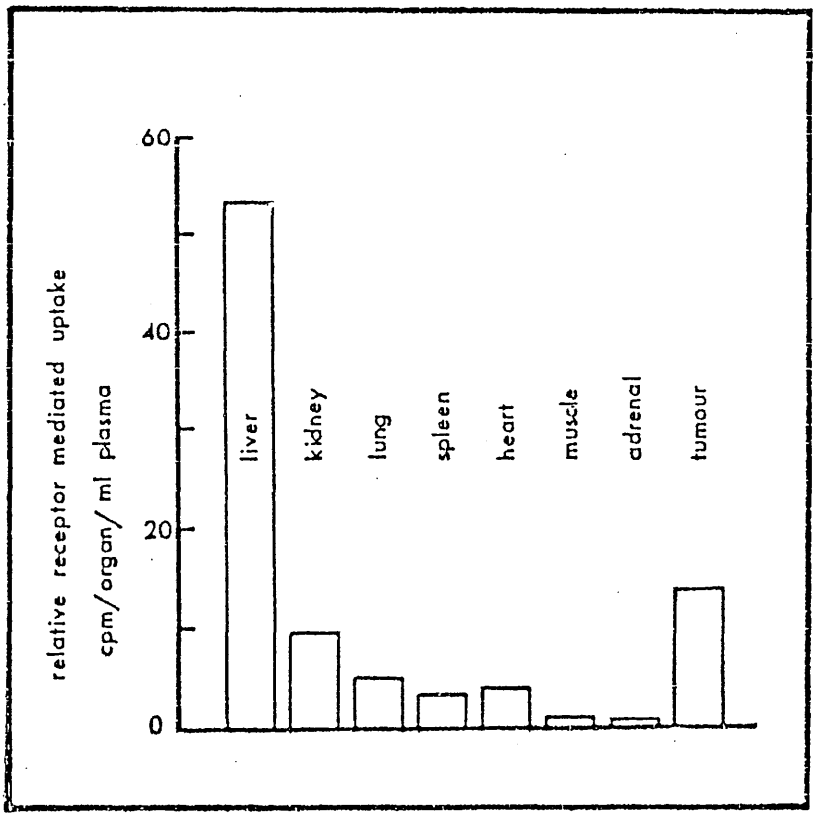



Figure 21: Uptake of LDL via the LDL receptor pathway, in CFLP mouse tissues and polyoma virus-induced tumours, measured as described in Section 2.1.14.

| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/GRAM TISSUE/mL PLASMA) | |
|---|---|---|
| | CONTROL GROUP (n=3) | TUMOUR GROUP (n=3) |
| Liver | 88±3 | 104±15* |
| Adrenal | 71±10 | 63±16 |
| Spleen | 58±13 | 57±10 |
| Ovary | 70±3 | 29±7 * |
| Lung | 72±13 | 50±8 # |
| Kidney | 70±6 | 83±24 |
| Gut | 64±2 | 64±12 |
| Heart | 28±5 | 26±6 |
| Muscle | 8±0.2 | 8±3 |
| Fat | 5±1 | 14±4 |
| Tumour | - | 160±35 |

Table 8 : Receptor-mediated uptake of LDL by the tissues of normal rats and those with dimethylhydrazine-induced colonic tumours.

* Wilcoxon rank test vs controls, $p<0.05$,

\# Not significant

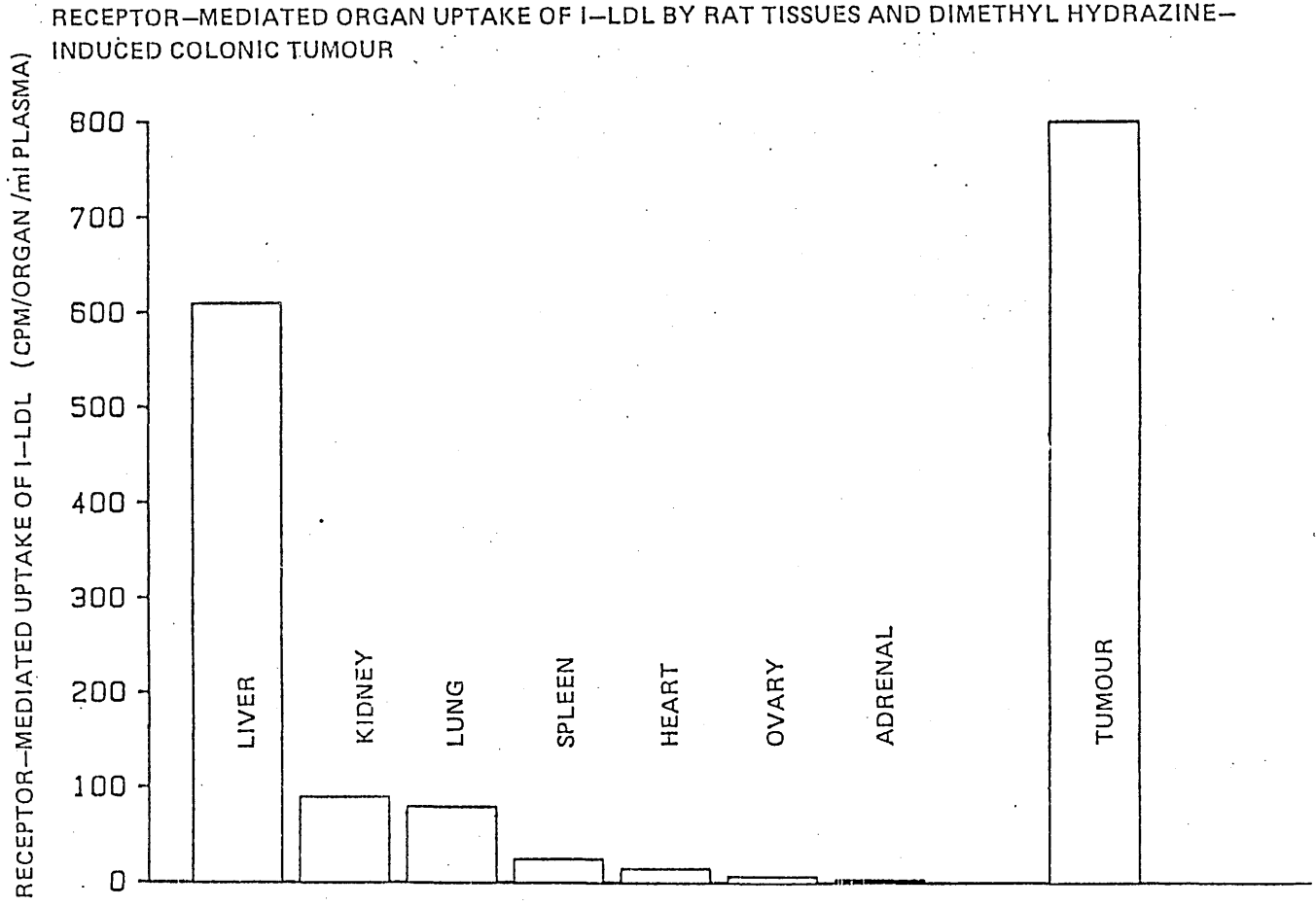



Figure 22: Receptor-mediated uptake of LDL by rat tissues and colonic tumours, measured as described in Section 2.1.14.

## D) WALKER-256 TUMOUR IN WISTAR RATS

This tumour was very active in receptor-mediated LDL assimilation. When compared to rat vital organs, tumour came second only to liver (figure 23) and gram for gram (table 9) tumour was more active than any other type of tissue assayed. Adrenals and spleen had high uptake per gram of tissue, but as organs they catabolised only a small proportion of the LDL injected (figure 23).

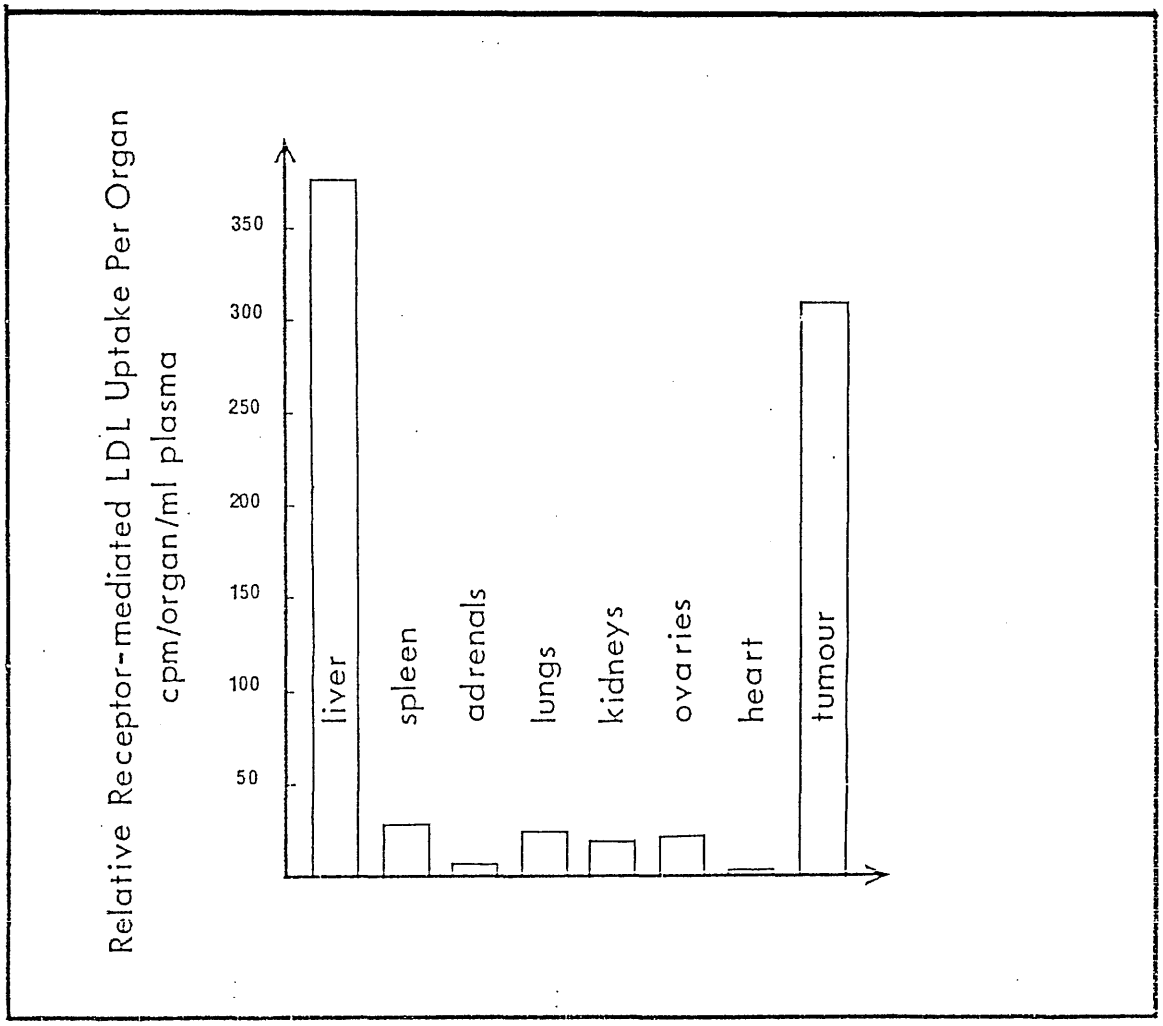



Figure 23: Relative receptor-mediated uptake of LDL by rat organs and Walker256 tumour implants.

| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/GRAM TISSUE/mL PLASMA) |
|---|---|
| Tumour | $36^{+}_{-}12$ |
| Liver | $29^{+}_{-}5$ |
| Spleen | $29^{+}_{-}5$ |
| Adrenal | $17^{+}_{-}10$ |
| Lung | $13^{+}_{-}5$ |
| Kidney | $10^{+}_{-}3$ |
| Ovary | $8^{+}_{-}2$ |
| Gut | $7^{+}_{-}10$ |
| Skin | $5^{+}_{-}4$ |
| Muscle | $1^{+}_{-}2$ |
| Fat | $1^{+}_{-}1$ |

Table 9 : Relative Receptor-Mediated uptake of LDL into tissues of 8 rats with Walker 256 Tumour implants.

## 2.2.2. MEASUREMENT OF LDL CATABOLISM USING CUMULATIVE NON-DEGRADABLE MARKERS

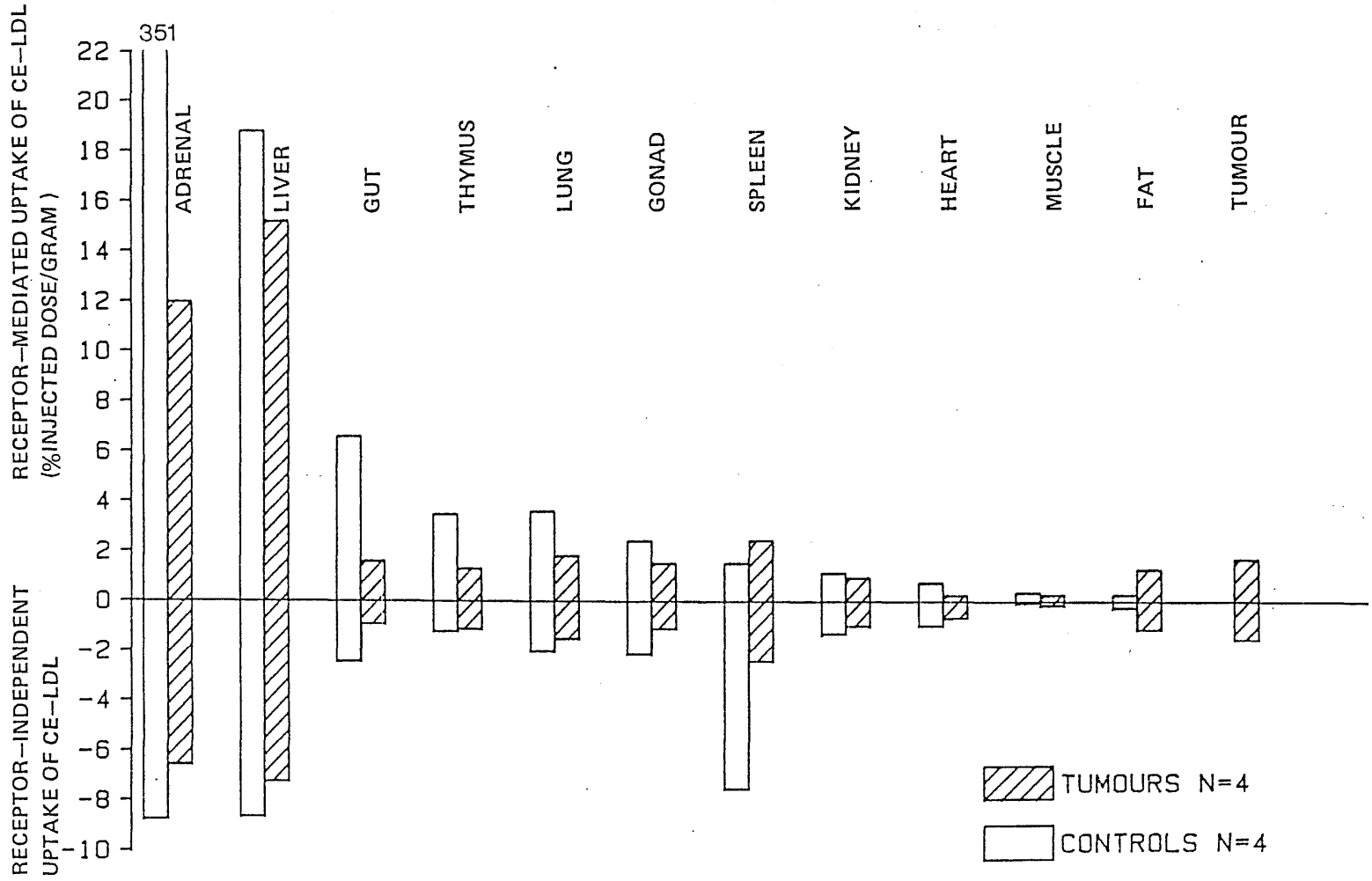
In an attempt to improve the assessment of LDL uptake into tumour tissues $^{125}$I-cellobiose-tyramine-LDL[105] and $^{125}$I-cholesteryl linoleyl ether-LDL[106] (figure 14), were used as probes. It has been reported[105,106] that, once assimilated by cells, the leakage rate of these tracers is less than 35% per day compared with the instantaneous release of iodotyrosine from cell-ingested radioiodinated LDL.

Animals were injected with native $^{125}$I-CT-LDL (2.1.7.) or $^{125}$I-CE-LDL (2.1.5.) and after 18 hours tumour and tissues were removed, rinsed, blotted and assayed for radioactivity. The uptake of labelled LDL into each tissue was expressed as a percentage of the injected dose per organ and per gram of tissue. In NMRI mice with MAC-13 tumours receptor activity was measured after injection of $^{125}$I-native- and $^{131}$I-CHD-modified-cholesteryl ether LDL.

A) MAC-13 TUMOURS

In the control group adrenal uptake of CE-LDL (per gram) by the receptor pathway was highest of all tissues (figure 24). Uptake by adrenals in tumour-inoculated animals was significantly lower ($p<0.05$ Wilcoxon Rank Test), table 10, a phenomenon which was not observed using I-LDL as a probe (figure 20). Most tissues in tumour animals showed slightly lower LDL assimilation by both receptor-dependent and receptor-independent routes. Using this marker of LDL catabolism, relative tumour LDL receptor activity was quite low, although it was second only to liver when considered as a whole.

Figure 24: UPTAKE OF CHOLESTERYL–ETHER–LDL PER GRAM OF NMRI MOUSE TISSUES AND MAC-13 TUMOUR



| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/ORGAN/DOSE) x 100 | |
|---|---|---|
| | CONTROL GROUP (n=6) | TUMOUR (n=3) |
| Liver | 14.1±1.1 | 10.7±0.2 * |
| Spleen | 0.35±0.51 | 0.44±0.03 |
| Kidneys | 0.12±0.01 | 0.08±0.009* |
| Adrenals | 0.05±0.005 | 0.02±0.007* |
| Heart | 0.03±0.007 | 0.02±0.002 |
| Lung | 0.09±0.008 | 0.13±0.01 |
| Thymus | 0.03±0.002 | 0.04±0.001 |
| Gonads | 0.018± 0.004 | 0.018± 0.002 |
| Tumour | - | 2.13± 0.18 |

Table 10 : Relative receptor-mediated uptake of Cholesteryl ether-LDL by NMRI mouse organs and MAC-13 tumour.

* Wilcoxon rank test vs controls, $p<0.05$.

B) COLONIC TUMOURS

In the rats, spleen had the highest uptake of $^{125}$I-CT-LDL/g (table 11). It is likely that this high activity largely represents the accumulation of denatured tracer material, which is less than 5% of the injected dose (figure 25) but is directed specifically at this organ and accumulates there. Next highest in LDL assimilation was liver, where there was comparatively higher hepatic LDL uptake in the tumour group than the control group ($p<0.05$, Wilcoxon Rank Test) - exactly the opposite of the situation in both mouse models studied (tables 5 and 7). Tumour uptake was surpassed only by liver and spleen (table 11).

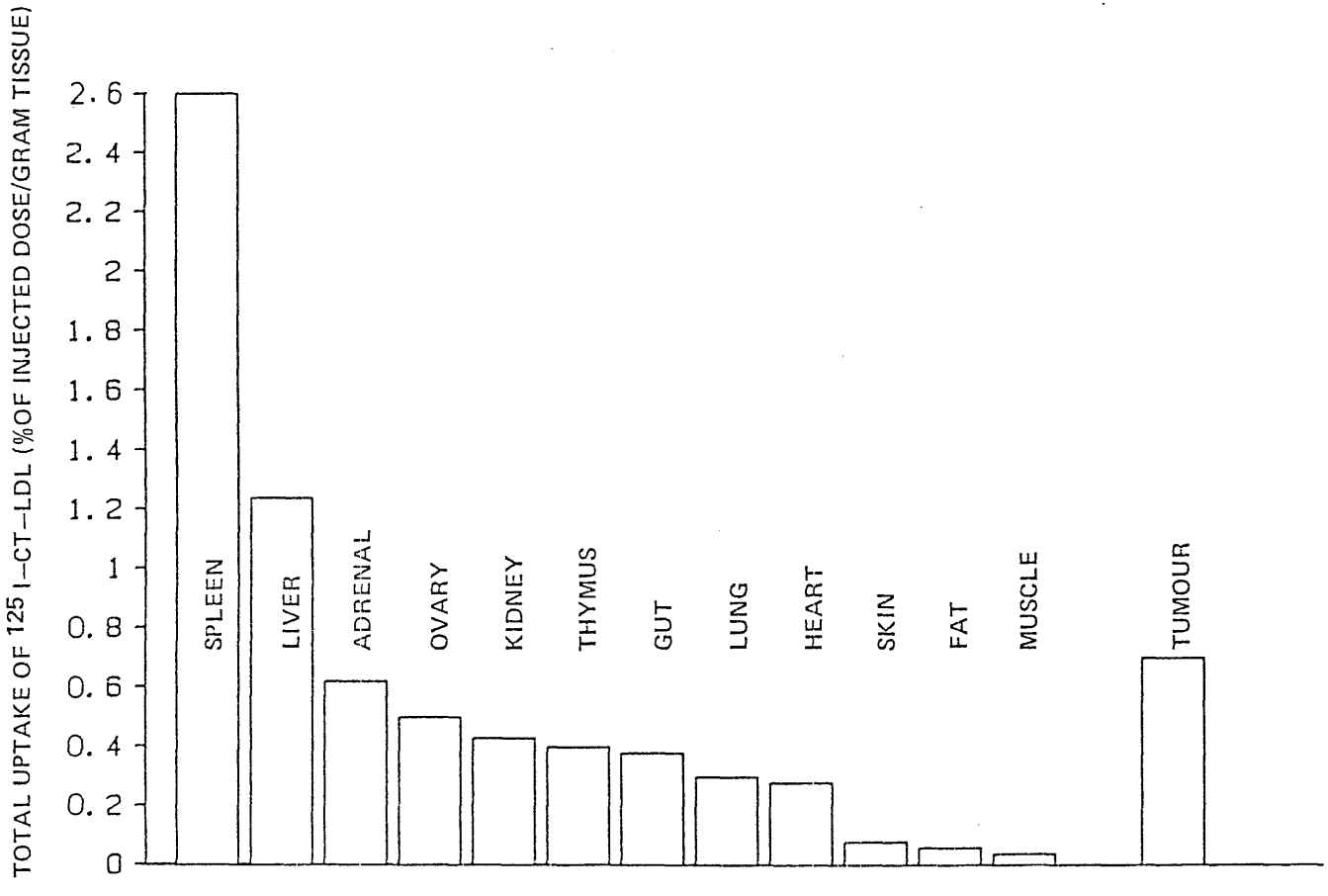
C) PANCREATIC TUMOURS

As a whole, liver had considerably higher overall LDL uptake than any other tissues in these animals (table 12). When uptakes were expressed per gram of tissue, spleen had the highest value (figure 26), as observed in the rat model above. In tumour animals splenic uptake was suppressed ($p<0.05$, Wilcoxon Rank Test) as compared to control rats. In both cases the overall contribution of the spleen to LDL catabolism was relatively small. Adrenals in the tumour group had high LDL assimilation, whereas the activity in control animals was considerably smaller ($p<0.05$, Wilcoxon Rank Test). In this model a number of tissues were quite active in assimilation of LDL (figure 26) and tumour appears to be only moderately active.

| TISSUE | TOTAL LDL UPTAKE (CPM/ORGAN/DOSE) X 100 | |
|---|---|---|
| | CONTROL GROUP (n=18) | TUMOUR GROUP (n=22) |
| Tumour | - | 0.70±0.23 |
| Spleen | 3.15±1.07 | 2.60±1.18 |
| Liver | 1.00±0.19 | 1.24±0.20* |
| Adrenal | 0.60±0.28 | 0.62±0.17 |
| Ovary | 0.33±0.24 | 0.51±0.28 |
| Kidney | 0.43±0.07 | 0.43±0.07 |
| Thymus | 0.23±0.09 | 0.40±0.09* |
| Gut | - | 0.38 0.22 |
| Lung | 0.40±0.13 | 0.31±0.07 |
| Heart | 0.19±0.09 | 0.18±0.04 |
| Skin | - | 0.08±0.02 |
| Fat | 0.09±0.07 | 0.06±0.06 |
| Muscle | - | 0.05±0.04 |

Table 11 : Uptake of $^{125}$I-cellobiose-tyramine-LDL by rat organs and dimethyl hydrazine-induced-colonic tumours.

Figure 25: UPTAKE OF $^{125}$I–CT–LDL BY RAT TISSUES AND COLONIC TUMOURS EXPRESSED AS A PERCENTAGE OF THE INJECTED DOSE, PER GRAM OF TISSUE

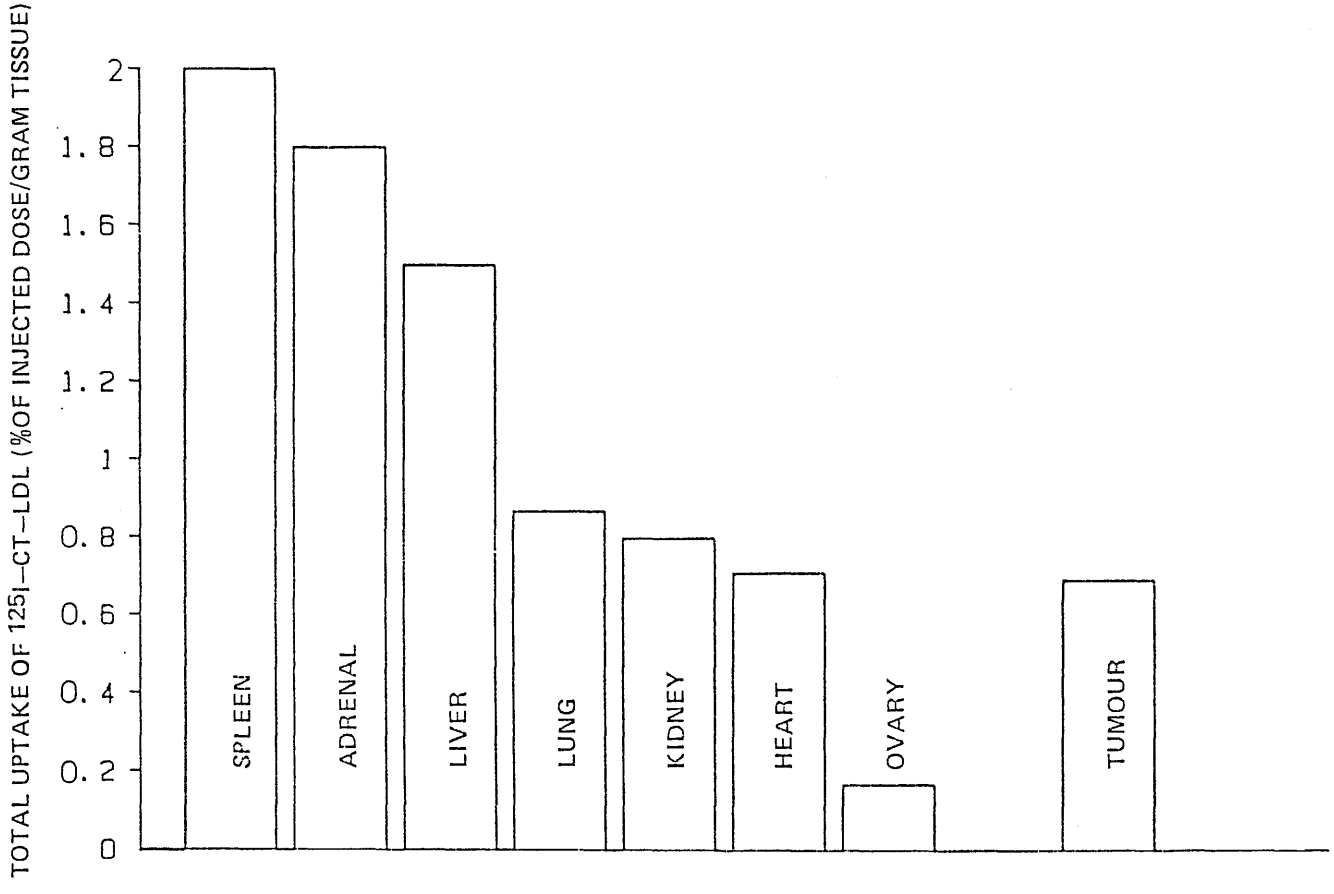


| TISSUE | TOTAL LDL UPTAKE (CPM/ORGAN/DOSE) X 100 | |
|---|---|---|
| | CONTROL GROUP (n=5) | TUMOUR GROUP (n=4) |
| Tumour | - | 0.88±0.77 |
| Liver | 3.68±2.13 | 6.2 ±1.6 |
| Spleen | 0.11±0.05 | 0.26±0.04* |
| Kidneys | 0.70±0.30 | 1.03±0.25 |
| Adrenals | 0.06±0.03 | 0.77±0.03* |
| Heart | 0.28±0.15 | 0.33±0.14 |
| Lungs | 0.69±0.26 | 0.72±0.07 |
| Thymus | 0.10±0.10 | 0.10±0.07 |
| Gonads | 0.11±0.02 | 0.11±0.01 |

Table 12 : Uptake of $^{125}$I-cellobiose-tyramine-LDL by Hamster organs and BOP-induced pancreatic tumours.

* Wilcoxon Rank Test vs. controls, $p<0.05$

Figure 26: TOTAL UPTAKE OF $^{125}$I–CT–LDL BY HAMSTER TISSUES AND PANCREATIC TUMOUR AS A PERCENTAGE OF THE INJECTED DOSE, PER GRAM OF EACH TISSUE



## 2.2.3. FEEDING EXPERIMENTS

In all of the preceding animal models, liver has been the major organ of receptor-mediated LDL catabolism, both as a whole organ and per gram of tissue. It is likely therefore that chemotherapy using LDL as a vehicle would be greatly damaging to this essential organ. In order to reduce the potential toxicity we have looked at a number of ways of minimising hepatic uptake of LDL during therapy (figure 27).

Also identified as organs at risk during "LDL chemotherapy" are the adrenal glands. Although they are very small and their overall contribution to LDL catabolism is relatively low, they rapidly utilise LDL in the synthesis of essential steroid hormones. As a result, adrenals have very high LDL receptor activity per gram, and are likely to be damaged during chemotherapy using LDL as a vehicle. Hydrocortisone has been used to reduce adrenal LDL uptake.

### A) CHOLESTEROL FEEDING

NMRI mice with and without tumours were fed a diet containing 1% cholesterol (g/g) (2.1.13.) for 7 days prior to the experiment. Plasma cholesterol concentrations were measured at the time of sacrifice in groups of animals fed the normal diet or the higher cholesterol diet and these are shown in (table 6). Despite the increase in plasma cholesterol levels, a similar pattern of LDL catabolism was observed in both groups of animals. No significant suppression of receptor-mediated assimilation was apparent in any of the mouse organs or tumour (table 13).

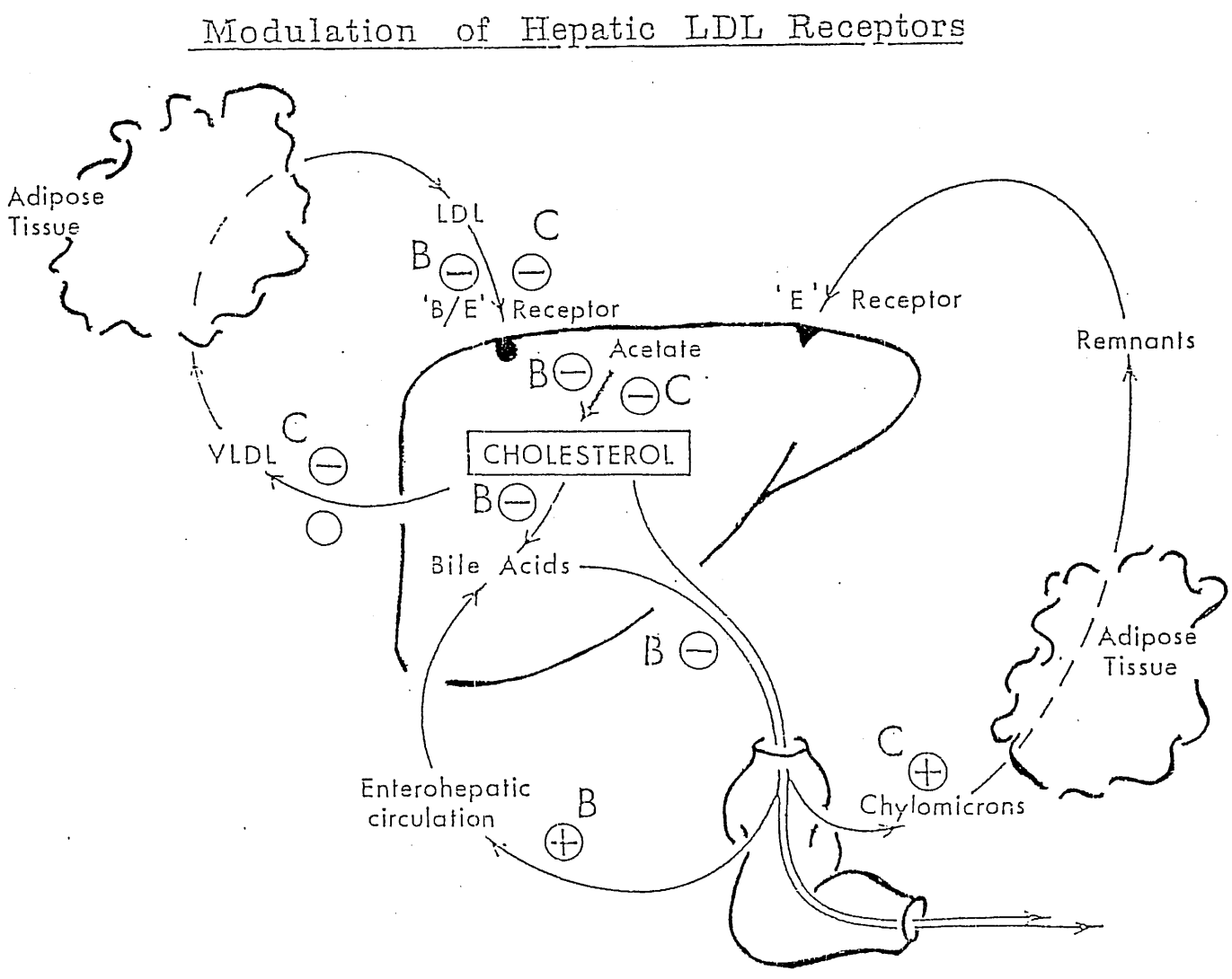



Figure 27: The effects of bile acid (B) and cholesterol (C) feeding on hepatic lipoprotein receptors.

| TISSUE | RELATIVE RECEPTOR-MEDIATED LDL UPTAKE (CPM/GRAM TISSUE/mL PLASMA) | |
|---|---|---|
| | CONTROL GROUP (n=12) | TUMOUR GROUP (n=15) |
| Liver | $26 \pm 7$ | $20 \pm 12$ |
| Spleen | $22 \pm 8$ | $17 \pm 7$ |
| Kidney | $53 \pm 32$ | $30 \pm 20$ |
| Heart | $21 \pm 6$ | $27 \pm 11$ |
| Muscle | $12 \pm 6$ | $10 \pm 7$ |
| Tumour | - | $20 \pm 10$ |

Table 13 : Effects of cholesterol feeding on receptor-mediated LDL assimilation by the tissues of control and MAC-13 tumour inoculated NMRI mice[152].

Both groups received a diet containing 1% cholesterol (W/W) for 7 days prior to the experiment. Plasma Cholesterol values for these animals are given in table 6.

B) BILE SALT SUPPLEMENTATION

It is known that bile acid feeding suppresses hepatic sterol synthesis and LDL receptor activity in the dog[195,196] and in the rat.[197] When sodium taurocholate was administered to NMRI mice (table 14) at a dose of 1% of the dietary intake (g/g) a fall incurred in receptor-mediated LDL uptake by the liver ($p < 0.05$, Wilcoxon Rank Test). As expected this agent did not affect LDL uptake into other organs, including the adrenal gland.

C) HYDROCORTISONE THERAPY

In an attempt to inhibit adrenal uptake of LDL, hydrocortisone was administered daily by intraperitoneal injection at a dose of 5mg per day to NMRI mice for 7 days. This effectively reduced ($p < 0.05$, Wilcoxon Rank Test), the receptor-mediated adrenal uptake of LDL (table 14) by ca. 50% in the control mice and by a similar amount in the tumour-inoculated group. In rats with colonic tumours, hydrocortisone reduced adrenal uptake by 40% in control animals and 30% in tumour bearing animals (table 15).

D) COMBINATION OF BILE SALTS AND HYDROCORTISONE

When sodium taurocholate feeding was combined with hydrocortisone injections in NMRI mice, adrenal receptor activity fell dramatically (by 80% in controls and 60% in tumour-bearing mice) table 14. In the rats, the fall in adrenal LDL receptor activity (table 15) remained the same whether hydrocortisone was administered alone or in conjunction with bile salts.

| GROUP | RELATIVE RECEPTOR MEDIATED TISSUE UPTAKE (cpm/gm/1.0 ml plasma) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LIVER | SPLEEN | KIDNEY | HEART | LUNG | MUSCLE | ADRENAL | TUMOUR |
| Control (N=4) | 64±5 | 26±8 | 33±12 | 32±3 | 31±12 | 5±3 | 66±16 | - |
| Control + Na taurocholate (n=4) | 48±9* | 26±13 | 44±22 | 36±17 | 31±18 | 6±4 | 65±4 | - |
| Control + hydrocortisone (n=4) | 91±2 | 43±11 | 52±16 | 47±18 | 53±20 | 11±2 | 34±16* | - |
| Control + Na taurocholate + hydrocortisone (n=4) | 61±1 | 41±2 | 57±13 | 37±10 | 29±15 | 16±10 | 13±16* | - |
| Tumour (n=5) | 53±8* | 30±8 | 42±9 | 50±34 | 45±7 | 24±18 | 71±14 | 78±15 |
| Tumour + Na taurocholate (n=5) | 44±2* | 27±7 | 34±19 | 23±5 | 35±15 | 7±3 | 41±22* | 99±56 |
| Tumour + hydrocortisone (n=5) | 54±17 | 28±10 | 47±12 | 32±6 | 33±13 | 11±5 | 50±8* | 84±25 |
| Tumour + Na taurocholate + hydrocortisone (n=2) | 34±2* | 14±6 | 30±5 | 20±3 | 29±6 | 5±2 | 29±3* | 59±17 |

The effects of diet or steroid supplementation were assessed by the Wilcoxon Rank Test.
* Significantly different from untreated group, $p<0.05$

Table 14 : Effects of Na taurocholate and hydrocortisone sodium succinate on relative receptor mediated LDL uptake by tissues of control and MAC-13 inoculated NMRI mice[152].

RELATIVE RECEPTOR-MEDIATED UPTAKE OF LDL BY RAT TISSUES AND COLONIC TUMOURS
(cpm/g tissue / ml plasma)

| GROUP | LIVER | OVARY | LUNGS | ADRENAL | SPLEEN | KIDNEY | GUT | SKIN | THYMUS | HEART | MUSCLE | FAT | TUMOUR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (n=4) | 53±10 | 53±12 | 49±6 | 48±12 | 46±4 | 38±12 | 35±11 | 28±15 | 27±9 | 20±5 | 5±4 | 3±0.6 | - |
| Control + hydrocortisone (n=4) | 43±1 | 16±2* | 38±7 | 29±5 | 38±3 | 28±1 | 21±2 | 12±2 | 3±1* | 14±1 | 4±0.7 | 2±0.2 | - |
| Control + sodium taurocholate + hydrocortisone (n=4) | 50±15 | 16±9* | 46±11 | 39±12 | 54±14 | 48±12 | 39±11 | 14±1 | 14±5 | 21±6 | 3±1 | 2-0.5 | - |
| Tumour + hydrocortisone (n=4) | 31±2* | 10±2* | 34±1* | 33±11 | 45±5 | 31±2* | 21±1 | 14±0.3 | 6±0.3* | 17±1 | 3±0.1 | 2±0.2 | 34±6 |
| Tumour + sodium taurocholate + hydrocortisone (n=4) | 43±3 | 18±3* | 37±3* | 29±1* | 39±4 | 40±4 | 11±16 | 15±1 | 21±5 | 18±1 | 4±0.1 | 4±0.7 | 39±9 |

* Wilcoxon rank test vs. controls, $p<0.05$.

Table 15 : Effects of sodium taurocholate and hydrocortisone sodium succinate on relative receptor-mediated uptake of LDL by rat tissues and dimethyl hydrazine-induced colonic tumours.

Assimilation of LDL by the tumour did not change significantly during either hydrocortisone or bile acid therapy, or as a result of their simultaneous administration.

## 2.3. METHODS II

The following section describes the methods employed for incorporation of cytotoxic drugs into LDL, and assessing the cytotoxicity of the drug-LDL complexes so formed.

### 2.3.1. SYNTHESIS OF CHLORAMBUCIL CHOLESTERYL ESTER (CBE)

Molar equivalents of chlorambucil, cholesterol and dicyclohexyl-carbodiimide and (when required) 1 mCi [7-$^{3}$H]-cholesterol were dissolved in dry diethyl ether in a pre-dried round-bottomed flask. The flask was flushed with nitrogen, sealed and its contents stirred at room temperature for 24 hours. The progress of the reaction was monitored by thin-layer chromatography (TLC) using chloroform : ethyl acetate 8 : 1 as eluant, on silica gel $F_{254}$ TLC plates. Chlorambucil remained near the origin ($R_f$ = 0.35) while the ester migrated near the solvent front ($R_f$= 0.92) and cholesterol between the two ($R_f$= 0.65). On completion of the reaction, precipitate was removed by filtration and the solvent evaporated from the remaining solution. The residue was dissolved in chloroform, prior to removal of unreacted starting materials on silica and basic alumina columns. Elution was achieved using chloroform and carbon tetrachloride alternatively in 5 mL aliquots. The purified cholesteryl ester was dried, weighed and infrared and $^{1}$H-NMR spectra obtained (figure 28). The infrared spectrum showed a characteristic peak for the ester carbonyl stretch at 1740 cm$^{-1}$ and confirmed that the product was free of unreacted cholesterol (no H-O absorption). The reaction yield was typically 60-80%.

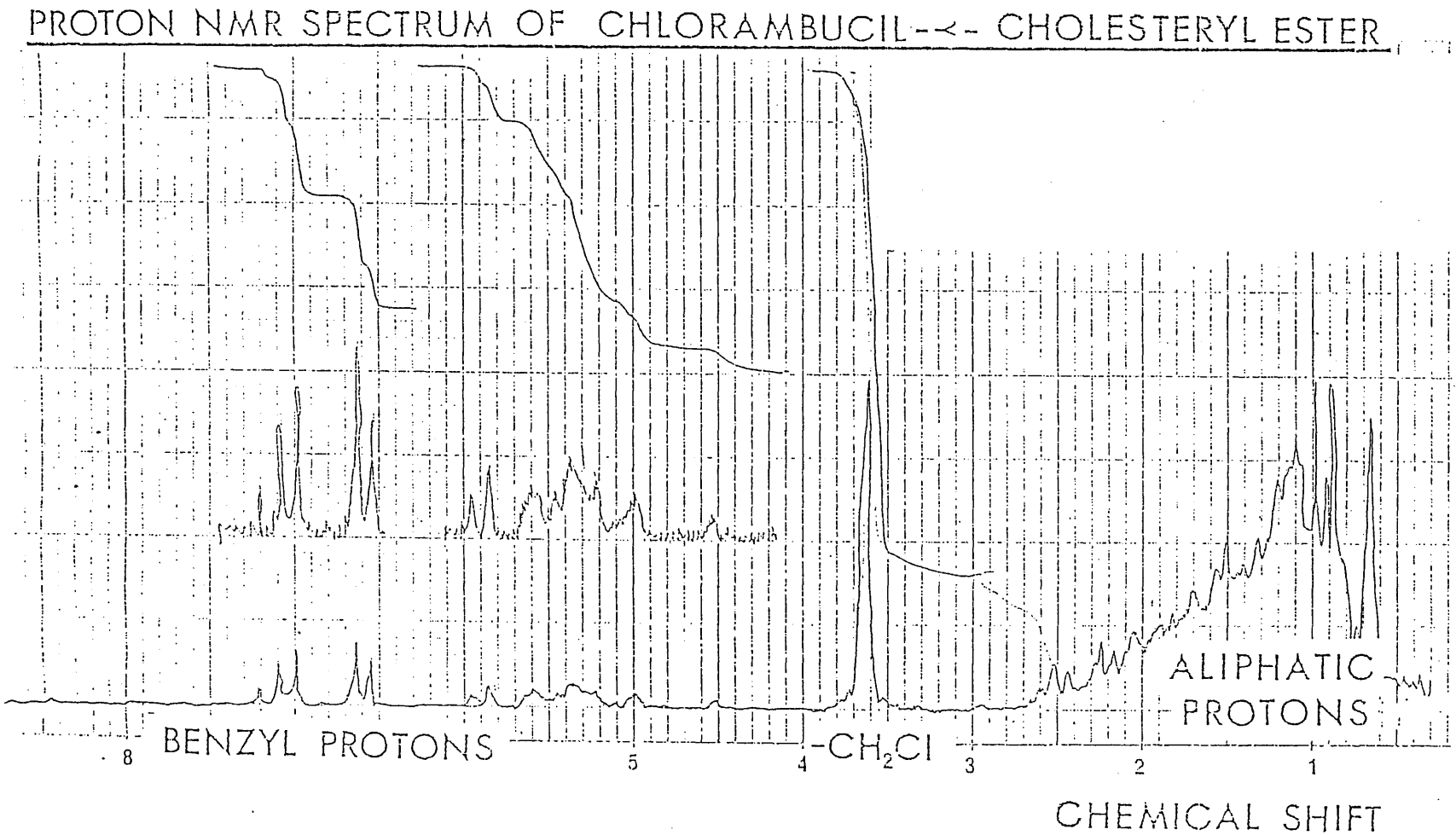



Figure 28: Proton NMR spectrum of the cholesteryl ester of chlorambucil prepared as described in Section 2.3.1.

## 2.3.2. INCORPORATION OF CBE INTO LDL BY EXCHANGE FROM CHOLATE/LIPID MICELLES

Initially we used an adaptation of Stoffel's method[190] for loading HDL with labelled cholesteryl esters. This procedure has been described in detail in Section 2.1.5. In order to improve the overall efficiency of drug incorporation, several other modifications were made to the method (figure 29).

### A) "RECONSTITUTED" HDL

HDL (d1.063 - 1.21Kg/L) was prepared from human plasma as described in Section 2.1.2. An aliquot containing 23mg of HDL protein was extracted twice with two volumes of chloroform : methanol (3:1), washed with ether and lyophilised overnight. Phospholipid, cholesterol, and [7-$^3$H]-cholesterol chlorambucil ester were added to give the molar ratios of each found in HDL (80:24:53, lipid:protein,1:1,W/W) and the mixture sonicated in 5 mL 0.02M tris, 0.02M NaCl, 0.02M EDTA buffer, pH 8.2 for 15 minutes at 0°C. For comparison, an aliquot of HDL containing the same amount of HDL protein was loaded with[$^3$H]-CBE by exchange from cholate micelles as outlined in Section 2.1.5., replacing I-cholesteryl ether with[$^3$H]-CBE. Both[$^3$H]-CBE preparations were purified by filtration through 0.22 μm millipore filters and centrifugation at 1.063 Kg/L. Fractions of 5 mL were removed from each centrifuge tube and assayed for CBE by[$^3$H]-specific activity.

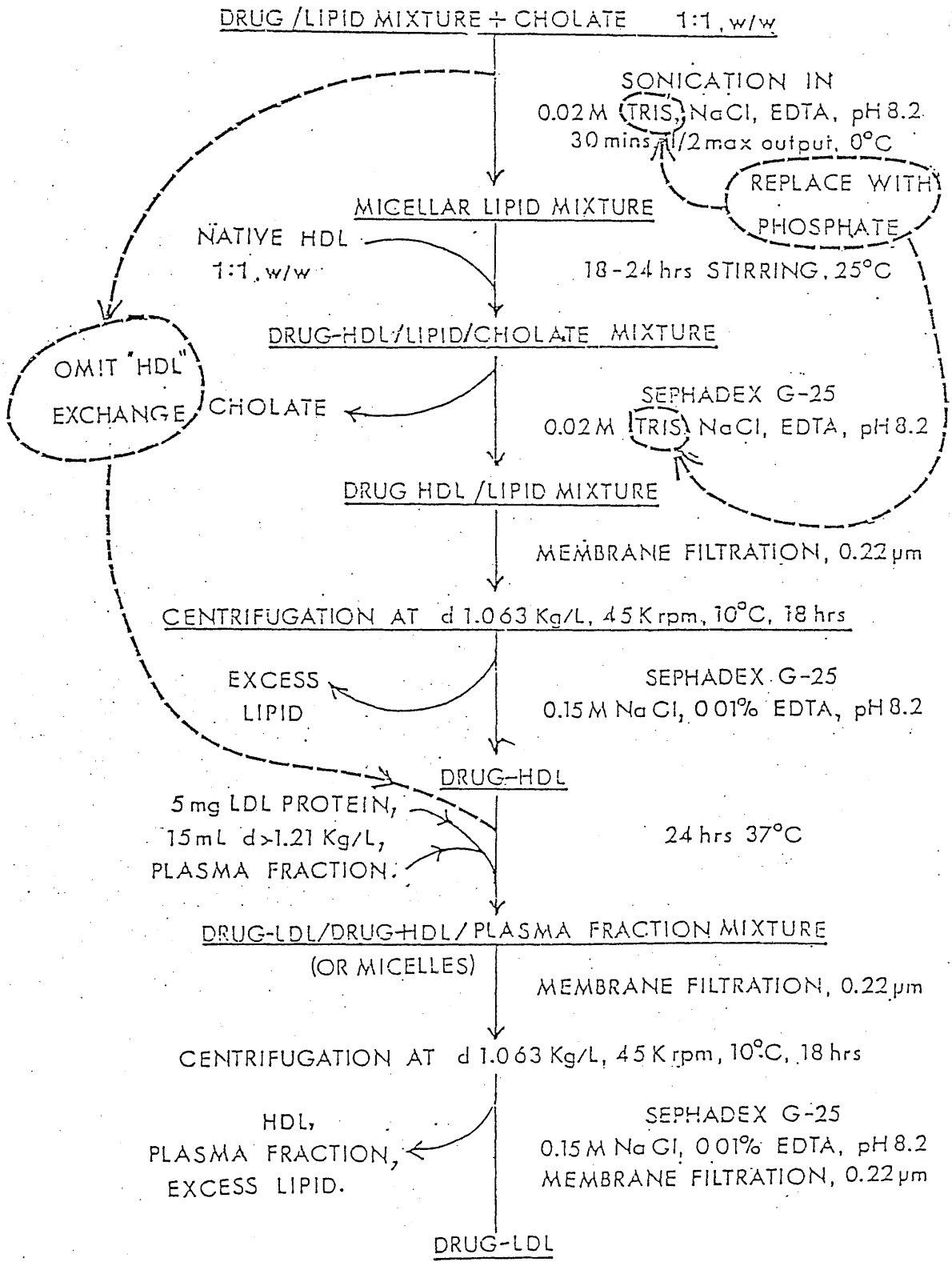



Figure 29: The modified Stoffel method, section 2.3.2, used to load LDL with chlorambucil cholesteryl ester (CBE).

B) EXCHANGE OF CBE FROM CHOLATE MICELLES DIRECTLY TO LDL

5 mL of drug-laden cholate micelles, Section 2.1.5, was stirred overnight at room temperature with 5 mg human LDL (protein) and 15 ml $d > 1.21$ Kg/L plasma fraction (prepared as described in Sections 2.1.1. and 2.1.2. respectively). Chlorambucil ester-laden LDL was purified as described for I-cholesteryl ether LDL (2.1.4.)

## 2.3.3. DEGRADATION OF CBE DURING INCUBATION WITH LDL

Mixtures contained 2mg of CBE in 1mL of micelles, Section 21.1.5. and 4mL of either PBS, heat-inactivated[198] d >1.21 Kg/L plasma fraction (prepared as in section 2.1.2., heated for 1 hour at 60°C and filtered through a 0.45 μm filter), or sodium iodoacetate-treated d >1.21 Kg/L plasma fraction (pre-incubated overnight with 5 mM NaIOAc and dialysed against PBS to remove free NaIOAc)[199]. Each mixture was stirred gently at room temperature for 24 hours and samples were removed at 0,1,2,4 and 24 hours and assayed for CBE by HPLC (2.3.6.)

## 2.3.4. TRANSFER OF CBE FROM SYNTHETIC MICROEMULSIONS TO LDL

As an alternative to the Stoffel[190] method (2.3.2.) drug/phospholipid/ cholesteryl oleate microemulsions were made by the method of Craig et al[200] (figure 30) using the molar proportions of each component suggested by Parks et al[201]. 2.4 mg each of dimyristoyl pholphatidyl choline (DMPC) and CBE were added to 2.37 mg of cholesteryl oleate, in chloroform, the solvent evaporated and the mixture dried under vacuum for at least 1 hour. The dried lipids were dissolved in 200 μL isopropanol (pre-dried over molecular sieves). Sixty-six μL of the lipid mixture were injected quickly into 1 mL of rapidly vortexing PBS, with the entire system kept at 46°C throughout the procedure. Several millilitres of microemulsions were prepared in this way and mixed with equal volumes of LDL (protein 2-6 mg/mL), prior to incubation for 5 hours at 37°C. Thereafter, solid KBr, 0.012 mg/mL, was added to the solution making its density 1.006 Kg/L. The preparation was overlayered with d1.006 solution in Beckman 40.3 centrifuge tubes and the drug-laden LDL separated from the microemulsions by overnight centrifugation a 45 K rpm and 25°C in Beckman 40.3 rotor.

PREPARATION OF CBE-LDL

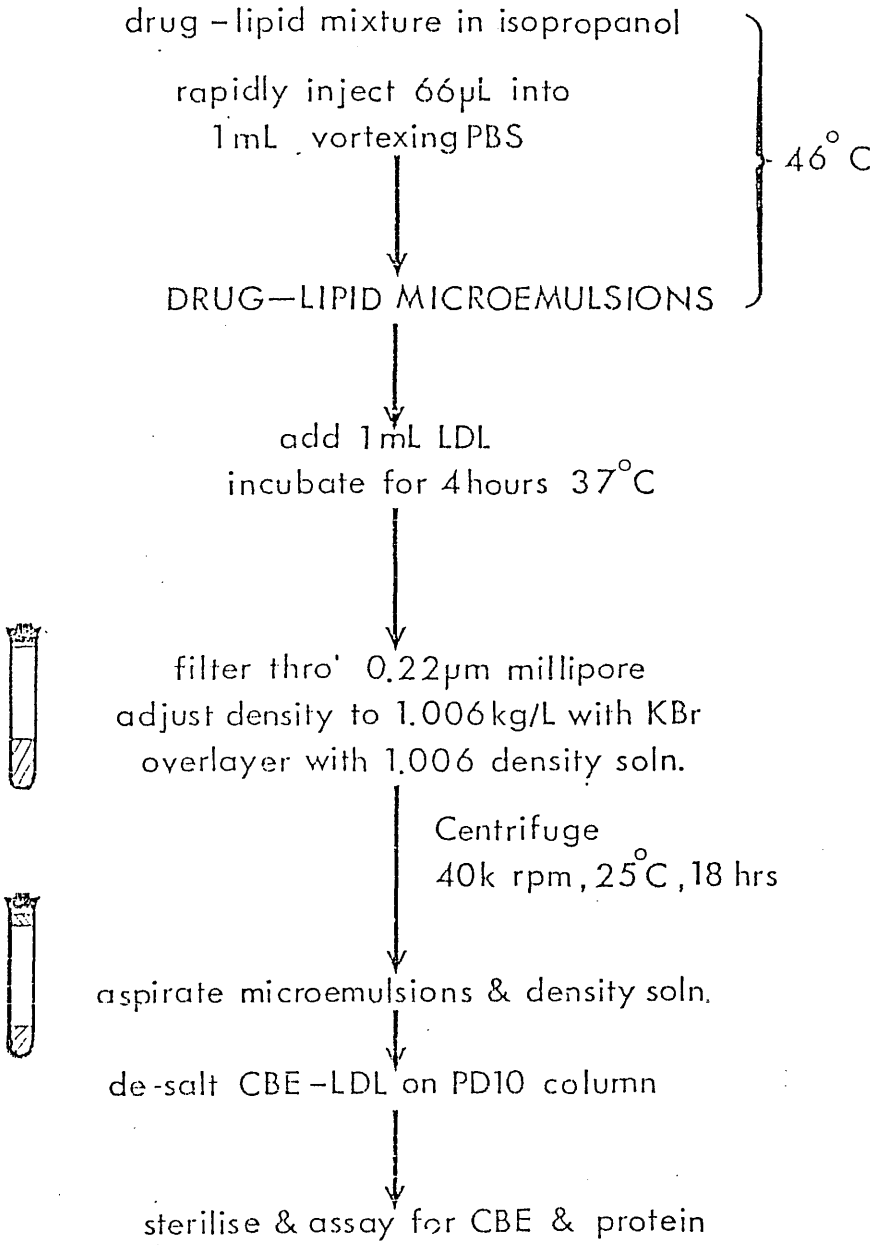



Figure 30: Protocol for the preparation of chlorambucil ester(CBE)-LDL using drug-lipid microemulsions as described in Section 2.3.4.

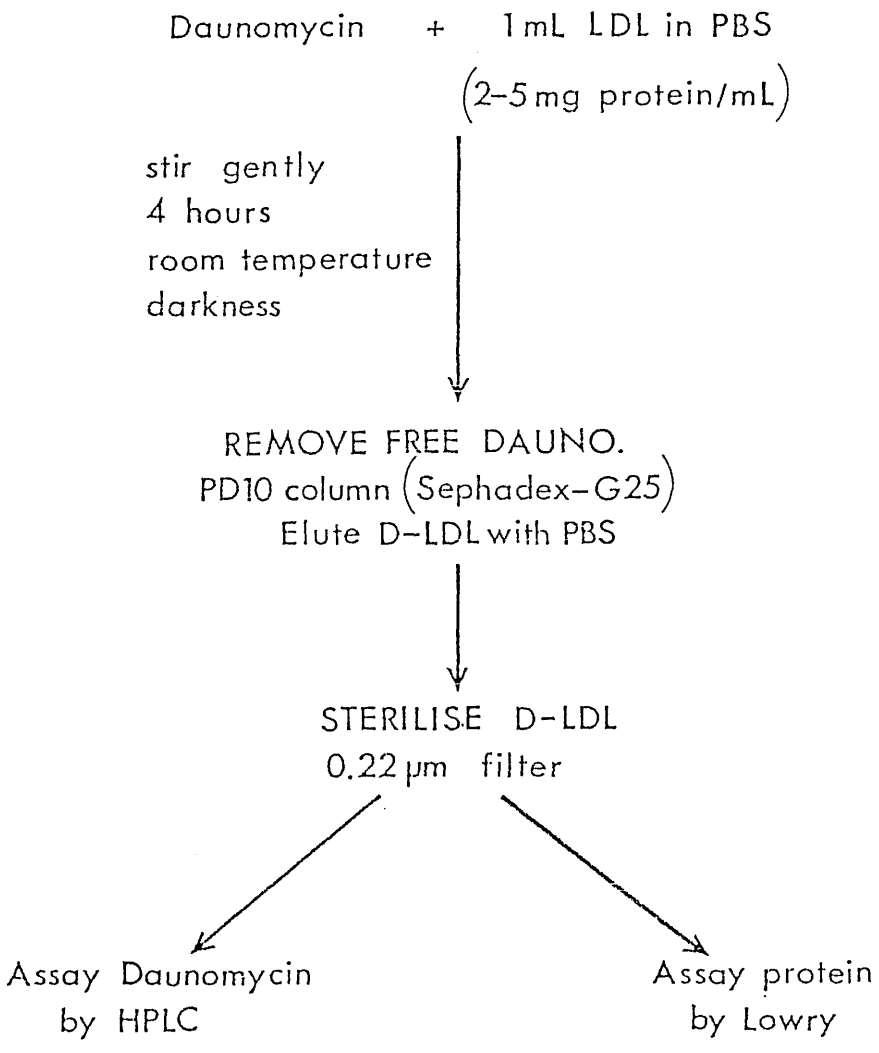
Microemulsions and density solution were aspirated and the infranatant CBE-LDL desalted by gel filtration on a PD10 column, eluting with PBS. The CBE-LDL was sterilised by filtration through a 0.22 μm millipore filter. This treatment also removed aggregated LDL and microemulsions which were of density greater than 1.006 g/mL. The protein and drug concentrations of the CBE-LDL complex were measured by Lowry protein assay (2.1.9.) and HPLC (2.3.6.) respectively. Identical incubations were performed in the absence of LDL and centrifuged as described above for the LDL-containing preparation. The infranatant was then filtered through a 0.22 μm millipore filter and assayed for CBE to ensure that no microemulsions remained after purification.

## 2.3.5. INCORPORATION OF ANTHRACYCLINES INTO LDL

A) Daunomycin was incorporated following the method of Iwanik et al[23] (figure 31). Drug (20-50 mg) was dissolved in methanol and dried onto the surface of a glass tube under $N_2$, 5 mL of LDL (protein 2-10 mg/mL) in buffer (5mM sodium phophate, 0.15M NaCl, 0.3 mM EDTA, pH7.3) was added and the mixture flushed with nitrogen and stirred gently at room temperature for 2-3 hours in darkness. The daunomycin-LDL complex was separated from the unbound drug by gel filtration through PD10 columns, using PBS as eluant. LDL-containing fractions were pooled, filtered through a 0.22 μm millipore filter and assayed for drug (2.3.6.) and protein (2.3.7.).

In procedures 2.3.5. B-G, LDL prepared by zonal (2.1.1.) was used without concentrating or de-salting in an attempt to reduce aggregation of the LDL during incubation with adriamycin.

PREPARATION OF DAUNOMYCIN - LDL




PBS: 5mM sodium phosphate, 0.15 M NaCl, 0.3mM EDTA, pH 7.3

Figure 31: Protocol for the preparation of daunomycin-LDL as described in Section 2.3.

B) Cholate Method: As described for CBE (2.3.2.), adriamycin, phospholipid, cholesterol, cholate micelles were prepared using 5 mg adriamycin, 10.9 mg lecithin and 1.6 mg cholesterol per 5 mL of cholic acid buffer. To each mL of micelles was added 1 mL of LDL (0.5 mg protein/mL) and the solution stirred overnight at room temperature.

C) DMSO Method: 2 mg adriamycin was dissolved in 2 mL of DMSO buffer[202] (0.15 M NaCl, 0.3 mM EDTA, pH 7.2 containing10% DMSO,v/v) and warmed to 37°C for 10 minutes before adding 2 mL LDL solution (protein 0.1-0.5 mg/mL). The mixture was incubated overnight at 37°C, in darkness.

D) PBS Method: Adriamycin (2 mg) was dissolved in 2 mL buffer (5 mM sodium phosphate, 0.15 M NaCl, 0.3 mM EDTA pH 7.3) and 2 mL LDL solution added (protein 0.1-0.5 mg/mL). The solution was stirred in darkness at room temperature for 2-3 hours.

E) Dialysis : LDL (0.1 mg/mL) in dialysis tubing, molecular weight cut-off 2,000-8,000 was surrounded by various concentrations of adriamycin (2-150 μg/mL) in DMSO buffer. The concentrations of free and bound adriamycin in the dialysis bags were measured at intervals by HPLC (2.3.6.). Free adriamycin was removed by chromatography on PD10 columns, with PBS as eluant.

F) Incubation in Glycine Buffer: Adriamycin-LDL was prepared by overnight incubation of 5 mg of drug with 2 mL LDL (0.5 mg protein/mL), and 5 mL NaCl/DMSO buffer, pH 7.2 or 5 mL 1M Glycine/DMSO buffer, pH 10. The complexes were concentrated by centrifugation at 38 K for 4 hours and at 10°C in Beckman SW41 rotor, after making the density of the solutions up to 1.4 g/mL by the addition of solid KBr. The drug-LDL complexes were desalted on PD10 columns using PBS as eluant.

G) Surfactants: Following the method of Tucker et al[203] surfactants were used to enlarge the LDL particles. Ten per cent solutions (w/v) of Brij-35, Triton x-100 and SDS were prepared in PBS and filtered through 0.22 µm filters prior to use. The surfactants were each added gradually to a different aliquot of LDL (protein 2 mg/mL in PBS), giving final surfactant to phosholipid molar ratios of 1:1. The mixtures were left to react for 30 minutes before adding two volumes of adriamycin solution (1mg/mL in PBS). After 24 hours incubation at room temperature, surfactant and free drug were removed by PD10 filtration.

In all of the above procedures, anthracyclines were assayed by HPLC (2.3.6.) and protein determined as described (2.3.7.)

2.3.6. HPLC ASSAYS

Chlorambucil[202], chlorambucil cholesteryl ester, adriamycin[203] and daunomycin were measured by reversed-phase high pressure liquid chromatography (HPLC). A 20 x 0.5 cm stainless steel column, slurry packed in the laboratory with 5 µm Hypersil-ODS, was used in all assays. Typical chromatograms and standard curves for each system are shown in figures 32 and 33.

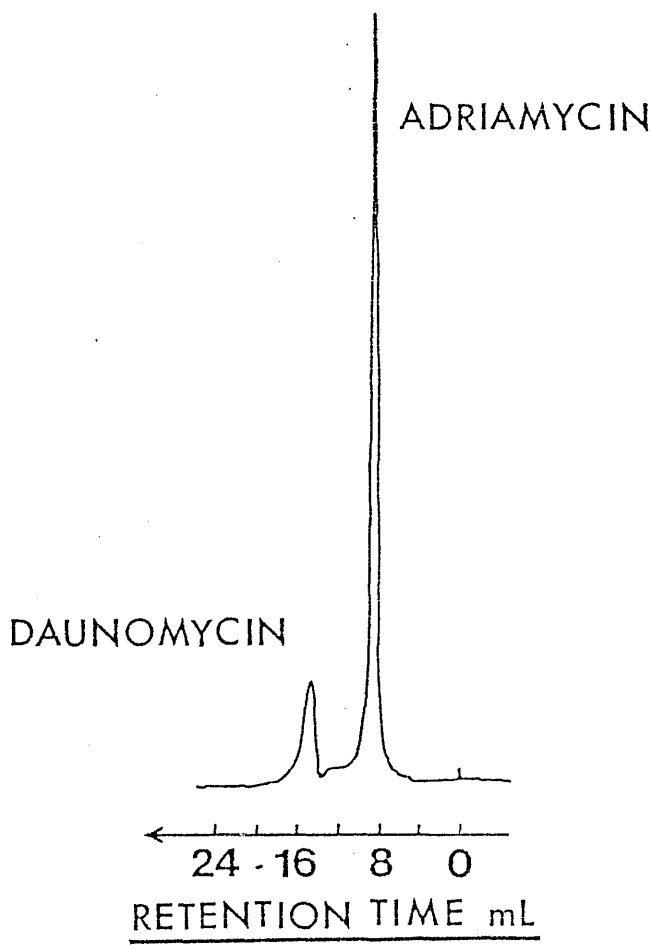



Figure 32: HPLC profiles of adriamycin and daunomycin

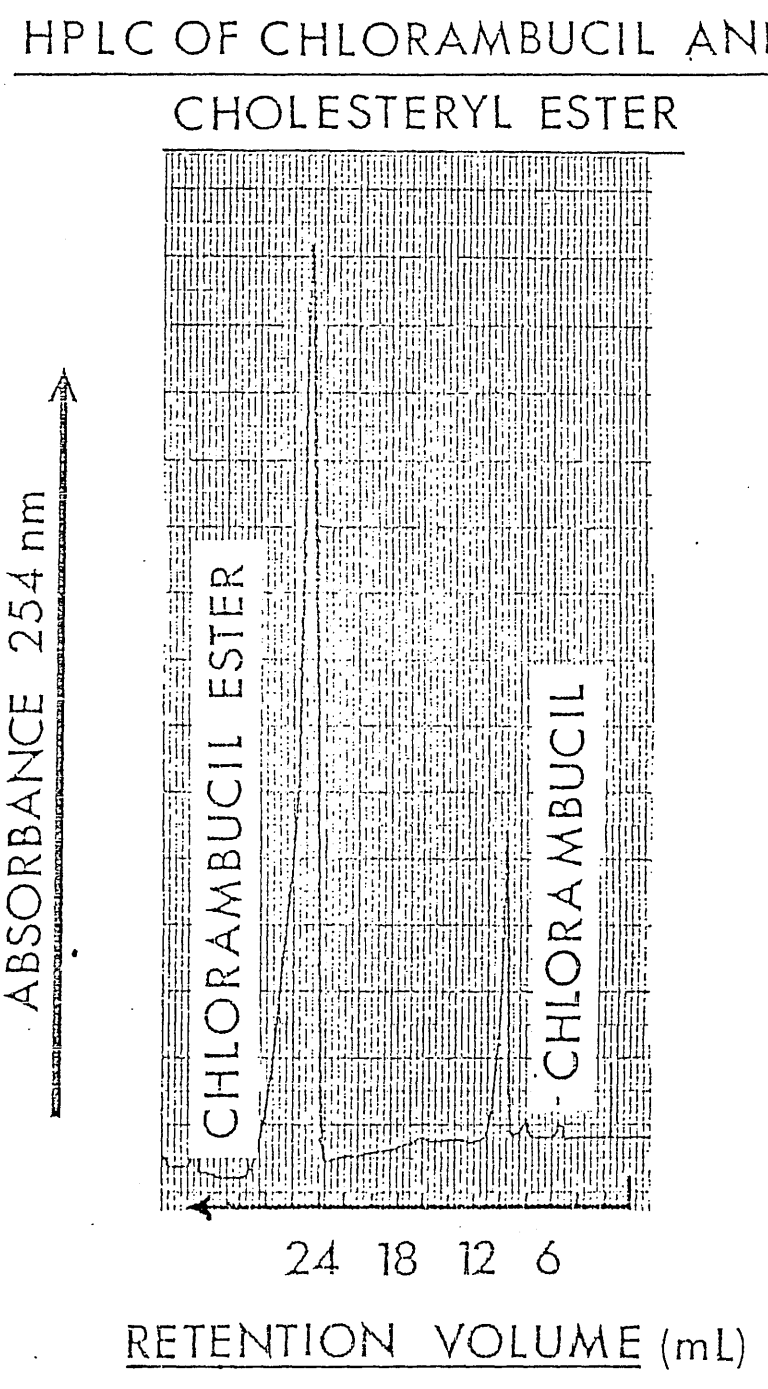



Figure 33 : HPLC profiles of chlorambucil and chlorambucil-∝-cholesteryl ester obtained using the system outlined in Section 2.3.6.(C).

A) ADRIAMYCIN ASSAY[203]

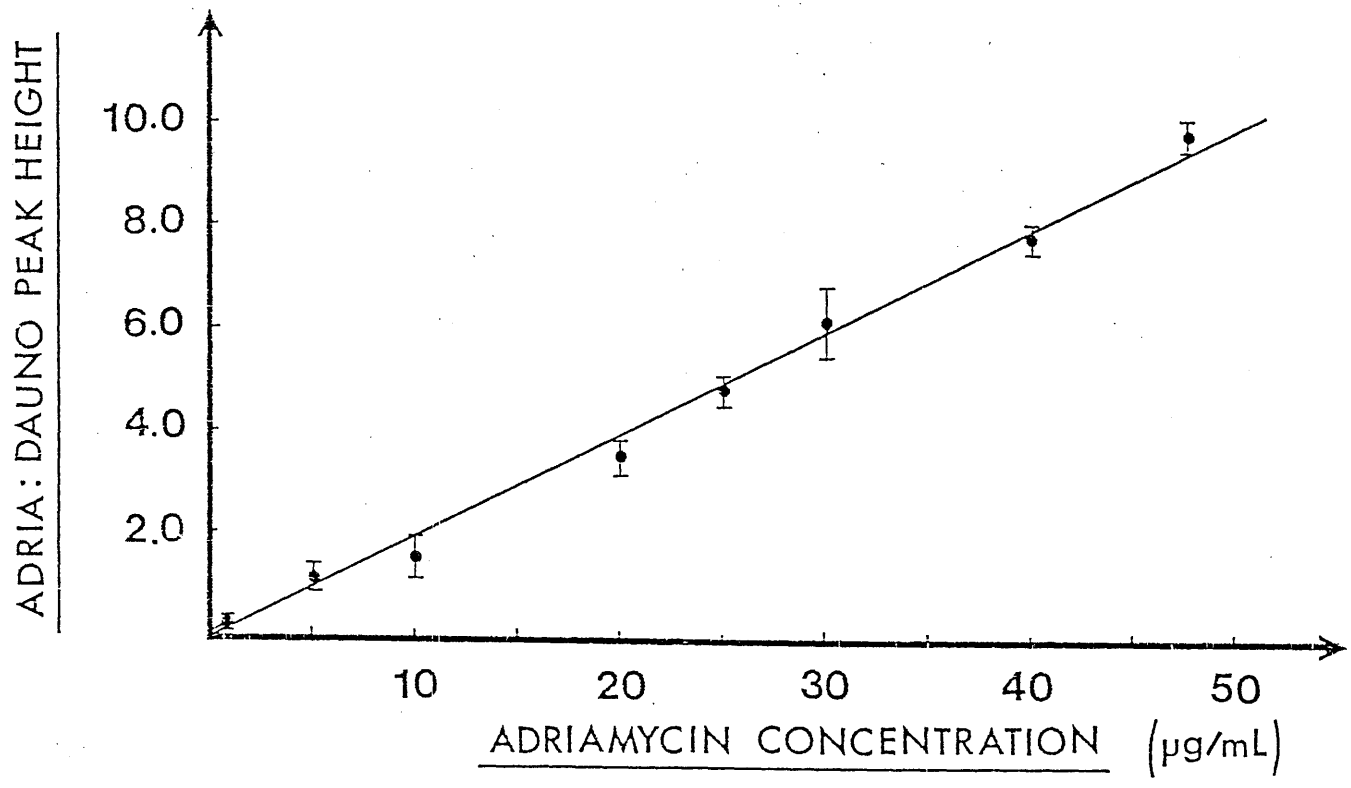
APPARATUS:

| | | |
|---|---|---|
| INJECTION VALVE | : | Ryeodyne 7125 with 20 µL loop. |
| PUMP | : | Varian 8500 |
| DETECTOR | : | Pye-Unicam LC-FL Fluorescence monitor, fitted with interference filters for excitation at 450 nm and emission at 550nm. |
| RECORDER | : | Servoscribe single pen chart recorder. |
| MOBILE PHASE | : | Acetonitrile: 10 mM Phosphoric acid (65:35) containing 6 mM Brij-35. |
| FLOW RATE | : | 2 mL/minute. |

EXTRACTION PROCEDURE: To 1 mL of solution was added 200 ng of daunomycin (as internal standard), in methylene chloride and the solution mixed with 1 mL 10 mM phosphate-buffered saline, pH7.8, followed by extraction with 2 x 5 mL methylene chloride. After centrifugation for 5 minutes at 2000 rpm, the organic layer was removed and evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in 100 µL of mobile phase and 20 µL injected onto the column.

STANDARDS: A standard solution of 10 µg adriamycin /mL was used to spike LDL solution, giving final drug concentrations of 10 ng-1µg/mL. Extraction of standards was carried out as described for samples and a standard curve plotted of adriamycin: daunomycin peak height vs adriamycin concentration (figure 34).

Figure 34: ADRIAMYCIN STANDARD CURVE*




* Each point is the mean of four assays.

B) DAUNOMYCIN ASSAY

Daunomycin was measured either on the system used for adriamycin or on the following system:

APPARATUS:

| | | |
|---|---|---|
| INJECTION VALVE | : | Rheodyne 7125 fitted with 100 µL loop |
| PUMP | : | Gilson 300 |
| DETECTOR | : | Gilson Holochrome U.V. Monitor, absorption measured at 330 nm. |
| RECORDER/INTEGRATOR | : | Schimadzu |
| MOBILE PHASE | : | Acetonitrile : 10 mM phosphoric acid (68:32) containing 6 mM Brij-35. |
| FLOW RATE | : | 2 mL/minute |

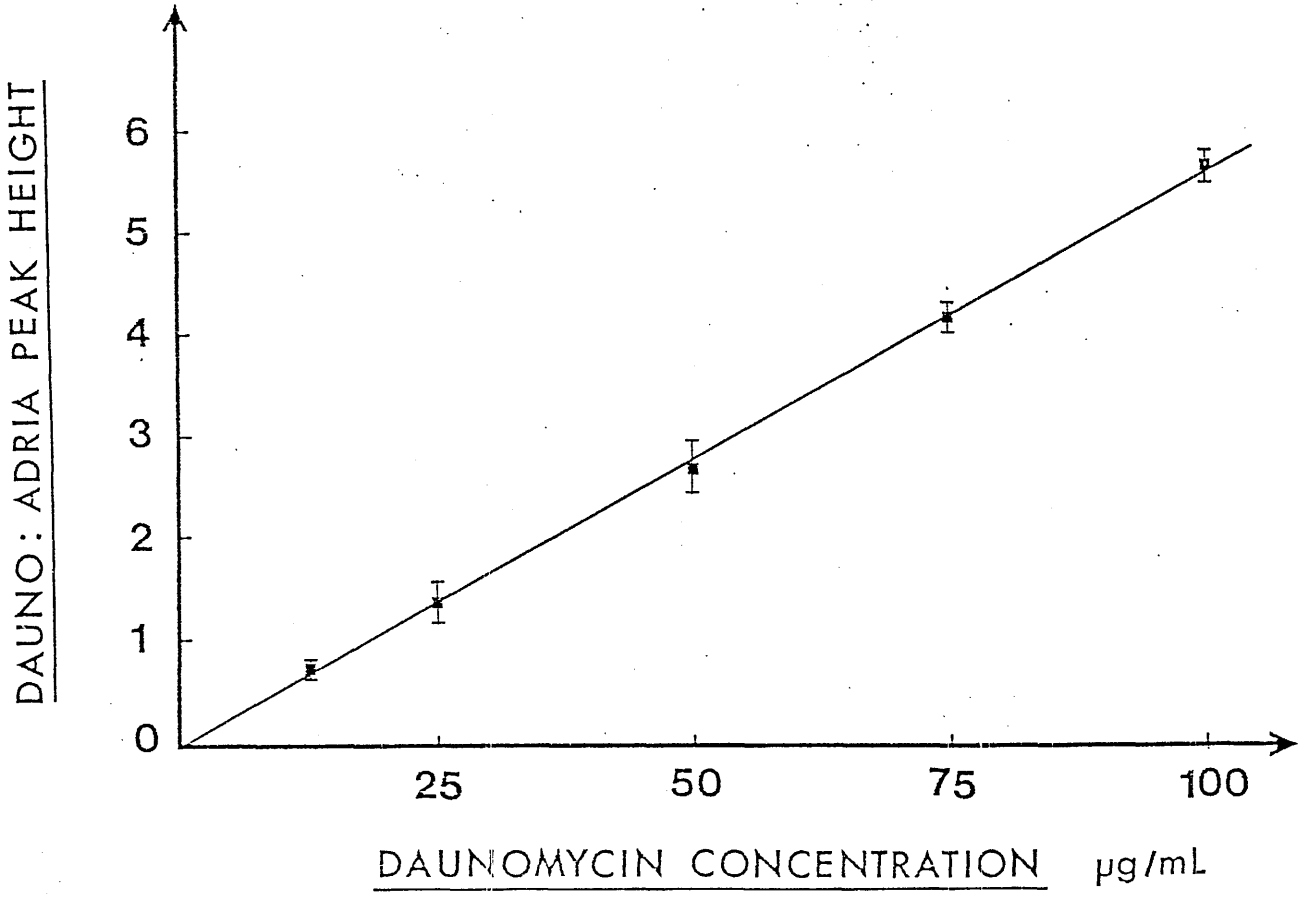
EXTRACTION PROCEDURE AND STANDARDS: Daunomycin was extracted from samples and standards as described for adriamycin (2.3.6 A) omitting the addition of 200ng of daunomycin. A standard curve was constructed by plotting daunomycin peak area against concentration (fig.35).

C) CHLORAMBUCIL AND CHLORAMBUCIL CHOLESTERYL ESTER ASSAY[202]

APPARATUS: as described for the daunomycin assay, with the following amendments:

| | | |
|---|---|---|
| DETECTION | : | 254 nm |
| MOBILE PHASE | : | Methanol:water (65:35) containing 10mM phosphoric acid |
| FLOW RATE | : | 1mL/minute |

Figure 35: DAUNOMYCIN STANDARD CURVE*



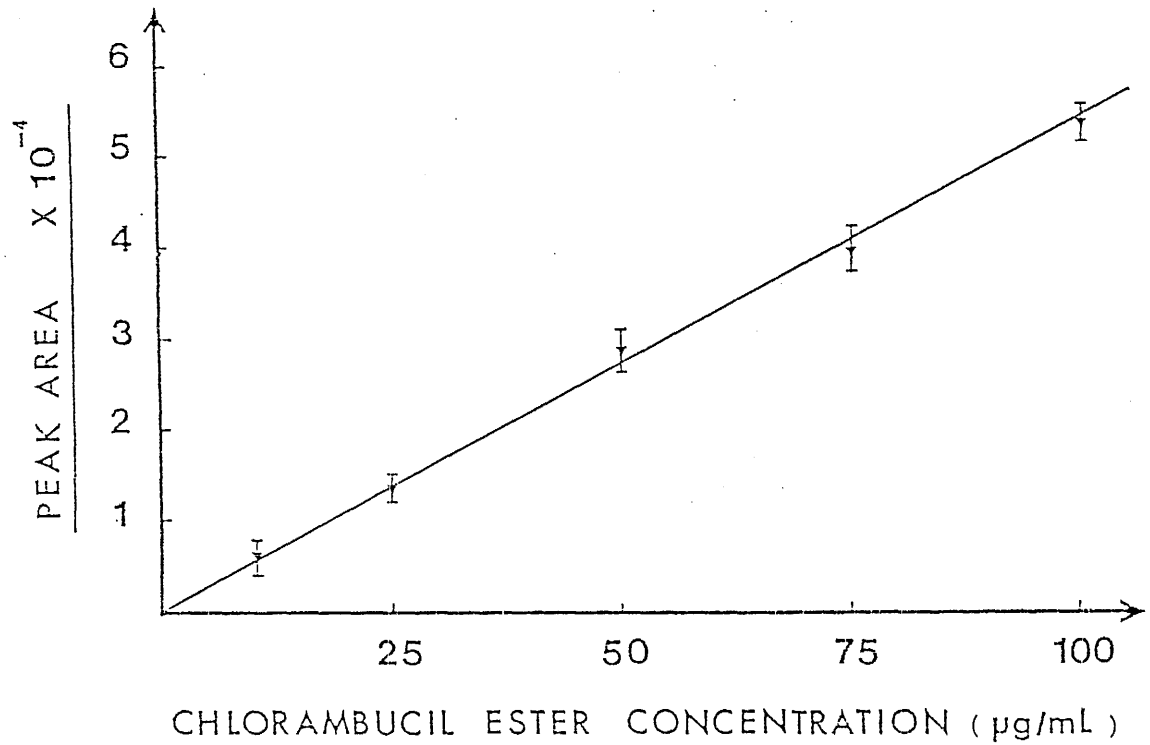

* Each point is the mean of four assays.

EXTRACTION PROCEDURE : Each mL of plasma or LDL solution was extracted with 2 volumes of chloroform and centrifuged, twice. The organic phase was aspirated and dried down under a stream of nitrogen at 40°C. The extract was dissolved in methanol and injected onto the column.
STANDARDS: Chlorambucil cholesteryl ester, purified by TLC, or chlorambucil was dissolved in chloroform and spiked into LDL solutions and extracted by the same procedure as the samples. Peak areas were plotted against drug concentration (figure 36).

## 2.3.7. LDL PROTEIN DETERMINATION IN SOLUTIONS CONTAINING ANTHRACYCLINES

The apoprotein content of the various lipoproteins prepared was assayed by the method of Lowry et al[188], as described in section 2.1.9. In the case of LDL containing anthracyclines, protein concentration was observed to be much higher than expected (figure 37). The Lowry assay conditions are basic and this changes the colour of the anthracyclines from orange to purple. A scan of adriamycin absorption in the ultraviolet and visible regions (figure 38) showed however that the high pH was not causing the "extra" absorbance at 750 nm. It is likely that the Lowry reagents form a complex with these drugs in much the same way as they do with peptides, so giving rise to an enhancement of the blue colour of the solution and consequently elevated estimates of protein concentration. Apoprotein B was therefore precipitated from solution using 1 volume of 1,1,3,3-tetramethyl urea, delipidated by extraction with 2 volumes of chloroform : methanol (2:1) and dried with ether before solvation in 0.5M NaOH and Lowry protein assay.

Figure 36: CHLORAMBUCIL CHOLESTERYL ESTER STANDARD CURVE *




* Each point is the mean of four assays

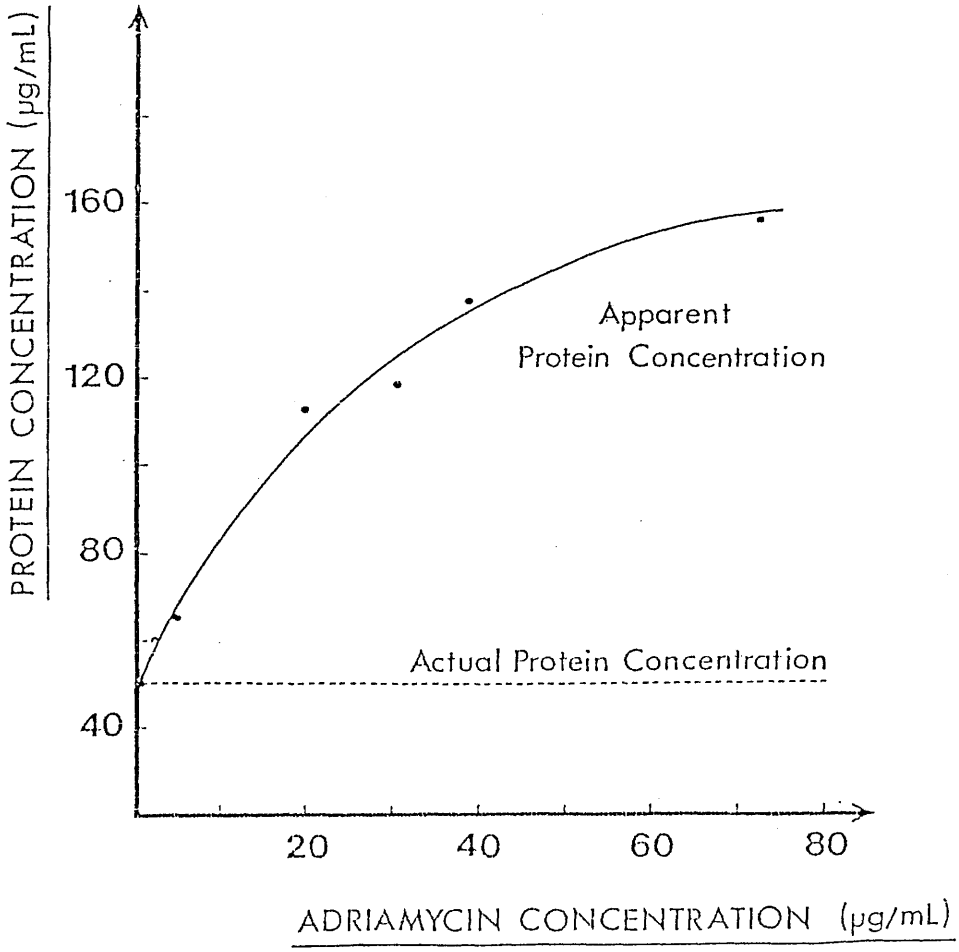
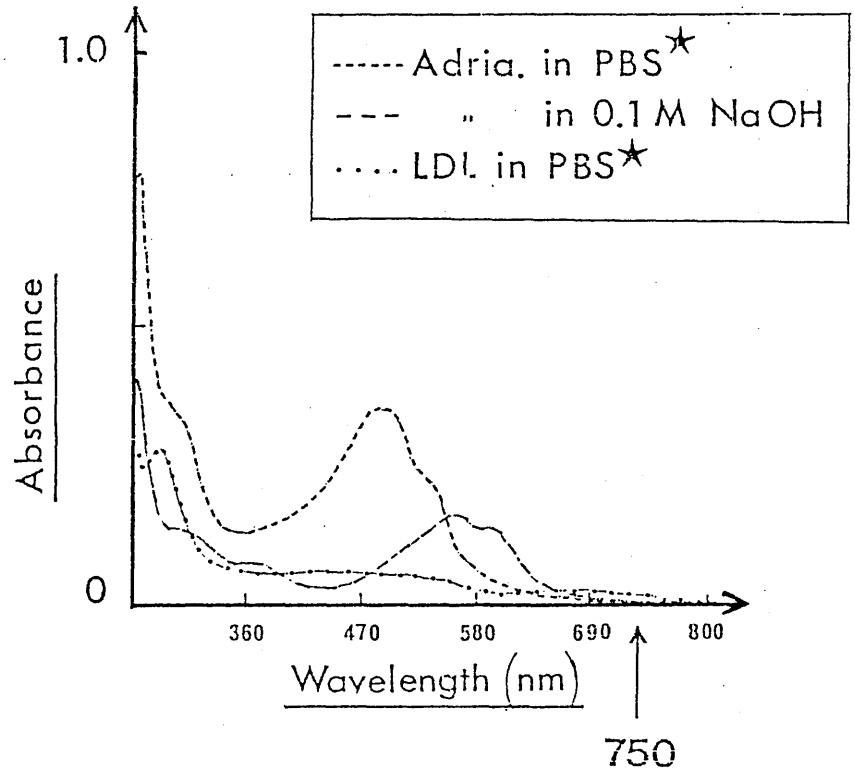



Figure 37: Apparent protein concentration in LDL solutions of constant LDL content and increasing adriamycin concentration.

# U.V./VISIBLE ABSORPTION OF ADRIAMYCIN IN NEUTRAL AND BASIC CONDITIONS




★ PBS: 5 mM sodium phosphate, 0.15 M NaCl, 0.3 mM EDTA, pH 7.3

Figure 38 : Absorption spectra of adriamycin in neutral and basic conditions. Note that there is no enhancement of the drug's absorption at 750nm on changing pH.

## 2.3.8 PLASMA CLEARANCE OF RADIOACTIVE LDL

$^{131}I$- or $^{125}I$- labelled LDL or drug were administered to New Zealand White rabbits by injection into a canulated marginal ear vein. Blood samples were taken after 5 minutes and at intervals over the following 24 hours. Isotope dilution in the 5-minute sample gave an indication of plasma volume. Plasma clearance of the radioactive species was directly related to the disappearance of radioactivity with time. Re-isolation of the labelled LDL was unnecessary since non-protein bound iodine has been reported to correspond to less than 1% of the total radioactivity and apoprotein B does not transfer to the other plasma lipoproteins[206]. Iodide was administered, 0.1g/L in drinking water, for two days prior to and throughout these studies. Radioactivity remaining in the plasma at each time point was expressed as a percentage of the level of radioactivity in the 5-minute sample.

## 2.3.9. CELL CULTURE MEDIA

HeLa cells were maintained in Ham's F10 medium containing 20mM HEPES and supplemented with 10% foetal bovine serum, 2mM glutamine, 5% $CO_2$ and 50 units/mL of penicillin and streptomycin. For culture of human glioma(G-CCM) and human lung carcinoma(L-DAN), medium containing 50% Ham's F10 and 50% Dulbecco's Modified Eagle's media supplemented with serum etc., as above, was used. Tumour samples were collected in balanced salt solution with antibiotics as above prior to dissection.

## 2.3.10 DETACHMENT OF CELLS FROM CULTURE FLASKS

Cells were detached from culture flasks using 0.25 % trypsin in balanced salt solution(BSS). After removing medium, cells were washed

with BSS and soaked in trypsin for one minute. The trypsin solution was aspirated and the flasks sealed and incubated at 37°C until the cells had detached from the surface of the flask. This usually required 10-15 minutes, after which the cells were re-suspended in medium prior to counting cell density using a Coulter Counter or haemocytometer.

### 2.3.11 PREPARATION OF LIPOPROTEIN-DEFICIENT SERUM

Fresh human blood was obtained from healthy volunteers and allowed to clot in 10mL glass tubes, and the serum was aspirated following centrifugation at 2500 rpm for 15 minutes. The density of the serum was adjusted to 1.25 Kg/L by the addition of 0.371g KBr/mL. This solution was overlayered with d1.25 density solution and centrifuged at 45000 rpm for 24 hours in a Beckman Ti60 rotor at 10°C. Lipoproteins and density solution were aspirated and the lipoprotein-deficient serum dialysed, first briefly against distilled water, and then against PBS, pH 7.4.

### 2.3.12 MICROTITRE DRUG SENSITIVITY ASSAY

Freshney and co-workers have established microtitre assays for testing cytotoxic drugs against human glioma[207] and HeLa[208] cell lines. This procedure has a number of advantages over the commomly used cloning experiments[209] for assessing cytotoxicity : large numbers of replicates can easily be handled, giving better reproducibility; and the long drug-exposure times enable all of the cells in culture to pass through the cell cycle at least once, thus excluding the cycle-dependence of some drugs as a variant in the assay. Protein synthesis was identified as a fundamental metabolic process without which the cells could not

survive. Since leucine is an essential amino acid it is unlikely that protein synthesis could carry on for long within the cell without the incorporation of leucine obtained from the surrounding medium. Thus, the failure of cells to incorporate [$^3$H]-leucine into protein several days after drug exposure will almost certainly correlate with cell death.

Protocols for HeLa and Glioma microtitre assays are shown in figure 39. Cells were seeded at a density of 5 X $10^3$ cells/mL, i.e. $10^3$ cells/well, in the central 60 wells of 96-well microtitre plates. The edge wells were filled with cell-free medium. Throughout the experiment the cells were incubated at 37°C in a humidified 5% $CO_2$ atmosphere. After being allowed to adhere to the surface for 3 days the cells were re-fed with fresh medium. Medium and drugs were administered on day 5 in the case of HeLa cells, and days 4, 5 and 6 for glioma cells. Drugs were added to the plate wells in 10-50 µL of PBS in serial dilutions of 1:10 or 1:5, with duplicate incubations at each concentration. Twelve wells of cells in each plate were left free of drugs to serve as controls. Following drug exposure the medium was replaced and the cells allowed to recover for 4 days(HeLa) or 5 days (glioma) before [4,5-$^3$H]-leucine (1 µCi/mL) was added in fresh medium. During this recovery period medium was replaced at least every 48 hours. The cells were exposed to [$^3$H]-leucine for 24 hours prior to washing with PBS, extraction with 10% (w/v) trichloroacetic acid (2 X 15 minutes) rinsing with distilled water, fixing with methanol and drying. The contents of each well were dissolved in 200µL of 0.3M NaOH containing SDS (1% w/v). An aliquot from each well was placed in a 1mL Sarstedt tube, neutralised and liquid scintillant added. Each tube was well mixed and counted for [$^3$H]-activity in a Packard β-Counter. The counts obtained for each well were corrected for background and expressed

GLIOMA MICROTITRE ASSAY[207]

| | |
|---|---|
| DAY 0 | Seed microtitre plates, $10^3$ cells per well |
| 1 | |
| 2 | |
| 3 | Replace medium |
| 4 | } Replace medium and add drugs |
| 5 | } Replace medium and add drugs |
| 6 | } Replace medium and add drugs |
| 7 | Replace medium |
| 8 | } Recovery Period |
| 9 | Replace medium } Recovery Period |
| 10 | } Recovery Period |
| 11 | } Recovery Period |
| 12 | Replace medium with medium containing [4,5-$^3$H]-leucine, 1 μCi/mL |
| 13 | Wash, TCA extract, fix and dry, dissolve cell residue in NaOH. |
| 14 | Measure $^3$H-incorporation by scintillation counting |

HeLa MICROTITRE ASSAY[208]

| | |
|---|---|
| DAY 0 | Seed microtitre plates, $10^3$ cells per well |
| 1 | |
| 2 | |
| 3 | Replace medium |
| 4 | |
| 5 | Replace medium and add drugs |
| 6 | Replace medium } Recovery Period |
| 7 | } Recovery Period |
| 8 | Replace medium } Recovery Period |
| 9 | } Recovery Period |
| 10 | Replace medium with medium containing [4,5-$^3$H]-leucine, 1 μCi/mL |
| 11 | Wash, TCA extract, fix and dry, dissolve cell residue in NaOH |
| 12 | Measure $^3$H-incorporation by scintillation counting |

Figure 39: Protocols for HeLa and Glioma Microtitre Drug Sensitivity Assays TCA : trichloroacetic acid

as disintegrations per minute (DPM). The mean DPM for controls was taken to be 100% survival and the $[^3H]$-leucine uptakes for other wells expressed as a percentage of this value. A plot of log(% cell survival) against log(drug concentration(M)) was constructed for each of the drugs and drug-LDL complexes studied. From these curves, the doses which corresponded to 50% inhibition ($ID_{50}$) and 90% inhibition($ID_{90}$) of cell growth, compared to controls, were calculated for each drug.

An extra plate of cells was seeded on day 0 for use in monitoring the cell growth rate. At intervals during the experiment several wells were trypsinised as described in Section 2.3.10 and the number of cells per mL measured using a haemocytometer. This was to verify that the majority of cells was in the logarithmic growth phase throughout the assay. This is essential if the maximum relative changes in $[^3H]$-leucine uptake between control and drug-treated cells are to be assessed. Biased results would be obtained if $[^3H]$-leucine incorporation was measured while controls had reached stationary phase and drug-treated cells were in log. phase. Growth curves for G-CCM and HeLa cells plotted from data obtained in this way are shown in figure 40.

### 2.3.13 L-DAN CLONOGENIC STEM CELL ASSAY AND SPHERIOD MODEL SYSTEM

This work was carried out with the collaboration of Dr. D.J. Kerr of the Department of Medical Oncology, Glasgow University. L-Dan cells were maintained in exponential growth in monolayer culture prior to disaggregation using trypsin (Section 2.3.10). Thereafter cells were seeded for clonogenic assay[209] or used for initiation of tumour spheroids[210] (fig. 42).

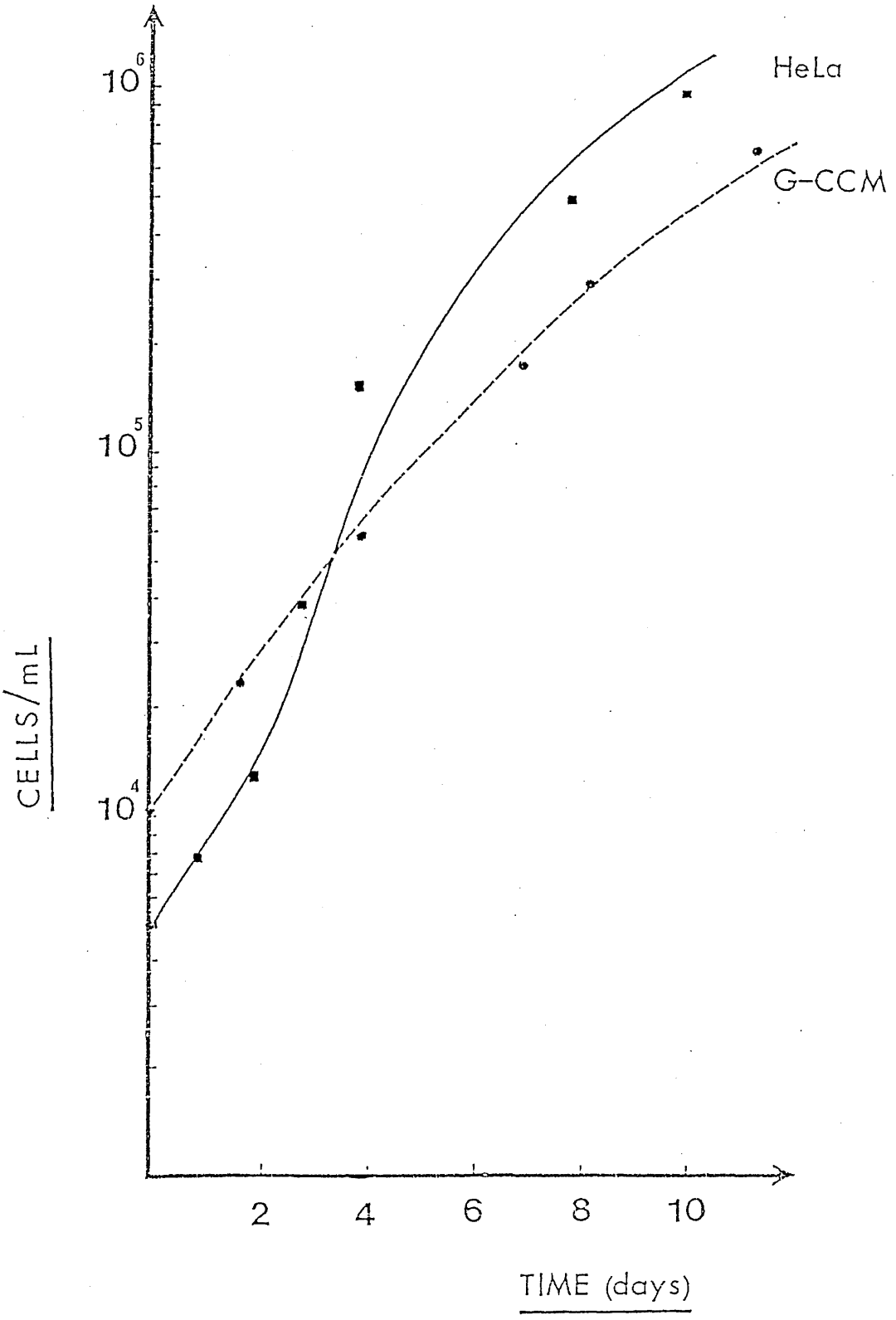



Figure 40: Growth curves for HeLa and glioma (G-CCM) cells in the microtitre assays (Section 2.3.12). Cell number was measured using a haemocytometer and each point is the mean of duplicate counts for a pool of the cells from twelve wells of the culture plate.

## CLONOGENIC ASSAY

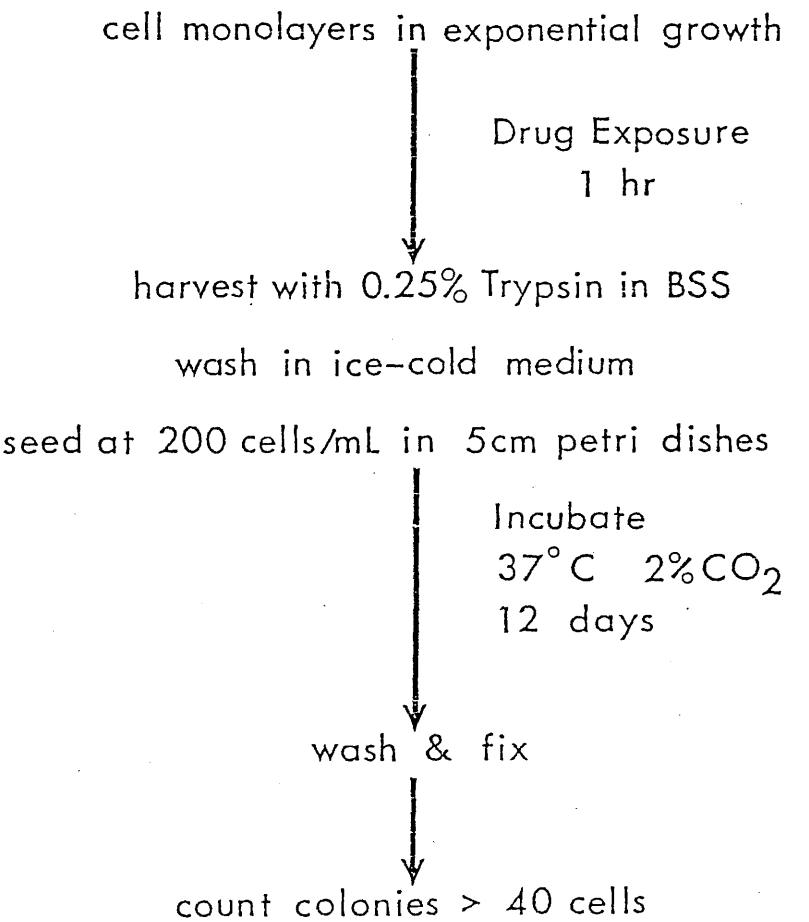



Figure 41: Protocol for the clonogenic stem cell assay used in the testing of free and LDL-encapsulated daunomycin against L-DAN lung tumour cells, as described in Section 2.3.13.

SPHEROID GROWTH DELAY & CLONOGENIC ASSAYS

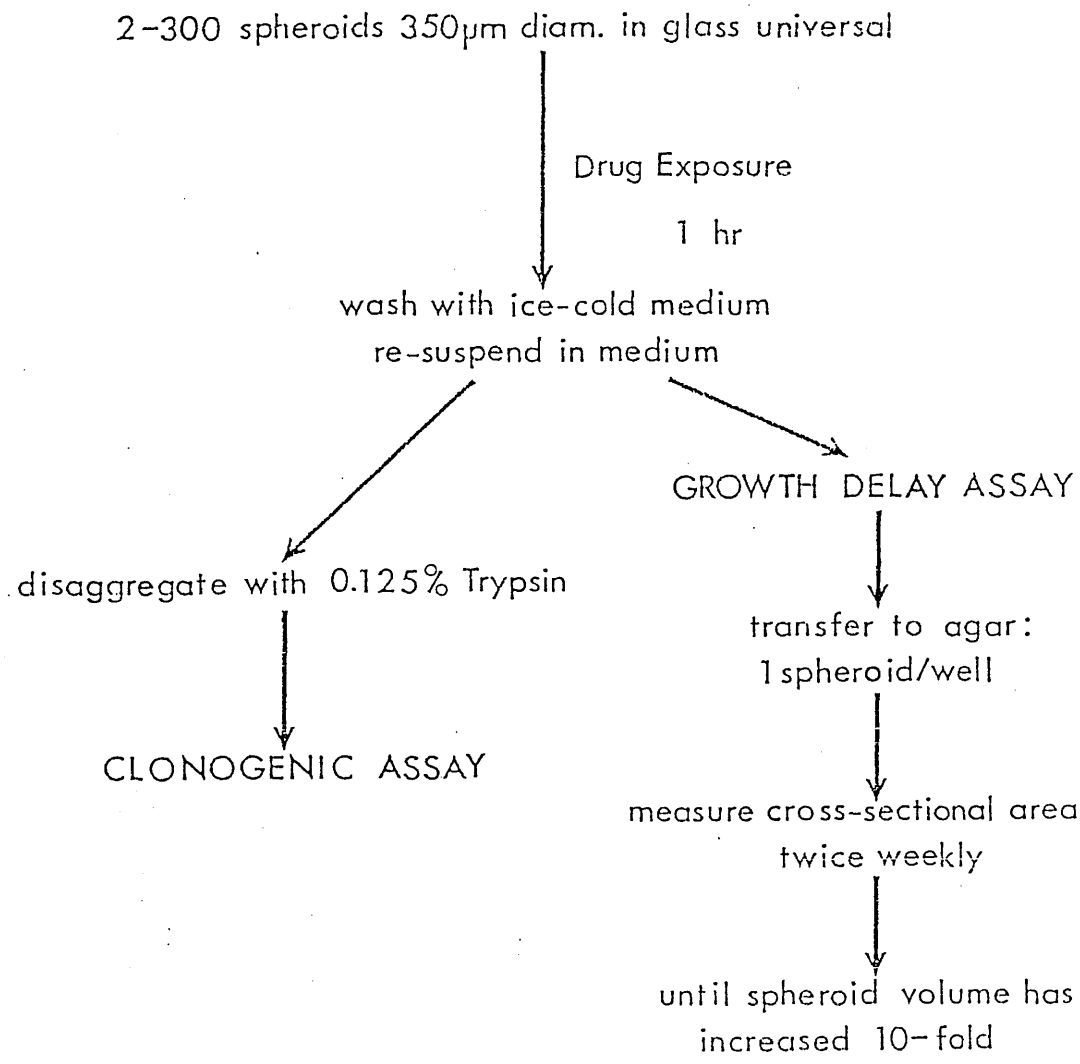



Figure 42 : Outline of the experiments performed with native and LDL-encapsulated daunomycin on lung tumour (L-DAN) cell spheroids as described in Section 2.3.13.

Monolayers were exposed to varying concentrations of daunomycin and daunomycin-LDL for one hour. Following treatment, the monolayers were harvested with trypsin, as described in Section 2.3.10, centrifuged, and washed with ice-cold medium, after which the cells were diluted in medium and seeded at 200 cells/mL in 5cm Petri dishes. The dishes were incubated for 12 days at 37°C in a 2% $CO_2$ atmosphere before fixing and staining of colonies with methylene blue. Colonies of more than 40 cells were counted. The cloning efficiency of the untreated cells was defined as 100% survival and the cloning efficiency of treated cells expressed as a percentage of control survival.

Spheroids were prepared[210], with a mean diameter of 350 µm, at 200-300 spheroids/glass universal tube and subjected to similar drug concentrations and exposure times as in monolayer, at 37°C with intermittent agitation. The spheroids were then allowed to sediment and washed with ice-cold medium, re-suspended in medium and subdivided for further analysis.

One aliquot of spheroids was incubated with 0.125% trypsin in PBS for 14 minutes at 37°C, after which the trypsin solution was replaced with medium. Disaggregation of the spheroids was acheived by repeated pipetting of the suspension. The clonogenic assay was performed as described above. A second aliquot of spheroids was used for a growth delay experiment. Intact spheriods were transferred to agar-coated wells on a plastic tissue culture multidish, 1 spheroid per well, using a pasteur pipette. The cross-sectional areas of 24 spheroids from each treatment group were measured twice weekly using a "micromeasurements" image analysis system coupled via a television camera to an inverted optical microscope[211]. These measurements were converted to volumes, assuming spherical geometry. Treatment-induced

growth delay was defined as the time taken for median spheroid volumes to increase by a factor of 10 above initial size.

### 2.3.14 THE SUBRENAL CAPSULAR (SRC) ASSAY

A six-day assay has been described by Bogden et al[212] for assessing the sensitivity of primary human tumour explants to chemotherapeutic agents in normal immunocompetent mice. Following this protocol (fig. 43), with the cooperation of Dr. D. Cunningham, Medical Oncology, G.R.I., the anti-tumour effects of some drug-LDL complexes have been tested.

Tumour samples were removed from patients or animals and immediately placed in sterile balanced salt solution containing penicillin and streptomycin, 50 units/mL. The tumour was cut into fragments measuring approximately $1mm^3$. The mice (NIC B6D2FI) were weighed and anaesthetised and a lateral incision was made over the left kidney, which was then exteriorised. Tumour was inserted under the renal membrane using a trocar, after a small incision had been made in the membrane with a Ziegler iridectomy knife. This procedure was carried out under magnification with a Zeiss stereoscopic microscope. Two perpendicular diameters of the tumour fragment were measured in situ using a micrometer scale, fitted to the microscope and calibrated so that 10 micrometer units were equivalent to 1mm. Kidney was gently replaced and the incision closed using 9mm autoclips.

Six animals were implanted with tumour in each treatment group. One group of six mice was left untreated to act as controls, and the rest received drugs by tail vein injection on the following day, i.e. day 1, and on days 3 and 5. On day 6 the animals were sacrificed and the tumour dimensions remeasured, before the kidneys were placed in

## SRC ASSAY

DAY 0 — Implant 1 $mm^3$ of tumour under renal capsule
Measure 2 perpendicular diameters of tumour in situ

DAY 1, DAY 3, DAY 5 — Inject drugs or LDL via a tail vein

DAY 6 — Sacrifice mice , re-measure tumour diameters
Calculate tumour volume : $0.5(l \times w^2)$
Express as % of initial volume

Figure 43: Protocol for the mouse Subrenal Capsular (SRC) assay.

formol saline, pending histological analysis. The change in tumour volume, ½(length X width$^2$), was expressed as a percentage of the original volume.

### 2.3.15 MEASUREMENT OF PLASMA WHITE BLOOD CELL DENSITY

Blood was removed from the mice used in the SRC assay, on day 6, via the vena cava, and placed in glass tubes containing $K_2$EDTA. White blood cell density was measured at the Department of Haematology, G.R.I. on an automated Coulter Counter system.

### 2.3.16 HISTOLOGICAL ANALYSIS OF TUMOUR SECTIONS

Three micron thick sections from mouse kidney and implanted tumour were stained with haematoxylin and eosin and examined by Dr. A. Jack of the Pathology Department, G.R.I.

### 2.3.17 LDL RECEPTOR ACTIVITY ASSAY IN VITRO

High-affinity LDL degradation was measured as the difference in cellular degradation of $^{125}$I-LDL in the absence and presence of an excess of unlabelled LDL (fig. 44).[213] Twenty-four-well culture plates were seeded on day 0 with cells in medium containing foetal bovine serum, Section 2.3.9, and incubated in a 5% $CO_2$ atmosphere at 37°C. Two wells of each plate were filled with medium only. On day 3, the medium was replaced with medium containing 10% lipoprotein-deficient serum, prepared as described in Section 2.3.11, instead of foetal bovine serum. Fresh lipoprotein-deficient medium was added to the cultures on day 6 and $^{125}$I-LDL was added at concentrations of 0-100 μg LDL protein/mL with duplicates of each concentration. . Identical incubations were set up

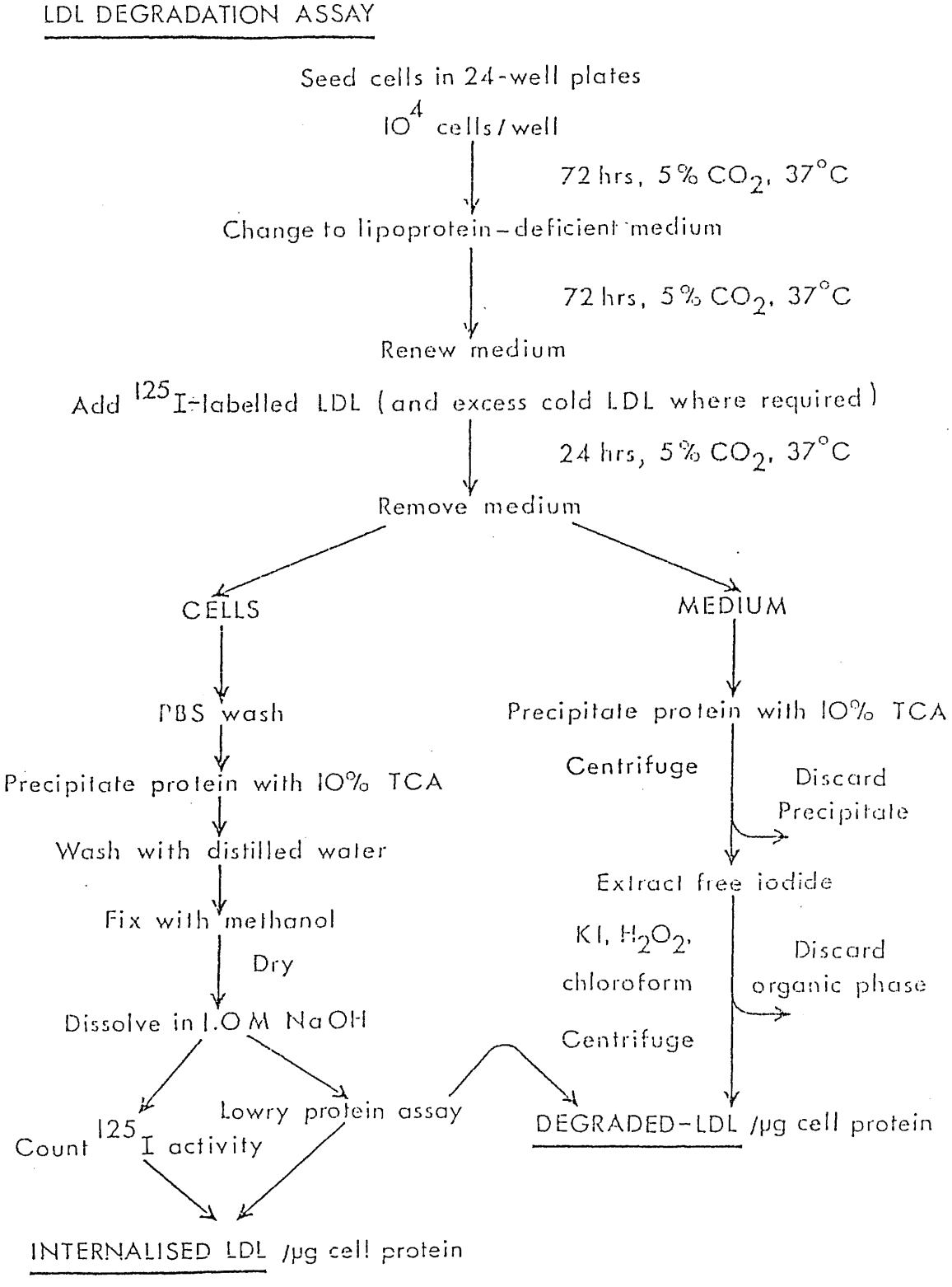



Figure 44: Scheme for assessing LDL degradation and internalisation in monolayer cell cultures.

and excess LDL added to a concentration of 0.5 mg LDL protein/mL in the culture medium. After a further 24 hours incubation, the medium was removed from the cells and its degraded LDL content measured. Cell-free incubations contained $^{125}$I-LDL at 100 µg LDL protein/mL and were subsequently analysed for spontaneous degradation of the tracer. This amounted to less than 3% of the total tracer used and was subtracted from the values obtained in other incubations. High molecular weight, presumably intact, LDL was precipitated by adding 1,1,1-trichloroacetic acid to the medium to a final concentration of 10% (w/v). The supernate was separated from the pellet by centrifugation and free iodide extracted from it by the addition of excess potassium iodide (10 µL, 40% solution) and 10 µL of 30% w/v hydrogen peroxide, followed immediately by 2mL chloroform. After thorough mixing and centrifugation, the aqueous phase, containing degraded LDL, was separated and assayed for $^{125}$I-activity in a Packard Autogamma Twin-Channel Scintillation Counter.

The cells remained attached to the surface of the culture wells and, after washing with PBS, extraction with 10% trichloroacetic acid (2 x 15 minutes), rinsing with distilled water, and fixing with methanol, the cell residue was dissolved in 1.0 M NaOH. Aliquots of the dissolved cellular material were assayed for protein by the Lowry method, Section 2.1.9, and for $^{125}$I-activity using a Packard Scintillation Counter as above. This procedure isolated intact intracellular lipoprotein and the amount of LDL internalised was expressed per microgram of cell protein for each incubation.

## 2.4 RESULTS II

### 2.4.1. CHLORAMBUCIL CHOLESTERYL ESTER-LDL

Chlorambucil cholesteryl ester (CBE) was loaded into LDL by the modified Stoffel method[190] described in Section 2.3.5. Approximately 50% of the drug-ester (as measured by specific [$^3$H] activity) was transferred at each stage (table 16), producing LDL which had approximately 10% of its cholesteryl ester core replaced with CBE.

In an effort to increase the net amount of drug incorporated into HDL and subsequently into LDL, reconstituted HDL containing increased amounts of CBE, was generated as described in Section 2.3.2 (A). The percentages of total drug-ester found in each fraction after centrifugation at 1.063 g/mL are shown in fig.45. The HDL formed by the reconstitution procedure was of a wide density range and would prove difficult to separate from LDL after the next step in the procedure. For this reason, reconstituted HDL was not used in any subsequent preparations.

The CBE-LDL complex was tested on human glioma cells in the microtitre assay outlined in Section 2.3.12. Results (fig.46) indicated that the drug-LDL complex was inactive. It is likely that the drug itself had become inactive during the incorporation procedure, since the G-CCM cells had already been reported[20] to express high LDL receptor activity under similar conditions. Possibly tris (tris(hydroxymethyl)-methylamine), a nucleophile in the cholate sonication buffer, Section 2.3.2., was attacking the carbon attached to the terminal chlorine of the drug and rendering it inactive. Replacing tris with phosphate in the sonication buffer appeared to reduce the inactivation of the drug

PREPARATION OF CHLORAMBUCIL-LDL BY STOFFEL'S METHOD AND OTHERS

| METHOD | APPARENT % CBE IN HDL | % CBE IN LDL | CONCENTRATION OF CBE IN LDL mg/mL | CONCENTRATION OF CBE IN LDL nmol/mL | LDL PROTEIN* CONCENTRATION mg/mL | LDL PROTEIN* CONCENTRATION nmol/mL | CBE TO LDL PROTEIN RATIO mg/mg | CBE TO LDL PROTEIN RATIO nmol/nmol |
|---|---|---|---|---|---|---|---|---|
| Cholate → HDL → LDL (Tris Buffer) | 61 | 29 | 0.58 | 861 | 3.0 | 6.0 | 0.19 | 143 |
| " " " (PBS Buffer) | 40 | 14 | 0.17 | 252 | 2.0 | 4.0 | 0.08 | 63 |
| " " " " " | 36 | 8 | 1.22 | 1813 | 5.0 | 10.0 | 0.24 | 181 |
| " " " " " | 15 | 10 | 0.57 | 847 | 2.3 | 4.6 | 0.25 | 184 |
| Cholate ——→ LDL (PBS Buffer) | - | 15 | 0.59 | 876 | 0.8 | 1.6 | 0.73 | 547 |

* Protein concentration was calculated assuming a molecular weight of 500,000 Daltons for the apo B content of each LDL particle.

Table 16 : Apparent concentration of Chlorambucil Cholesteryl Ester (CBE) in LDL and HDL at various stages of the preparation of CBE-LDL by the methods described in section 2.3.2.

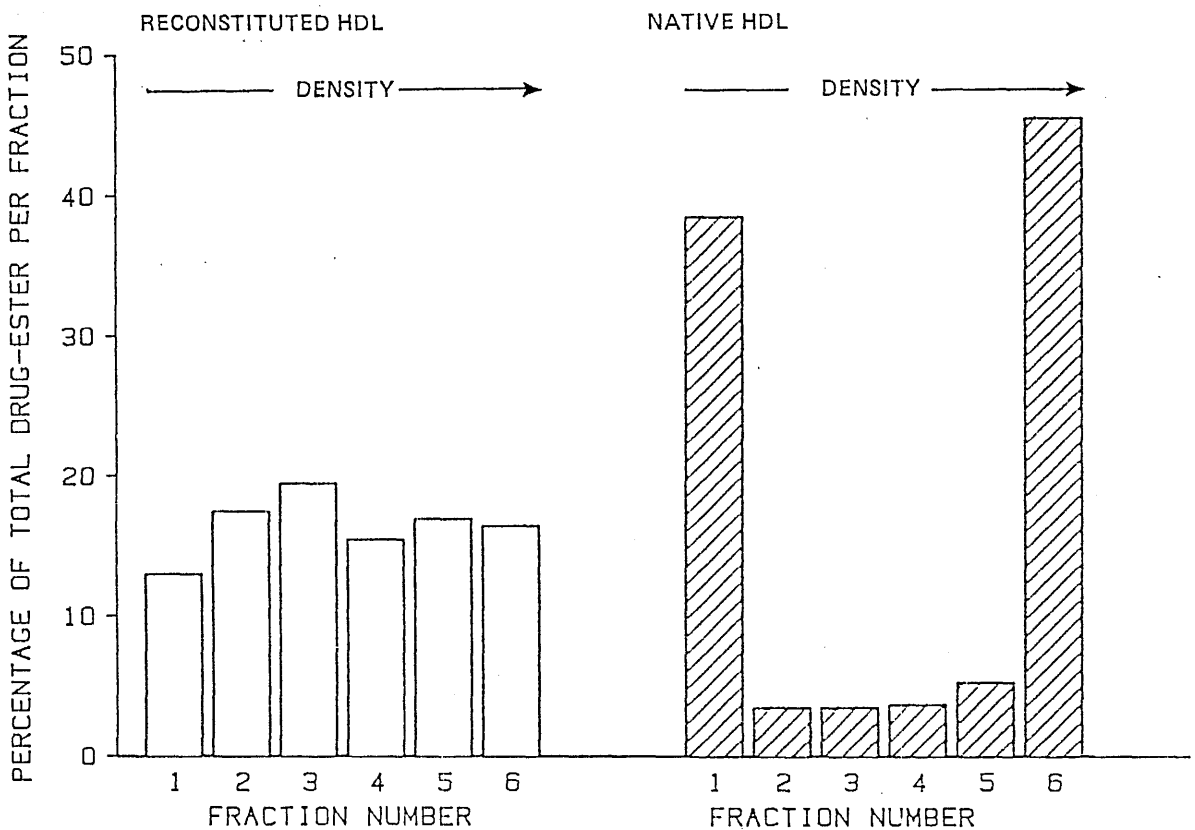



Figure 45: Relative chlorambucil ester content of fractions from the centrifugal separation of ester-laden HDL, prepared using native and "reconstituted" lipoprotein.

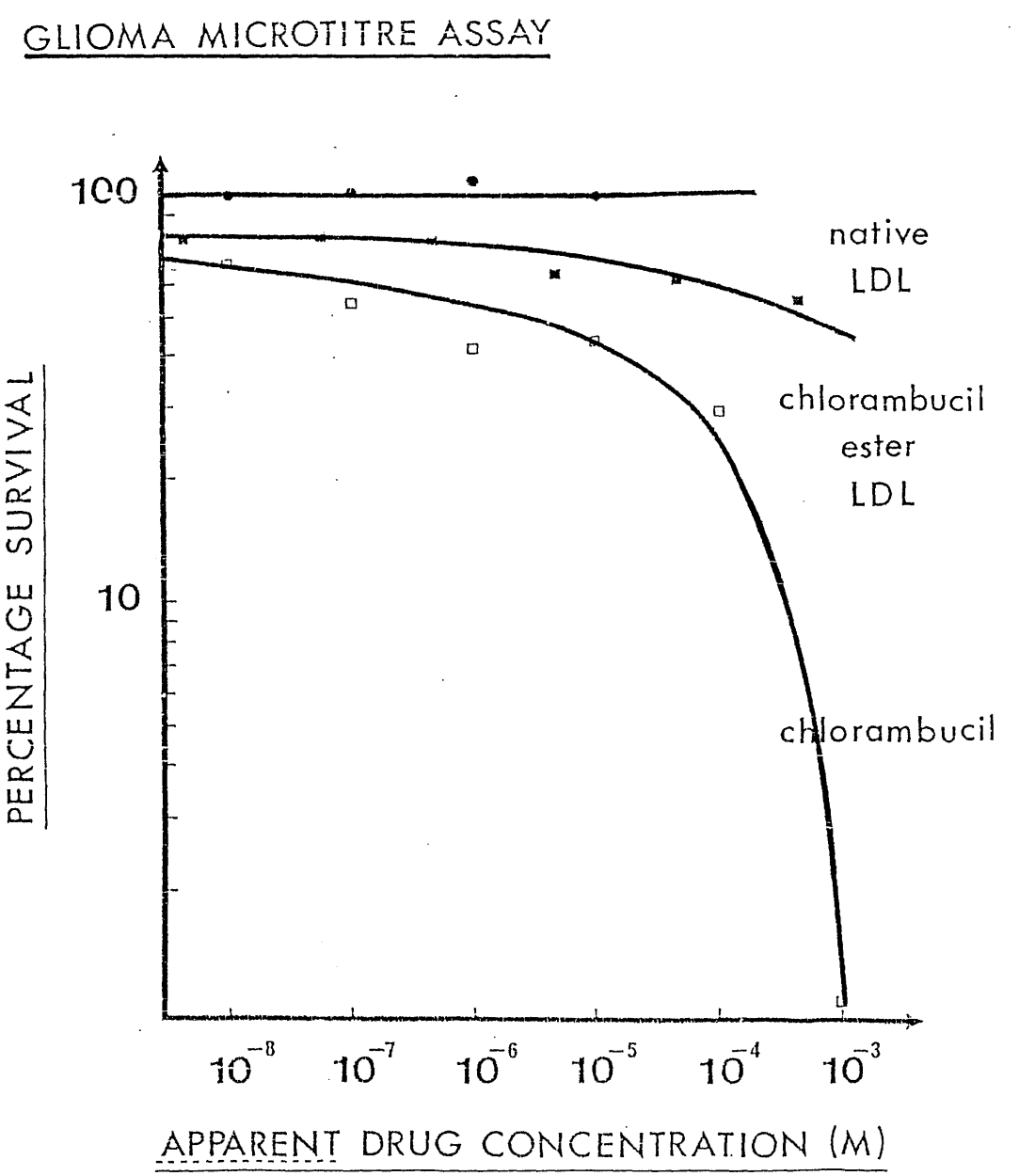



Figure 46: Comparative cytotoxicities of LDL, chlorambucil, and chlorambucil ester-LDL (prepared by the method used to prepare radioiodinated cholesteryl ether-LDL, fig. 15) on human glioma cells in the microtitre assay. Each point is the mean of duplicate incubations.

since the drug-LDL complex prepared in this way had cytotoxic activity (fig. 47) against human glioma cells in the microtitre assay, Section 2.3.12.) However, the buffer change also reduced the overall efficiency of the LDL loading procedure by a factor of 2 (table 16). Thus the 0.02M phosphate buffered saline, 0.02M EDTA, pH 8.2 was used in all of the following CBE-LDL preparations by Stoffel's method. In order to increase the net amount of drug recovered in LDL, the exchange step from cholate micelles to HDL was omitted, that is, the drug-laden micelles were incubated immediately with LDL and d>1.21 Kg/L bottom fraction, Section 2.3.2. This increased the overall incorporation of the drug-ester 3-fold (Table 16), with no obvious effects on the integrity of LDL. CBE-LDL prepared in this way was not obviously different from either native-LDL or CBE-LDL prepared by transfer of drug to LDL from cholate micelles via HDL. Plasma decays (figs. 48(a) and (b)) for each of the aforementioned LDL species made using radioiodinated LDL, Section 2.1.3, were measured in rabbits as described in Section 2.3.8. and simultaneously compared with the plasma decay of native LDL.

## 2.4.2. COMPARISON OF CBE CONCENTRATION MEASURED BY SPECIFIC $[^3H]$-ACTIVITY AND HPLC.

Until equipment was available for the assay of chlorambucil by HPLC, the drug-ester was synthesised with trace amounts of $[7-^3H]$- cholesterol and "apparent" drug concentration was measured by specific $[^3H]$-activity. This approach assumed that the drug-ester link was not being hydrolysed during the incorporation procedure - an assumption which later proved to be false.

Chlorambucil-ester-laden LDL was prepared as described in Section 2.3.2.

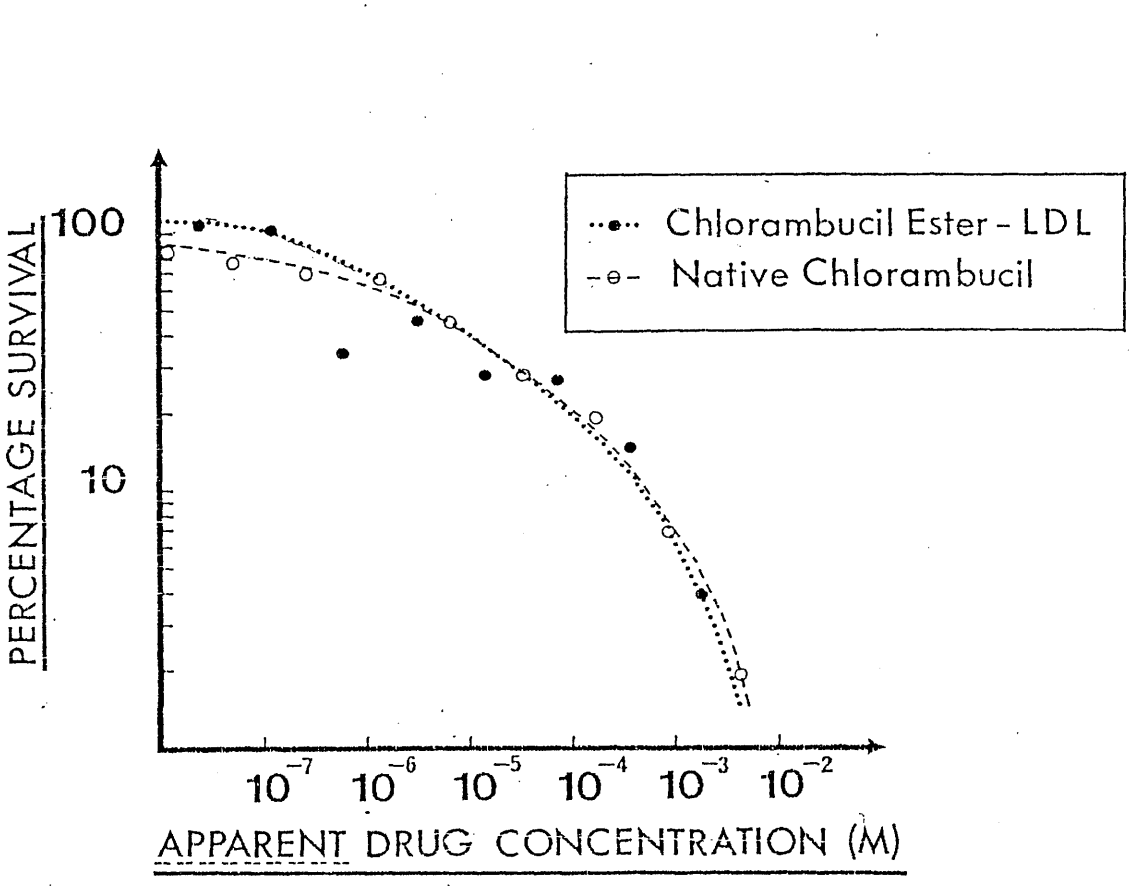



Figure 47: Comparative cytotoxicities of chlorambucil and chlorambucil ester-LDL (prepared by the method outlined in fig. 15, using phosphate buffered- instead of tris buffered- saline. Each point is the mean of duplicate incubations.

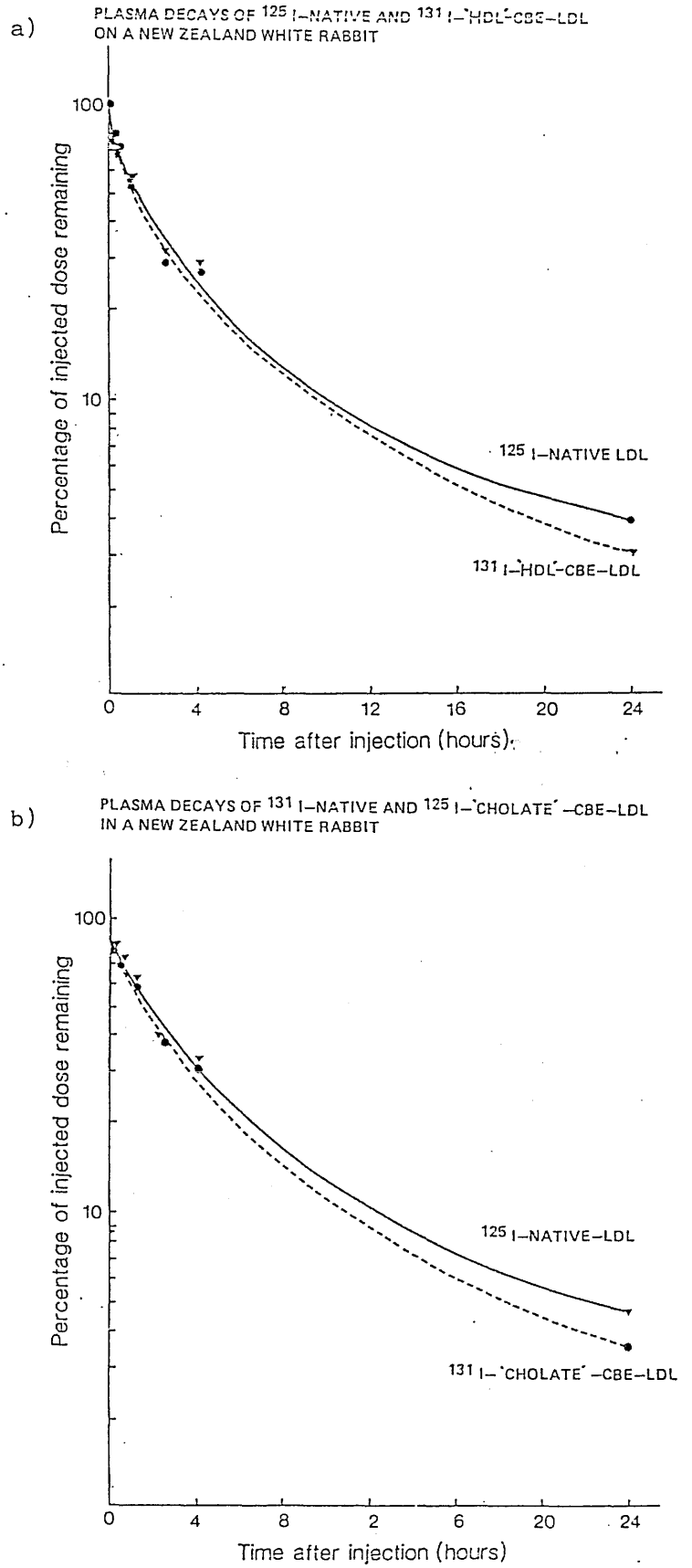



Figure 48: Plasma decays of native-LDL and chlorambucilester(CBE)-LDL prepared by exchange of drug from a) micelles to LDL via HDL ('HDL'-CBE-LDL) or b) micelles to LDL ('cholate'-CBE-LDL)

and aliquots of the solution were assayed for CBE by [$^3$H]-activity and HPLC and found to contain 0.72 mg/mL and 2.5 μg/mL by the respective methods. Lowry protein assay showed the solutions to have a protein concentration of 0.56 mg/mL. Thus only 3 molecules of drug had been incorporated per LDL particle, not 950 as calculated by specific activity. The CBE experiments already reported (figs. 45-48, table 16) then gave overestimates of the drug-ester incorporation into lipoproteins and consequently the results of these studies may be interpreted only qualitatively.

In all subsequent experiments with CBE, the drug-ester concentration was measured by HPLC, Section 2.3.6.

### 2.4.3. DEGRADATION OF CBE DURING INCUBATION PROCEDURES

Measurement of the CBE concentration in the cholate micelle mixture during the course of its incubation with LDL, showed that a large proportion of the ester had been degraded, leaving only 20-40% of the original amount of ester in the solution after 24 hours.

The d>1.21 Kg/L fraction of human plasma, Section 2.1.2., was used in the incubation as a crude preparation of cholesteryl-ester transfer protein, a plasma constituent which mediates the transfer of cholesteryl esters between LDL and other lipoproteins. Since this fraction contains numerous other enzymes, including LCAT, it was thought possible that the drug-ester was being hydrolised enzymically. Sodium iodoacetate (NaIOAc), an inhibitor of LCAT [199] was added to the LDL/cholate/d>1.21 Kg/L fraction mixture in an attempt to reduce the hydrolysis rate, Section 2.1.3. Another approach to reduce enzyme degradation of the compound was to inactivate a number of enzymes by

heating the d>1.21 Kg/L plasma fraction to 60°C, which leaves cholesteryl ester transfer protein unaffected[198].

The degradation of the drug-ester with time under each of the conditions used is shown in fig. 49. None of the agents gave any increase in the amount of intact drug-ester remaining after 24 hours, on the contrary, they increased its degradation. It is interesting to note that there was no concurrent increase in the concentration of the free chlorambucil in any of the incubations, suggesting that the drug-ester degradation was not simply a hydrolysis procedure.

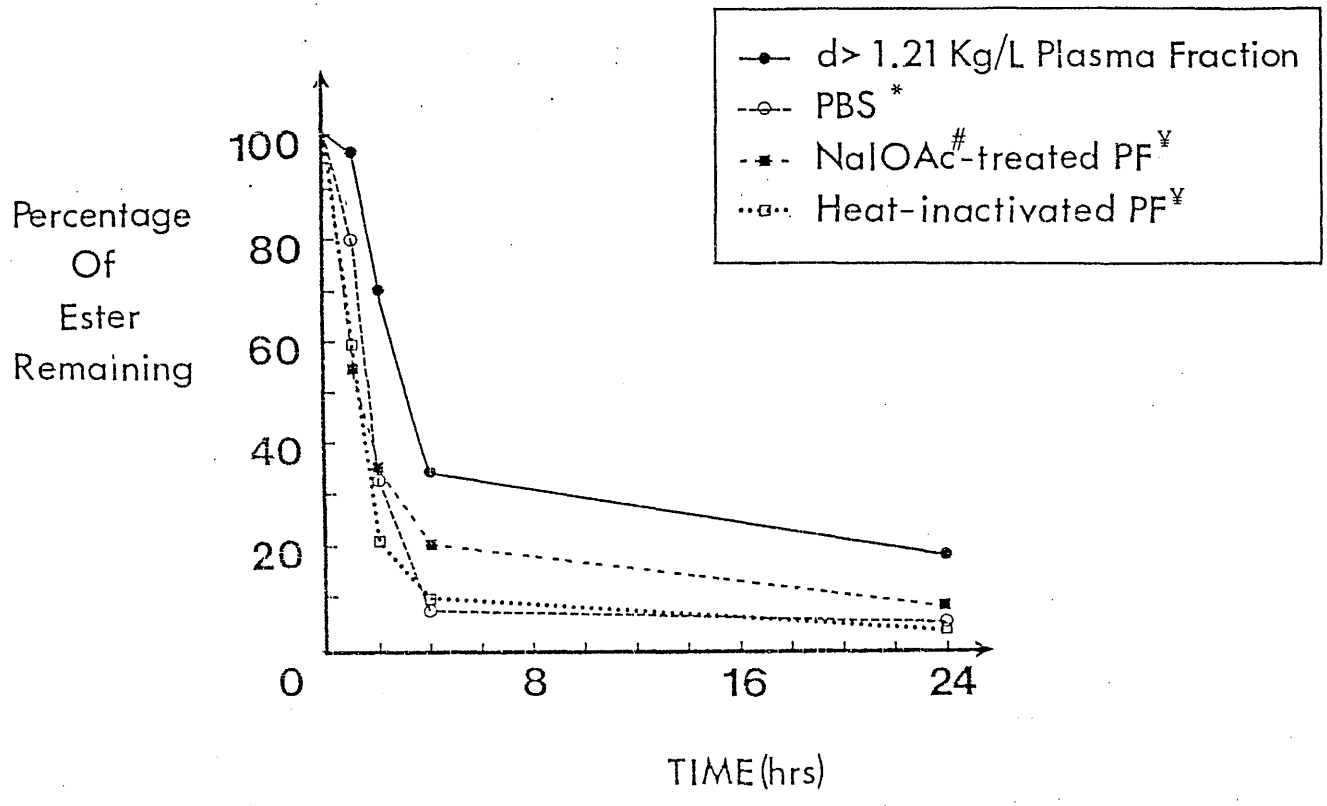
If therapeutic amounts of CBE are to be incorporated into LDL, other methods of incorporation must be considered, since the cholate method gives a final concentration of only a few micrograms of CBE per mg of LDL protein.

### 2.4.4. TRANSFER OF CBE FROM SYNTHETIC MICROEMULSIONS TO LDL

CBE-microemulsions were prepared as outlined in Section 2.3.4., using either DMPC or DPPC as the phospholipid component. Using DMPC emulsions, the final amount of drug in LDL was slightly higher, ∴ DMPC was used in all subsequent preparations of CBE-containing microemulsions. Recovery of the drug-ester at each stage of the preparation of CBE-LDL using these particles is shown in table 17. Even when contained within microemulsions, and in the absence of plasma proteins, 50% of the drug-ester is degraded during overnight incubation, table 17.

Typical recoveries of drug and LDL are shown, for each stage of the microemulsion/LDL preparation, in table 18. Less than 15% of the initial drug used is recovered in LDL but this is 100-times more than

Figure 49: DEGRADATION OF CBE UNDER VARIOUS CONDITIONS




CBE - chlorambucil cholesteryl ester, * 0.15 M phosphate buffered saline, pH 7.4,
# sodium iodoacetate, ¥ plasma fraction

| STAGE OF PREPARATION | PERCENTAGE OF CHLORAMBUCIL ESTER REMAINING |
|---|---|
| DMPC MICROEMULSIONS | |
| Incubation Mixture (t= 0 hrs.) | 100 |
| " " (t= 4 hrs.) | 60 |
| " " (t=24 hrs.) | 48.6 |
| Microemulsions only (t=24 hrs.) | 49 |
| Chlorambucil-Ester-LDL (after centrifugation) | 48 |
| DPPC MICROEMULSIONS | |
| Incubation Mixture (t= 0 hrs.) | 100 |
| " " (t= 4 hrs.) | 59.5 |
| " " (t=24 hrs.) | 51.9 |
| Microemulsions only (t=24 hrs.) | 49 |
| Chlorambucil-Ester-LDL (after centrifugation) | 40.8 |

Table 17 : Comparison of incorporation of chlorambucil cholesteryl ester into LDL by exchange from microemulsions containing drug, cholesterol oleate and Dimyristoyl Phosphatidyl Choline (DMPC) or Dipalmitoyl Phosphatidyl Choline (DPPC).

| STAGE OF PREPARATION | % CBE REMAINING | % LDL PROTEIN REMAINING |
|---|---|---|
| Incubation mixture (t= 0 hrs.) | 100 | 100 |
| Incubation mixture (t= 5 hrs.) | 78 | 100 |
| CBE-LDL (after centrifugation) | 44 | 75 |
| CBE-LDL (De-salted) | 35 | 62 |
| CBE-LDL (after 0.22μm filtration) | 28 | 55 |

Table 18 : Recoveries of chlorambucil cholesteryl ester (CBE) and LDL protein at each stage of the preparation of CBE-LDL as described in section 2.3.4.
Values shown are the mean of 3 preparations.

the drug recovery from the cholate procedure.

The proportions of drug and LDL protein in several preparations of CBE/LDL by this method are variable, as shown in table 19. On average, 100 drug molecules ($92\pm63$) are incorporated per LDL particle.

## 2.4.5. DEGRADATION OF CBE IN LDL DURING STORAGE AT 4°C

Aliquots of CBE-LDL (135 μg CBE/mg LDL protein) were stored at 4°C. Every 24 hours for 3 days, an aliquot was removed and assayed for CBE by HPLC, Section 2.3.6. The decay profile for the drug-ester under these conditions is shown in figure 50. In the first 24 hours, the ester decayed rapidly, leaving only 35% of the original ester in solution. Thereafter, a further 5% of the drug is lost over the following 48 hours. The absence of free chlorambucil in the solution at any time implies that the drug-ester is not being hydrolysed to yield free drug.

## 2.4.6. ADRIAMYCIN-LDL

Several methods of incorporating adriamycin into LDL were employed, Section 2.1.5. As reported by Iwanik et al[23], incubation of LDL with Adriamycin in sodium chloride/phosphate buffer causes immediate aggregation of LDL and drug. In an attempt to reduce this precipitation we used LDL solution immediately from the zonal preparation, Section 2.1.1., without concentrating it or removing any of the sodium bromide. This did not completely prevent aggregation in any of the buffers used but about 10% of the LDL and some drug remained complexed and in solution, and was subsequently purified and assayed for drug, Section 2.3.6, and protein, Section 2.3.7. Proportions of drug and

| CHLORAMBUCIL ESTER CONCENTRATION | | LDL PROTEIN* CONCENTRATION | | DRUG TO PROTEIN RATIO | |
|---|---|---|---|---|---|
| (μg/mL) | (nmol/mL) | (mg/mL) | (nmol/mL) | (gram/gram) | (nmol/nmol) |
| 181 | 269 | 5.5 | 11.0 | 0.033 | 24 |
| 752 | 1120 | 7.8 | 15.6 | 0.096 | 72 |
| 530 | 788 | 4.6 | 9.2 | 0.115 | 86 |
| 221 | 328 | 1.7 | 3.4 | 0.130 | 97 |
| 1130 | 1679 | 5.0 | 10.0 | 0.226 | 168 |
| 180 | 267 | 7.5 | 15.0 | 0.024 | 18 |
| 1580 | 2348 | 6.5 | 13.0 | 0.243 | 180 |

* LDL protein concentration calculated assuming a molecular weight of 500,000 Daltons for the apoprotein B content of each LDL particle.

Table 19 : Proportions of chlorambucil cholesteryl ester and protein in CBE-LDL complexes prepared as described in section 2.3.4. On average, $92 \pm 63$ drug molecules were incorporated per LDL particle.

Degradation Of Chlorambucil Ester Encapsulated In LDL And stored At 4°C For Several Days

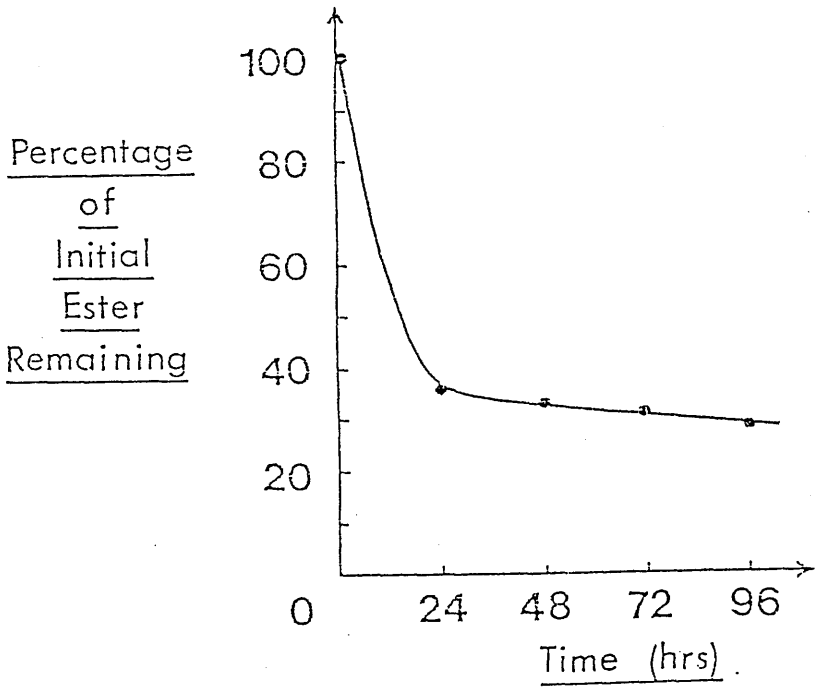



Figure 50: Degradation of chlorambucil ester in a preparation of the drug-LDL complex containing 371 μg of drug ester and 3.8 mg of LDL protein per mL of solution. The complex was stored at 4°C for several days and aliquots removed at intervals for drug quantitation by HPLC as described in Section 2.3.6.(C).

protein in adriamycin-LDL complexes prepared by the methods described in Section 2.3.5 are shown in table 2o. After 4 hours incubation in DMSO and PBS buffers, only a few drug molecules had partitioned into each LDL particle. This number was increased by prolonging the incubation time to 24 hours. On the basis of these experiments, 24 hours incubation of drug in DMSO buffer, Section 2.3.5.(c), produced LDL with the highest adriamycin content. Incubation of drug and LDL at higher pH, Section 2.3.5.(f), produced no increase in incorporation. On the contrary, slightly less drug was included, table 20, and significant amounts of adriamycin degradation products were apparent, fig. 51, in the pH 10 incubation mixture when analysed by HPLC, Section 2.3.6. after 24 hours. However, it is interesting to note that only Adriamycin had been incorporated into LDL. Surfactants were used to enlarge the LDL particle and so facilitate the entry of adriamycin into the core space. Tucker et al[203] reported that several different surfactants, including those used here, caused rapid increases in the Stokes radius of the LDL particle. Adriamycin-LDL complexes prepared after incubation of LDL with Brij-35, Triton X-100, and SDS, Section 2.3.5.(g), were,disappointingly, not much higher in drug content than those prepared without surfactants, table 20. As with the other methods, the incorporation of the drug was quite erratic. SDS caused around 80% of the LDL protein to precipitate, before the adriamycin was added. No aggregation was observed in any of the other surfactant/LDL mixtures prior to drug addition.

By subjecting LDL to gradually increasing concentrations of adriamycin, via dialysis, it was hoped that the drug would be incorporated into the LDL particle without such a high degree of aggregation. The actual amount of drug which bound to LDL was less than 1% of the amount available, even in the most concentrated adriamycin solutions, fig. 52.

ADRIAMYCIN-LDL

| METHOD | INCUBATION TIME | ADRIAMYCIN CONCENTRATION | | LDL PROTEIN* CONCENTRATION | | DRUG TO PROTEIN RATIO | |
|---|---|---|---|---|---|---|---|
| | Hours | (μg/mL) | (nmol/mL) | (μg/mL) | (nmol/mL) | (gram/gram) | (nmol/nmol) |
| PBS | 4 | 0.15 | 0.27 | 60 | 0.12 | 0.0027 | 2.3 |
| PBS | 24 | 1.40 | 2.41 | 40 | 0.08 | 0.035 | 30.1 |
| DMSO | 4 | 0.08 | 0.14 | 29 | 0.06 | 0.0028 | 2.3 |
| DMSO | 24 | 2.75 | 4.74 | 24 | 0.05 | 0.114 | 94.8 |
| CHOLATE | 24 | 3.20 | 5.51 | 43 | 0.09 | 0.074 | 61.2 |
| DMSO/GLYCINE pH 10 | 24 | 0.40 | 0.69 | 260 | 0.52 | 0.0015 | 1.32 |
| DMSO/NaCl pH 7.2 | 24 | 1.1 | 1.89 | 300 | 0.60 | 0.0036 | 3.15 |
| DMSO | 24 | 0.20 | 0.34 | 30.4 | 0.06 | 0.0067 | 5.6 |
| SURFACTANTS | | | | | | | |
| BRIJ. 35 | 24 | 5.52 | 9.51 | 192 | 0.38 | 0.029 | 25 |
| BRIJ. 35 | 24 | 1.40 | 2.4 | 260 | 0.52 | 0.005 | 4.6 |
| TRITON X-100 | 24 | 2.92 | 5.03 | 180 | 0.36 | 0.016 | 14 |
| TRITON X-100 | 24 | 1.30 | 2.30 | 264 | 0.53 | 0.006 | 4.3 |
| SDS | 24 | 0.29 | 0.50 | 50 | 0.10 | 0.005 | 58 |

* Protein concentration was calculated assuming a molecular weight of 500,000 Daltons for the apo B content of each LDL particle.

Table 20 : Adriamycin and protein content of adriamycin-LDL complexes prepared by the various methods described in section 2.3.5.

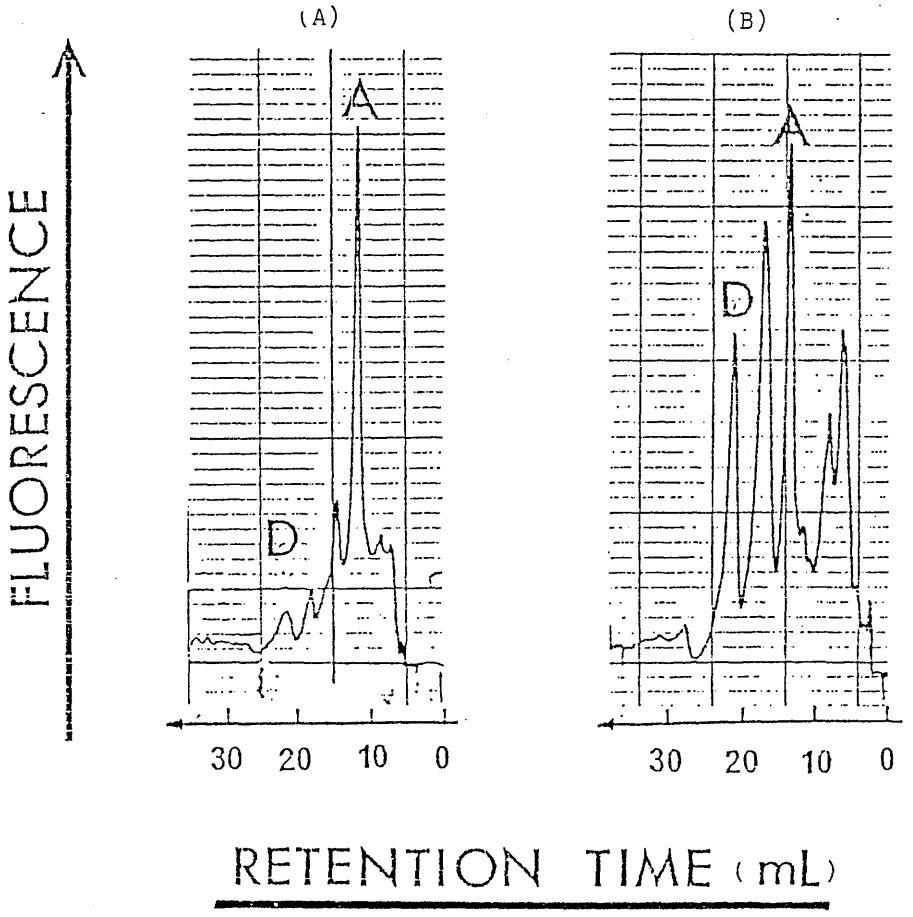



Figure 51: HPLC profiles of adriamycin/LDL incubation mixtures after 24 hours at 25 °C in buffers containing 10% dimethyl sulphoxide and

(A) 0.15 M NaCl, pH 7.2 (sample diluted 1: 10)

or

(B) 0.10 M glycine, pH 10.

A : adriamycin, B : daunomycin internal standard

At higher concentrations, the incorporation of drug into LDL as a function of the free adriamycin concentration, fig. 53, began to reach a plateau. The aggregation of LDL was not significantly reduced by the dialysis procedure, which yielded adriamycin-LDL containing up to 18 drug molecules per particle.

### 2.4.7. DAUNOMYCIN-LDL

Daunomycin-LDL was prepared as described in Section 2.3.5.(a). The degree of daunomycin incorporation was variable, table 21, as with the other drugs studied. On average, 120 daunomycin molecules (range 40-190) were sequestered within each particle, giving drug concentrations up to 0.73 mg/mL.

### 2.4.8. IN VITRO TESTING OF CYTOTOXICITY OF DRUG-LDL COMPLEXES

Daunomycin-LDL (D-LDL), adriamycin-LDL (A-LDL) and chlorambucil-ester-LDL (CBE-LDL) complexes were prepared, as outlined in Sections 2.3.4 and 2.3.5. The cytotoxicities of these complexes were compared to those of the native drugs. In all experiments, controls were set up containing no drugs. Identical plates were prepared with native LDL or LDL which had undergone the drug incorporation procedure, in the absence of drug. These controls induced no significant difference in cell survival from that seen in the cells to which neither drug nor LDL had been added. Additionally LDL and uncomplexed drug, added separately to the medium induced dose response exactly like cells treated only with native drug. All of the complexes studied had cytotoxic effects, although the extent of enhancement of cell kill conferred by the use of LDL as a vehicle was variable.

In G-CCM glioma cells, daunomycin-LDL was less toxic than an equal

## Incorporation Of Adriamycin Into LDL By Dialysis

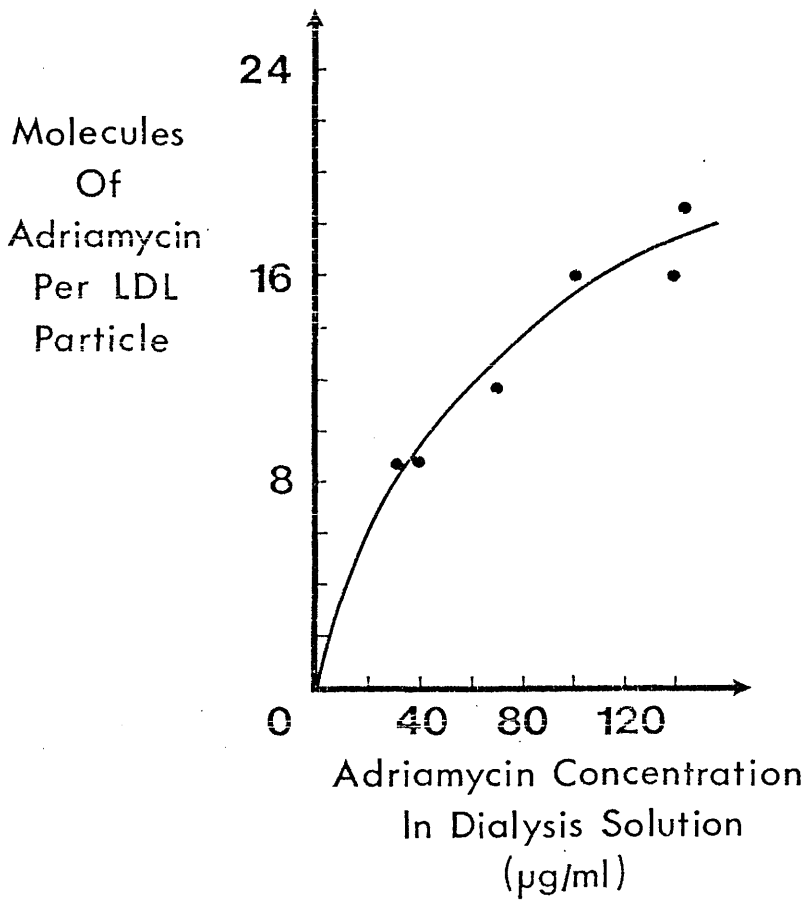



Figure 53: Incorporation of adriamycin into LDL by dialysis into the lipoprotein solution, as a function of the free adriamycin concentration.

| DAUNOMYCIN CONCENTRATION | | LDL PROTEIN* CONCENTRATION | | DRUG TO PROTEIN RATIO | |
|---|---|---|---|---|---|
| (μg/mL) | (nmol/mL) | (μg/mL) | (nmol/mL) | (gram/gram) | (nmol/nmol) |
| 95 | 168 | 650 | 1.3 | 0.146 | 130 |
| 300 | 531 | 1600 | 3.2 | 0.188 | 166 |
| 280 | 496 | 6500 | 13.0 | 0.043 | 38 |
| 400 | 709 | 2500 | 5.0 | 0.160 | 142 |
| 379 | 673 | 5000 | 10.0 | 0.076 | 67 |
| 725 | 1285 | 3400 | 6.8 | 0.213 | 189 |

* LDL protein concentration calculated assuming a molecular weight of 500,000 Daltons for the apoprotein B content of each LDL particle.

Table 21 : Proportions of daunomycin and protein in daunomycin-LDL complexes prepared as described in section 2.3.4. On average $122 \pm 58$ drug molecules were incorporated per LDL particle.

dose of native drug, fig.54. When tested in the microtitre system, Section 2.3.12, complex and drug had $ID_{90}$'s of $3.5 \times 10^{-6}$M and $5.6 \times 10^{-7}$M respectively. When tested on L-DAN cells by clonogenic stem cell assay,Section 2.3.13, free and LDL-bourne drug were almost indistinguishable in their cytotoxicity, table 22, despite the presence of high receptor-mediated LDL assimilation in L-DAN cells, fig. 55, Section 2.3.17. The clonogenic $ID_{50}$'s for intact spheroids which were disaggregated after treatment, Section 2.3.13, showed that spheroids were significantly less sensitive to both daunomycin and daunomycin-LDL than their monolayer counterparts, table 22. Spheroid growth delay Section 2.3.13, was significantly longer for daunomycin-LDL compared to native daunomycin at a drug concentration of 5 μg/mL, table 22. Qualitative fluorescence microscopy on the spheroids after drug exposure showed that D-LDL, fig.56 had diffused further into the spheroids than free daunomycin, fig. 57. In HeLa cells (microtitre assay, Section 2.3.12) the D-LDL complex was marginally more cytotoxic than the free drug, fig. 58.

Adriamycin, when carried in LDL, was active at a lower concentration than free drug, fig. 59, with $ID_{90}$ $1.1 \times 10^{-8}$M compared to $1.6 \times 10^{-7}$M for native adriamycin, when tested on HeLa cells in the microtitre assay Section 2.3.12.

Chlorambucil was more effective as a cytotoxic agent when carried in LDL, fig.60. The $ID_{50}$ of the drug fell from $1 \times 10^{-5}$M to $2 \times 10^{-7}$M when transported within LDL.

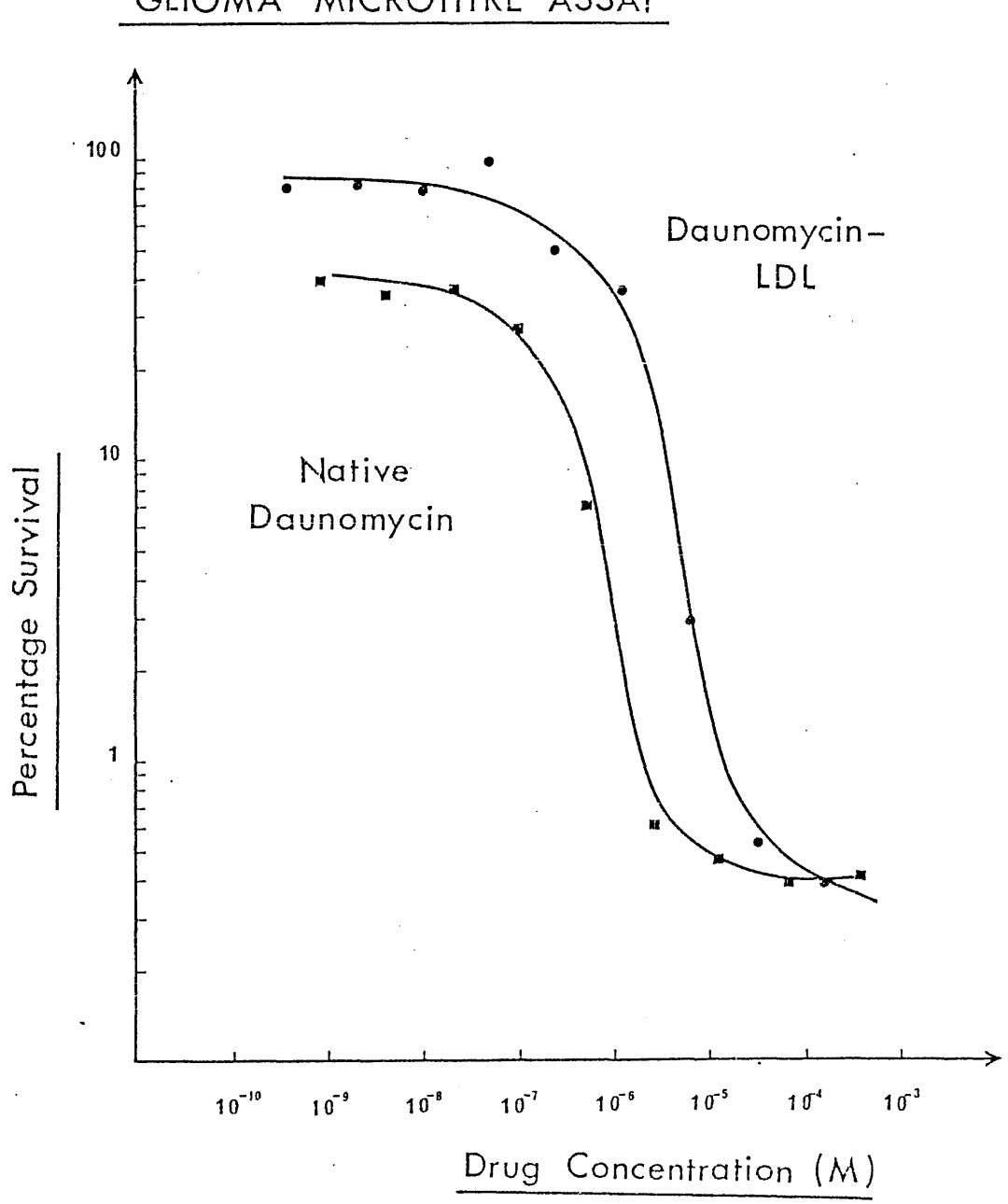



Figure 54: Comparative cytotoxicities of daunomycin and daunomycin-LDL on human glioma cells in the microtitre assay. Each point is the mean of duplicate incubations in three experiments.

L-DAN (Human Non-Small-Cell Lung Carcinoma)

| | EXPOSURE TO | |
|---|---|---|
| | Daunomycin | Daunomycin-LDL |
| MONOLAYER | | |
| CLONOGENIC SURVIVAL ($ID_{90}$) | 1.5 μg/mL | 1.6 μg/mL |
| SPHEROIDS | | |
| CLONOGENIC SURVICAL ($ID_{50}$) | 1.9 μg/mL | 1.3 μg/mL |
| GROWTH DELAY | 20 days | 23.2 days |

Table 22 : Comparative cytotoxicity of daunomycin and daunomycin-LDL, assessed in monolayer and spheroid culture as described in section 2.3.13.

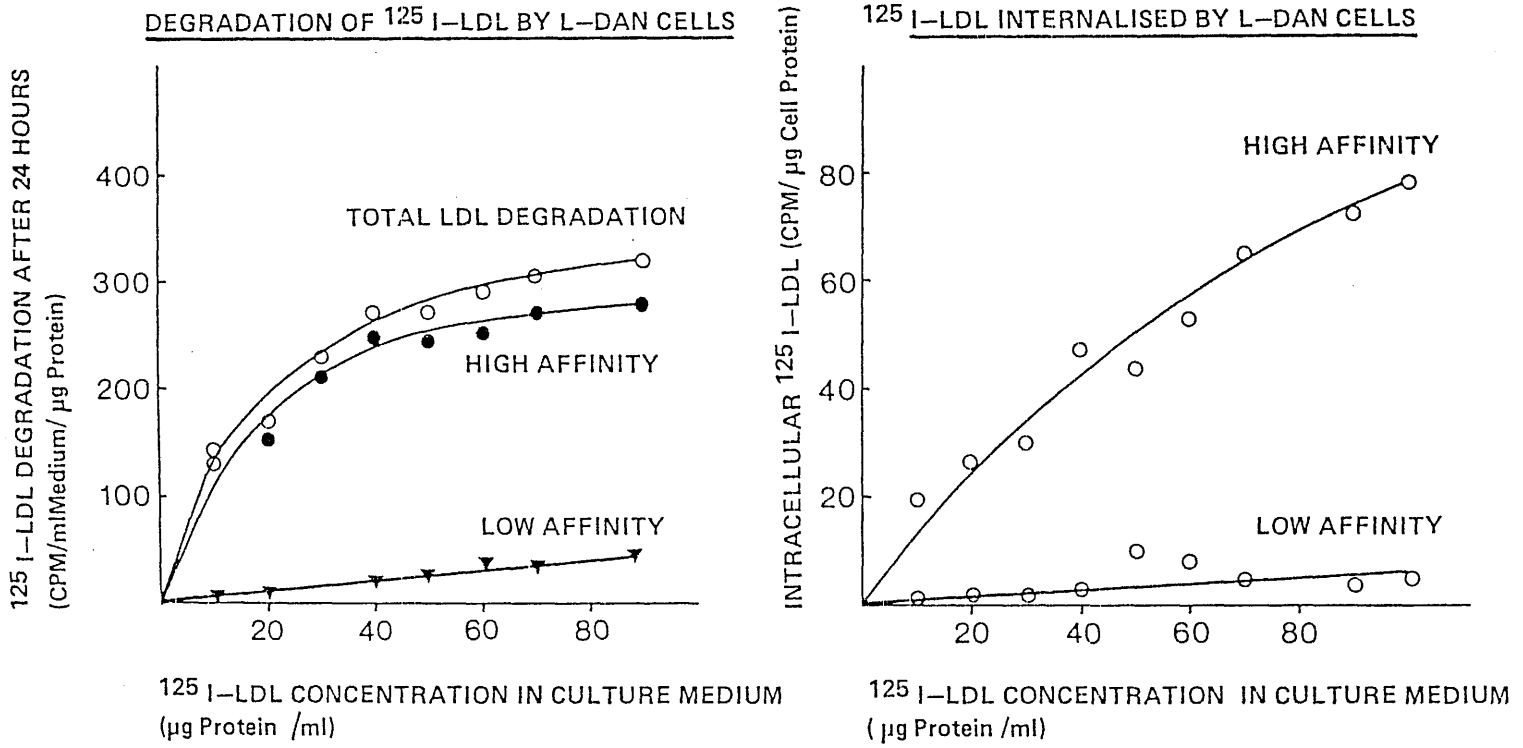
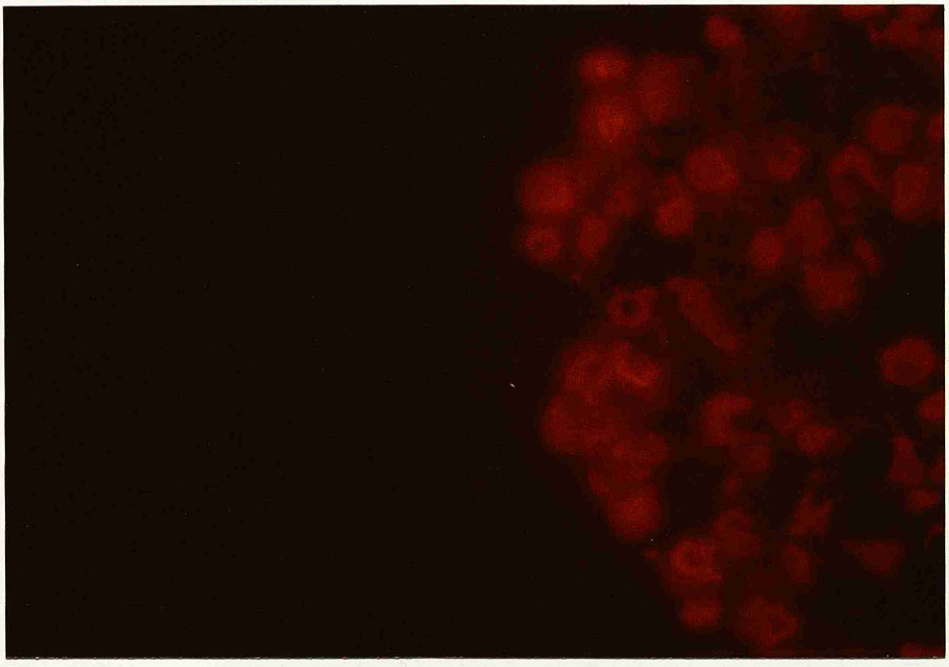
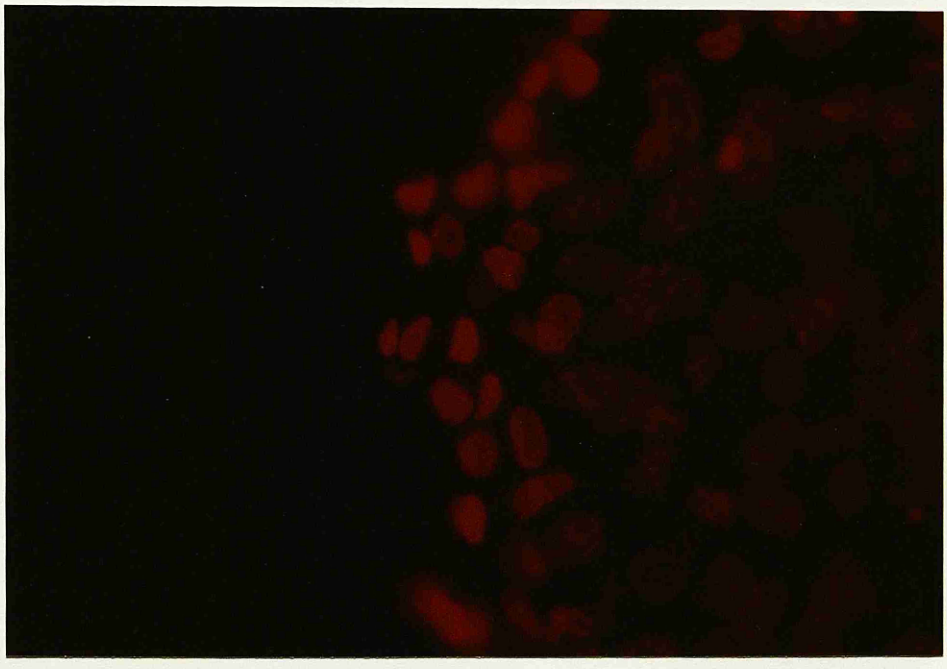



Figure 55: Internalisation and degradation of $^{125}$I-labelled-LDL via high- and low- affinity routes in L-DAN cells in monolayer culture.

Figure 56: Fluorescence micrograph of an L-DAN spheroid following exposure to daunomycin-LDL.

Figure 57: Fluorescence micrograph of an L-DAN spheroid following exposure to native daunomycin.





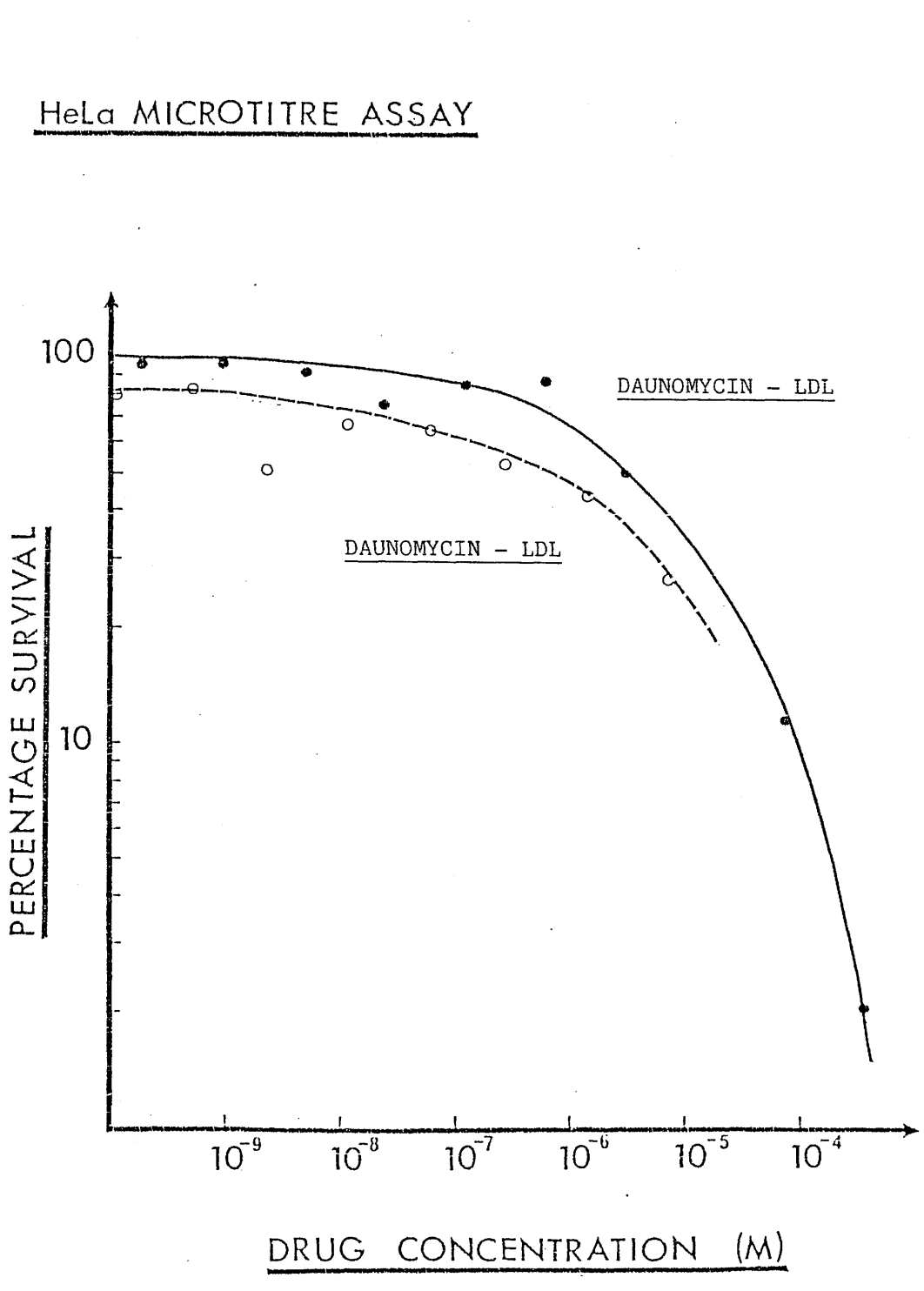



Figure 58: Relative cytotoxicities of native and LDL-encapsulated-daunomycin against HeLa cells in the microtitre drug sensitivity assay. Each point is the mean of duplicate incubations in three experiments.

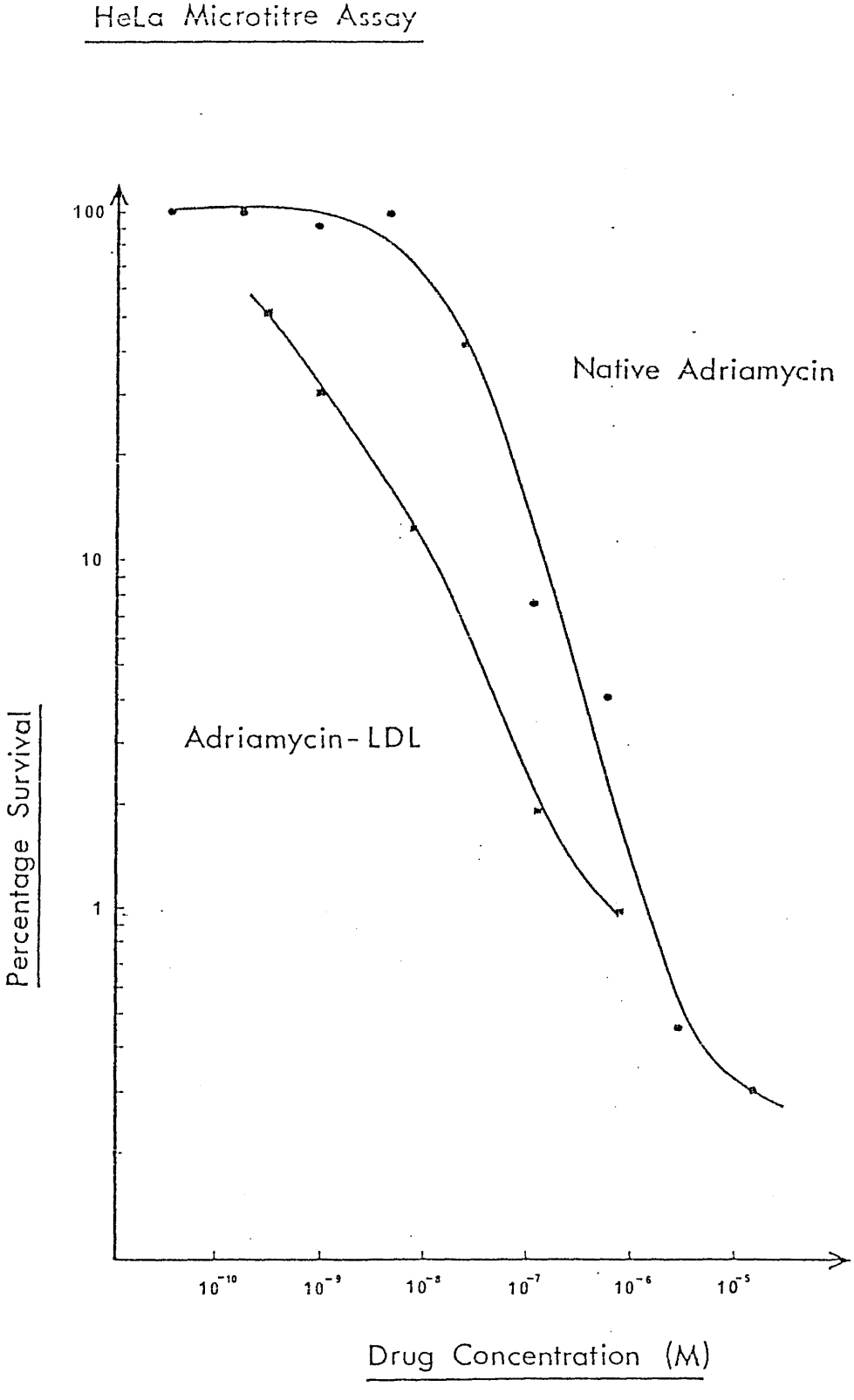



Figure 59: Relative cytotoxicities of native- and LDL encapsulated-adriamycin against HeLa cells in the microtitre drug sensitivity assay. Each point is the mean of duplicate incubations in three experiments.

## 2.4.9. IN VIVO TESTING OF DRUG-LDL COMPLEXES IN THE SRC ASSAY

Daunomycin-LDL or daunomycin were given by IV injection, at a dose of 4 mg/Kg, to groups of mice implanted with primary gastric tumour, as described in Section 2.3.14. The apparent increases in tumour size for each group are shown, along with those for untreated animals, in fig. 61. A significant inhibition in tumour growth ($p<0.05$, Wilcoxon) was observed only in the mice treated with D-LDL. However, histological examination of tumour sections revealed that the apparent tumour growth was actually a measure of lymphocyte infiltration, and very few viable tumour cells were present on day 6 in any of the tumour implants, whether treated or untreated, fig. 62. Thus, the observed reduction in "tumour" size in mice treated with D-LDL was actually a result of drug induced immunosuppression. The SRC assay has since been shown to be invalid for primary gastric tumours[214].
A second group of mice was implanted with rat Walker256 tumour (obtained as described in Section 2.1.12) and treated with daunomycin, D-LDL, LDL or left untreated. Despite the presence of a large number of viable tumour cells and only a small proportion of lymphocytes, no difference in tumour growth was observed in any of the drug or LDL treated mice when compared to controls.

Mice implanted with Walker256 tumour were treated with chlorambucil, CBE-LDL (8 mg of chlorambucil per Kg body weight) or LDL. Results obtained are shown in fig. 63. Both chlorambucil and chlorambucil-LDL were successful in suppressing growth of tumour. The LDL-treated group did not show any significant difference in tumour size when compared to the control group. Histology showed viable tumour cells to be present in all tumour implants, but variable degrees of lymphocyte infiltration, necrosis and fibrosis, fig. 64, were observed in a

Glioma Microtitre Assay

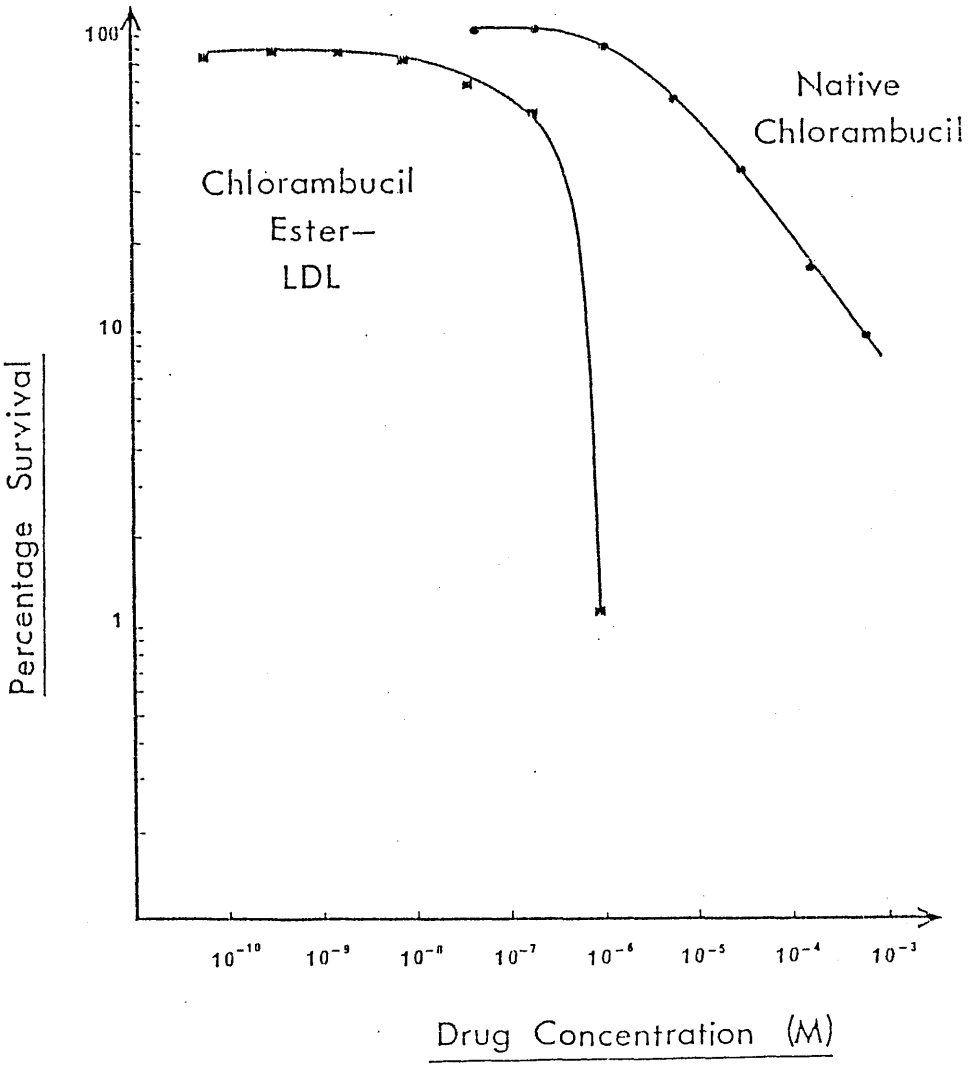



Figure 60: Comparative cytotoxicities of native chlorambucil and chlorambucil ester-LDL against human glioma cells in the microtitre drug sensitivity assay. Each point is the mean of duplicate incubations in three experiments.

## 2.4.9. IN VIVO TESTING OF DRUG-LDL COMPLEXES IN THE SRC ASSAY

Daunomycin-LDL or daunomycin were given by IV injection, at a dose of 4 mg/Kg, to groups of mice implanted with primary gastric tumour, as described in Section 2.3.14. The apparent increases in tumour size for each group are shown, along with those for untreated animals, in fig. 61. A significant inhibition in tumour growth ($p<0.05$, Wilcoxon) was observed only in the mice treated with D-LDL. However, histological examination of tumour sections revealed that the apparent tumour growth was actually a measure of lymphocyte infiltration, and very few viable tumour cells were present on day 6 in any of the tumour implants, whether treated or untreated, fig. 62. Thus, the observed reduction in "tumour" size in mice treated with D-LDL was actually a result of drug induced immunosuppression. The SRC assay has since been shown to be invalid for primary gastric tumours[214].
A second group of mice was implanted with rat Walker256 tumour (obtained as described in Section 2.1.12) and treated with daunomycin, D-LDL, LDL or left untreated. Despite the presence of a large number of viable tumour cells and only a small proportion of lymphocytes, no difference in tumour growth was observed in any of the drug or LDL treated mice when compared to controls.

Mice implanted with Walker256 tumour were treated with chlorambucil, CBE-LDL (8 mg of chlorambucil per Kg body weight) or LDL. Results obtained are shown in fig. 63. Both chlorambucil and chlorambucil-LDL were successful in suppressing growth of tumour. The LDL-treated group did not show any significant difference in tumour size when compared to the control group. Histology showed viable tumour cells to be present in all tumour implants, but variable degrees of lymphocyte infiltration, necrosis and fibrosis, fig. 64, were observed in a

Subrenal Capsular Assay - Human Primary Gastric Tumour

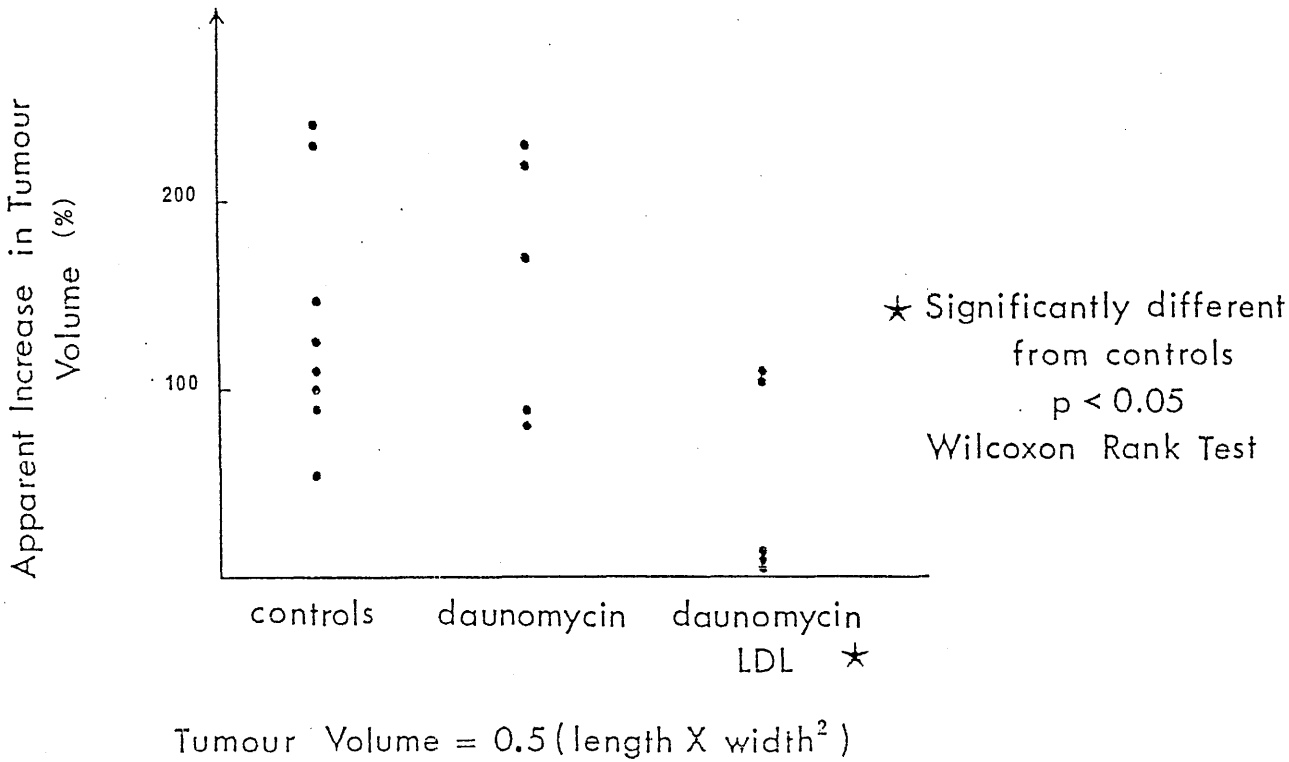



Figure 61: Apparent growth-inhibitory effect of daunomycin-LDL compared to daunomycin on mice implanted with human primary gastric tumour in the subrenal capsular assay.

Figure 62: Haematoxylin and eosin stained section of mouse kidney and gastric tumour implant on day 6 of the SRC assay. Note the large degree of lymphocyte infiltration (A) and lack of viable tumour cells (B).

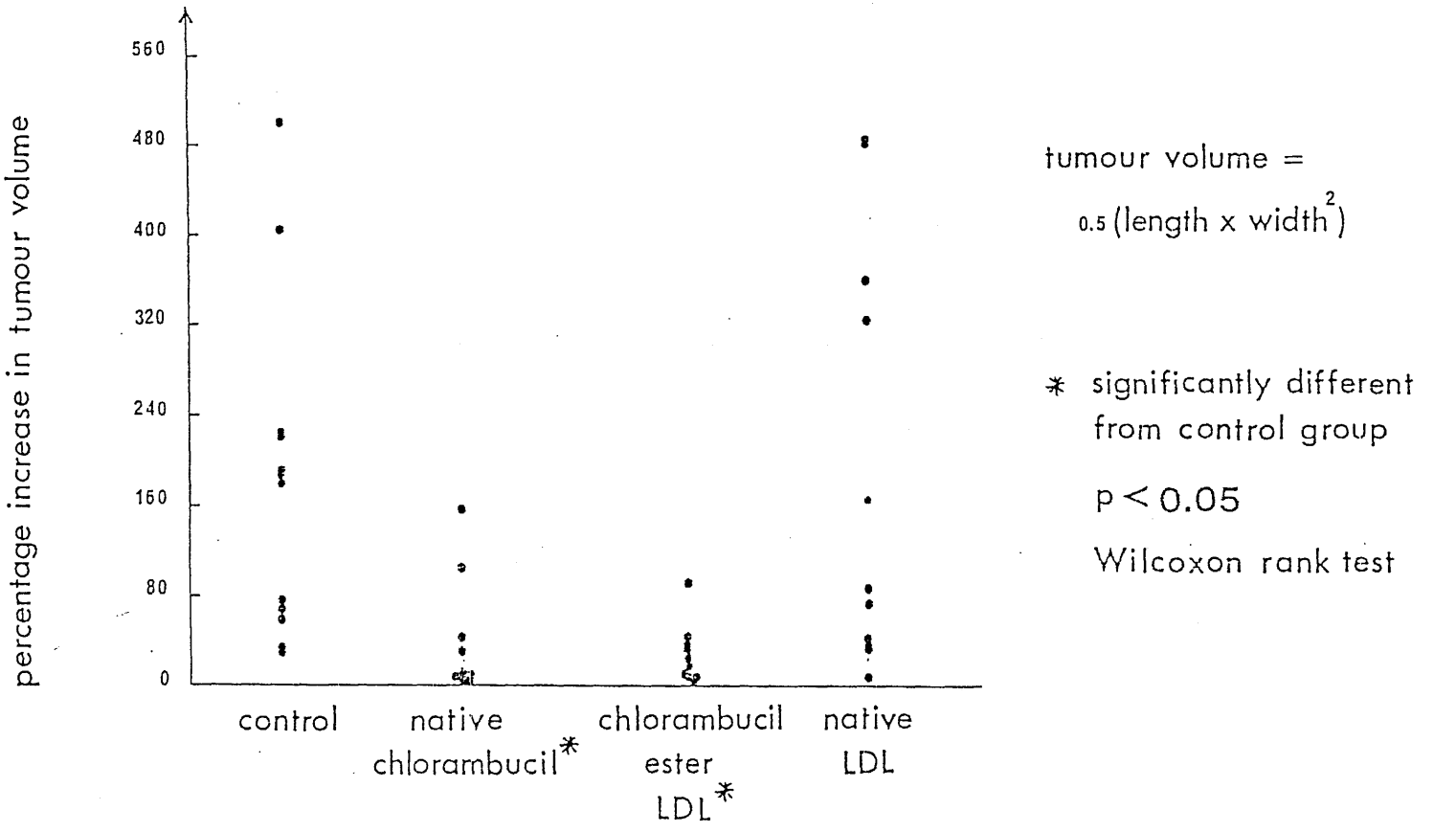



Figure 63: Growth-inhibitory effects of chlorambucil, chlorambucil ester-LDL, and native-LDL on Walker256 tumour implants in the mouse subrenal capsular assay.

FIGURE 62

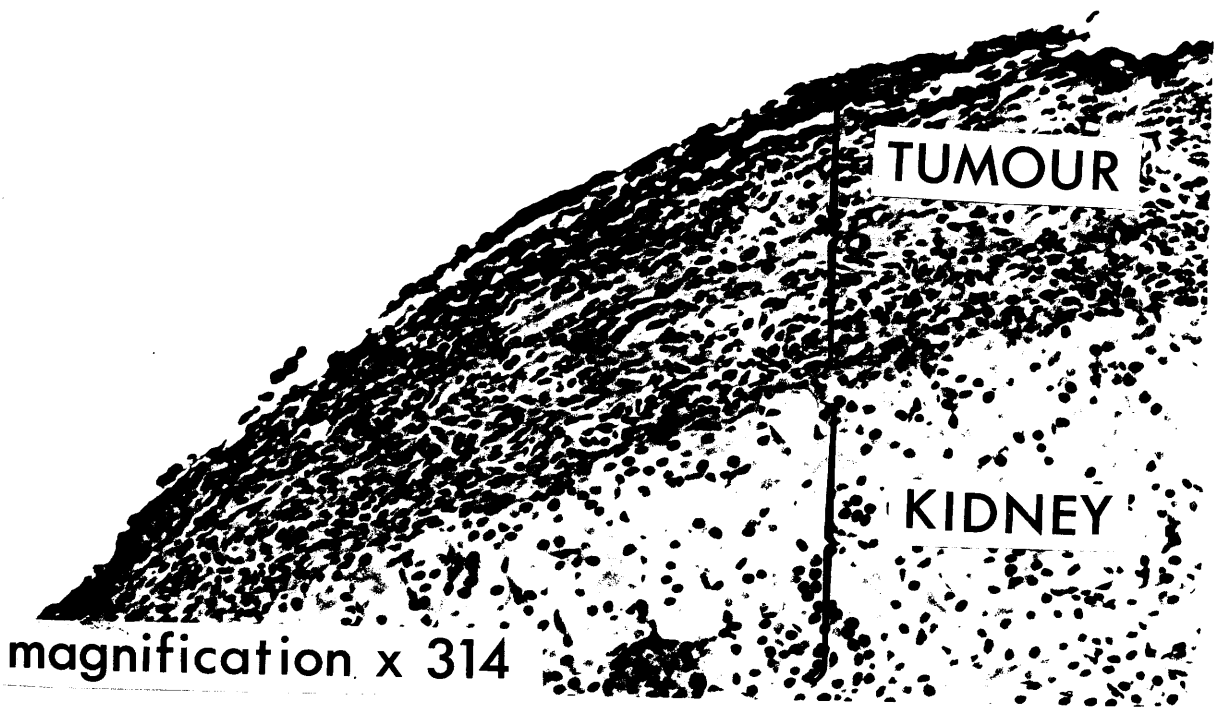


Figure 64 (I-IV): Haematoxylin and eosin stained sections of mouse kidney and Walker256 tumour implants on day 6 of the SRC assay.

Note the areas of :-

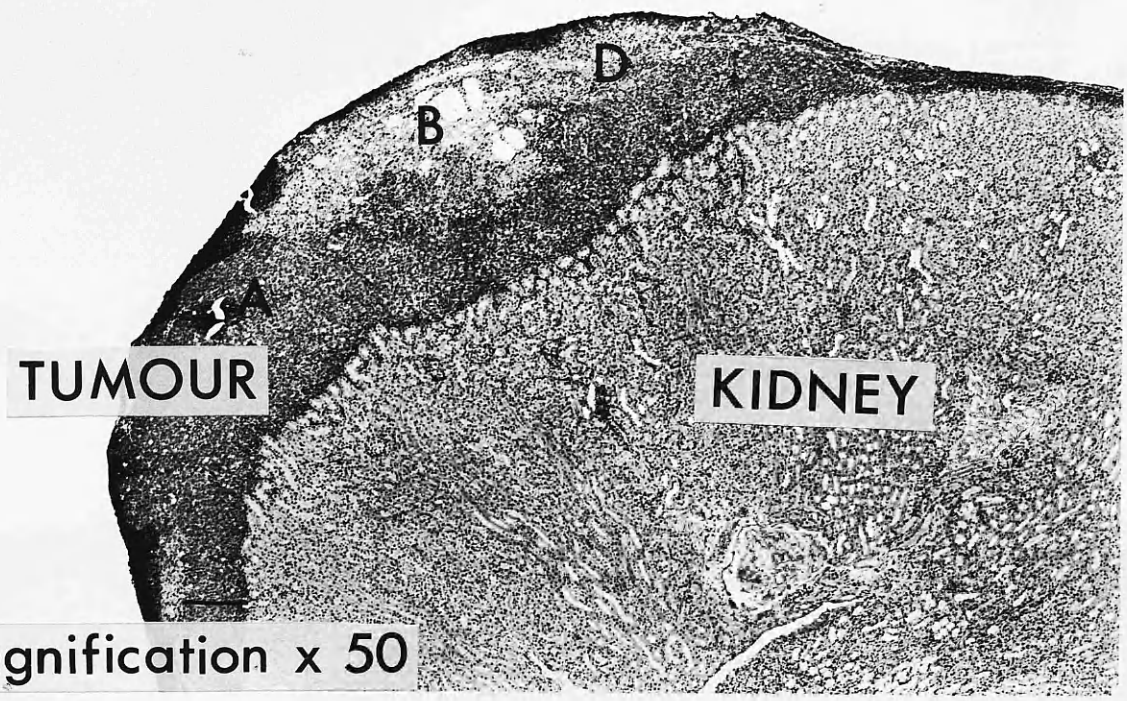
(A) lymphocyte infiltration

(B) necrosis

(C) fibrosis

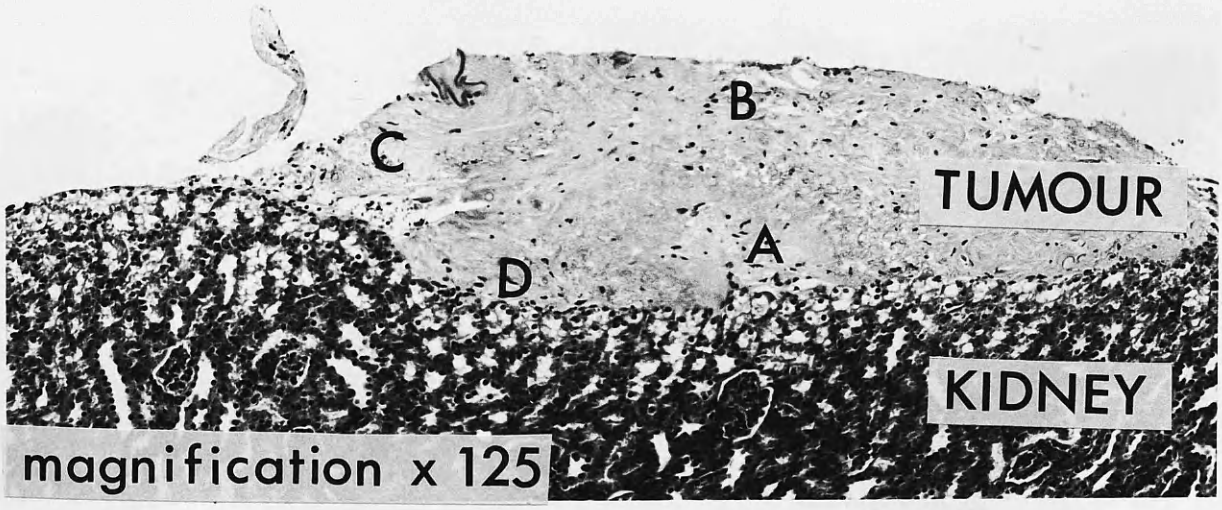
(D) viable tumour cells

I




magnification x 50

II

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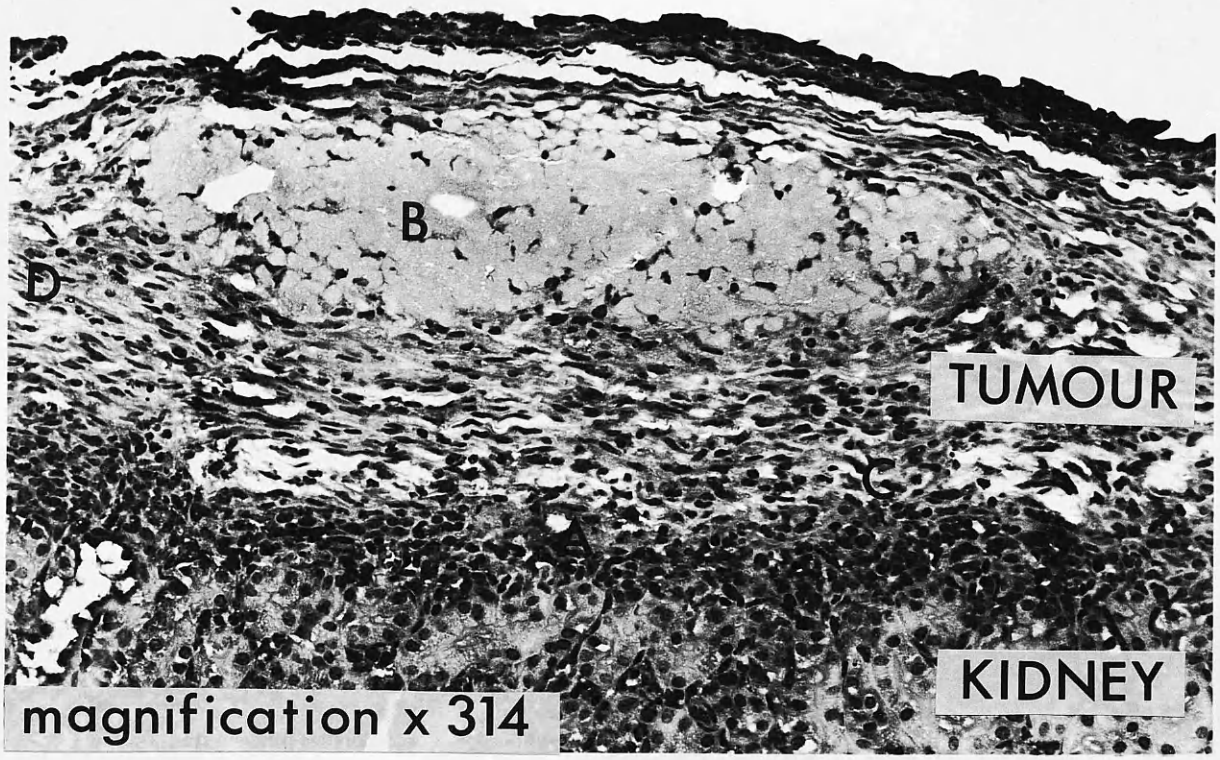
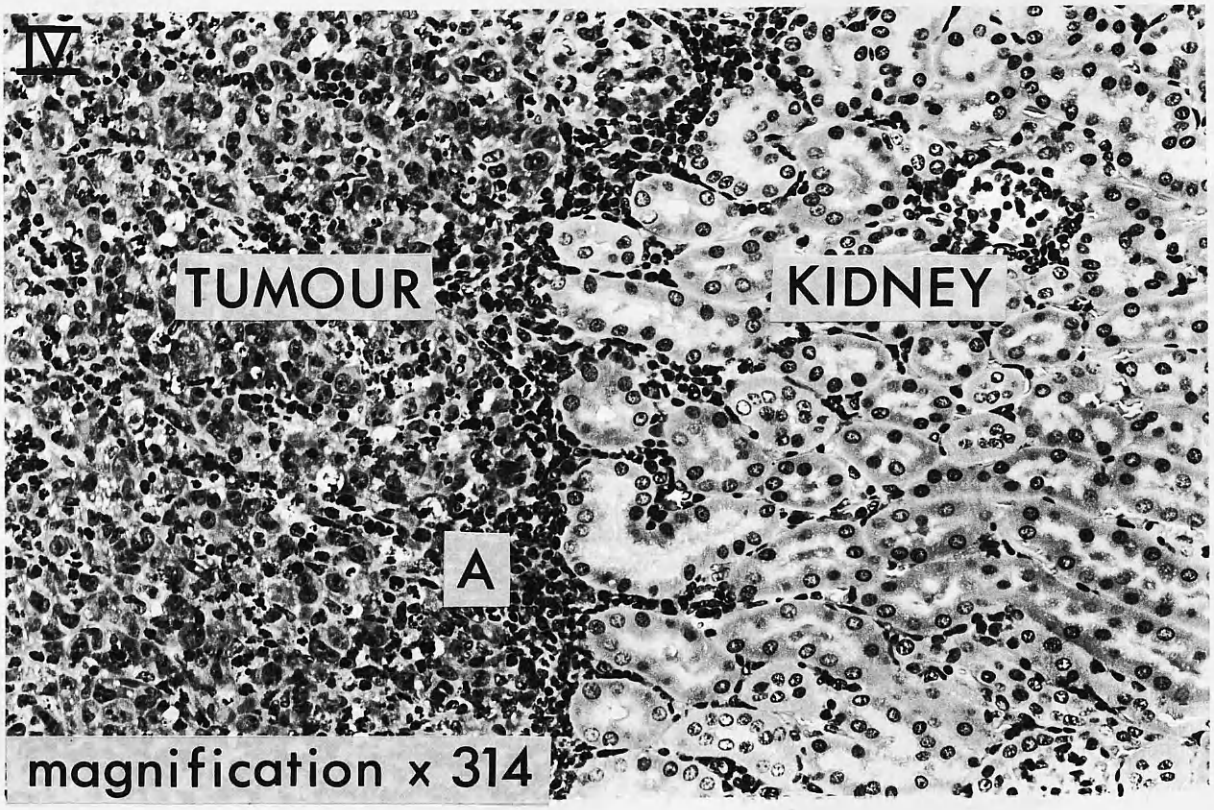
III


B
D
TUMOUR
C
A
KIDNEY
magnification x 314

IV


TUMOUR
KIDNEY
A
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random manner in each group, making assessment of the tumour size data qualitative rather than quantitative. In this group of animals the opportunity was taken to measure white blood cell counts, Section 2.3.15. Chlorambucil-treated animals had significant immunosuppression, fig.65, with the mean value only 25% of control. As expected, native LDL-treated mice showed no reduction in white blood cell number. The drug carried in LDL appears to have reduced the extent of immunosuppression with mean white blood cell count 81% of control(not significant, Wilcoxon) in CBE-LDL treated animals, while maintaining the same degree of tumour kill as an equivalent dose of native chlorambucil.

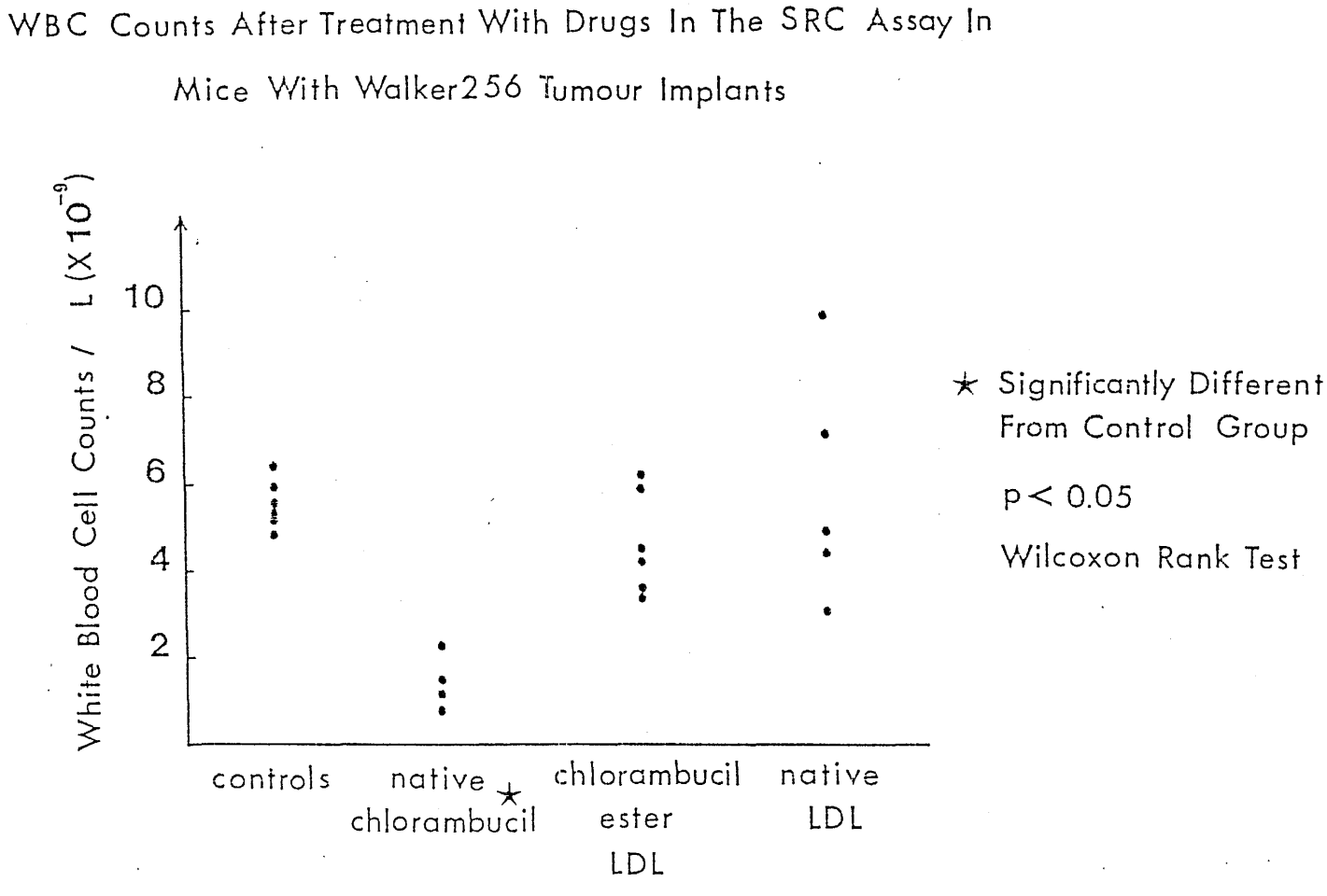


Figure 65: Effects of native chlorambucil, chlorambucil ester-LDL, and native-LDL on plasma white cell counts of mice treated with these agents in the subrenal capsular assay. Tumour size data for these animals are shown in figure 63.

# SECTION 3 : DISCUSSION

# 3. DISCUSSION AND CONCLUSIONS

## 3.1. LDL RECEPTOR ACTIVITY IN VIVO

Numerous *in vitro* experiments[12-22] have shown that LDL may be a useful vehicle for the selective delivery of drugs or radioisotopes to cancer cells. However, until recently, little was known of the likely degree of specificity which LDL would have as a selective targeting vehicle *in vivo*. Growing tumour cells, like other rapidly dividing cells, require a considerable amount of cholesterol for membrane synthesis. In many cell types it has been reported that LDL, the major cholesterol-carrying lipoprotein in human plasma, is the preferred source of the sterol when available.[25] Alternatively cellular cholesterol requirements are met by de novo synthesis from acetate[25]. In this study a double-isotope procedure was used to assess relative receptor-mediated and receptor-independent catabolism of LDL by individual organs and tumours in mouse and rat models. This approach has been shown previously[67,93,94] to be adequate to detect changes in LDL catabolism induced by diet or lipid-lowering drugs. In the animals examined in the present study, as in the human, the liver and adrenal gland are by far the most active in assimilating LDL via both receptor and non-receptor routes. Because of its size, the liver is responsible for the catabolism of a large proportion of plasma LDL and so plays a dominant role in LDL metabolism in control and tumour-bearing animals. Nevertheless, tumour cells were active in taking up LDL. The Mac-13 tumour exhibited substantial receptor-mediated uptake of the lipoprotein (fig.19) and was second only to liver in activity. This was also true of the polyoma virus-induced adenoma in mice (fig.21). Colonic tumours generated by administration dimethylhydrazine[193] to rats was considerably more active in LDL assimilation via receptors

than any other tissue examined, including liver (fig.22) and Walker-256 rat tumour was also active in receptor-mediated LDL catabolism, assimilating by this route almost as much LDL as liver (fig.23). In the experiments using hamster pancreatic tumours (fig.26) only total LDL uptake (i.e. uptake by both receptor-independent and receptor-mediated routes) was measured. Consequently no definite conclusion can be drawn regarding the receptor activity of these tissues. Here the tumours' acquisition of LDL was modest in comparison to the relatively high levels of LDL assimilation in other hamster tissues. In the rat model (fig.22), total LDL uptake by colonic tumours was surpassed only by liver and spleen. The high activity of the spleen probably reflects the removal of denatured tracer material, which is mediated by cells of the R.E. system. Although this corresponds to less than 5% of the injected dose, the organ appears to be particularly avid since the denatured tracer is directed specifically at it.

Thus all of these tumours, although of varying aetiology, exhibited substantial LDL catabolic activity and are potentially susceptible to LDL-mediated cytotoxic therapy. However, specificity remains a problem and it is likely than the liver and adrenal glands would suffer during chemotherapy, although it should be noted that the presence of tumour in mice appeared, by some undefined mechanism, to reduce receptor-mediated hepatic uptake of LDL (tables 5 & 7). Certainly, there were no macroscopically apparent metastases in the animals' livers which could have contributed to this effect. Consequently, it is necessary to protect these organs from the toxic effect of LDL therapy by reducing their receptor expression. This is possible since the number of receptors expressed by the cells of each organ can be influenced by changing the intracellular pool of cholesterol. Kovanen et al[40] have shown that, in mice, adrenal LDL receptor activity is

markedly activated by injections of ACTH and, in the dog, it has been demonstrated that hepatic receptor activity can be suppressed by dietary supplements of cholesterol[196] or bile acids[195-197]. However, in the groups of mice examined here there was little impact of cholesterol feeding on receptor-mediated hepatic LDL uptake (table 13) despite a substantial increase in plasma cholesterol in response to diet (table 6). Interspecies variation in the ability of cholesterol to inhibit receptors is well recognised. For example, dietary sterol supplementation is ineffective in changing rat plasma cholesterol levels since the bulk of excess sterol is excreted in the bile[29]. In man[215] on the other hand, the primary response appears to be to export cholesterol into the plasma as LDL rather than retain it in the liver and modulate receptor activity; and bile acid feeding in the mice used in the present investigation was more successful in reducing the expression of hepatic LDL receptors than was dietary cholesterol supplementation (table 14). This parameter fell by 25% ($p<0.05$, Wilcoxon rank test) in the control mice and by 17% ($p<0.05$) in the tumour-bearing animals. Presumably the reduced activity is consequent upon suppression of bile acid production from cholesterol, leading to an increase in the hepatic sterol pool and downregulation of the liver membrane receptors (fig.27). In rats bearing colonic tumours however, bile acid feeding did not produce a similar effect (table 15). Taurocholate-treated tumour-bearing mice also showed reduced receptor-mediated adrenal LDL uptake, a response which was not observed in the control group (table 14). This unexpected phenomenon, like the reduced hepatic receptor activity seen in tumour-bearing mice, presumably reflects a change in lipoprotein metabolism which is secondary to the presence of tumour.

LDL uptake by the adrenal gland was successfully suppressed by the

administration of hydrocortisone (table 14). In the control and tumour-inoculated mice, injection of the steroid lowered adrenal LDL receptor activity by 48% and 30% respectively ($p<0.05$, Wilcoxon rank test, in both cases). In rats with colonic tumours (table 15) hepatic uptake of LDL was reduced by 40% ($p<0.05$, Wilcoxon rank test) after hydrocortisone administration and adrenal receptor activity was lowered by 40% and 30% (both $p<0.05$) in control and tumour animals respectively.

When hydrocortisone was combined with taurocholate feeding, both hepatic and adrenal LDL uptakes were lowered in the mice without concomitant lowering of the activity of the tumour (table 14). This regimen also successfully lowered adrenal uptake in the tumour-bearing rat model (table 15). It is interesting to note that the ovaries of rats receiving hydrocortisone alone or with bile salts had significantly suppressed LDL receptor activity. This was not merely a secondary response to tumour presence since it also occurred in the matched control group (table 15). The mechanism for this effect is unclear.

## 3.2 EVIDENCE FOR LDL UPTAKE IN TUMOURS USING CUMULATIVE MARKERS OF LDL CATABOLISM

LDL labelled with radioiodinated cholesteryl linoleoyl ether[106] or radioiodinated cellobiose-tyramine[105] was used to obtain a cumulative index of the extent of catabolism of the lipoprotein by the tumour. These tracers have been reported to be internalised by the cell in an identical manner to native LDL, but once inside lysosomes they resist degradation and are retained there. Less than 35% leaks back into the circulation over a period of 24 hours, in contrast to the immediate release into the plasma of iodotyrosine from conventionally labelled

I-apo B-LDL. In MAC-13-inoculated mice, the tumour as a whole was second only to liver (fig.24) in assimilating labelled LDL. Hepatic uptake of I-CE-LDL by the receptor pathway was again suppressed by the presence of tumour ($p<0.05$, Wilcoxon rank test), as was originally observed using I-apo B-LDL as tracer (fig.19). LDL receptor activity in kidneys and adrenals was also suppressed in the tumour-bearing animals.

The uptake of LDL via both receptor-dependent and receptor-independent pathways by rat colonic tumours (fig.25) and hamster pancreatic tumours (fig.26) was measured using $^{125}$I-CT-LDL. In both models tumours were highly active in this regard, although several normal tissues in the hamster model showed a similar response.

In assessing the above results, it must be borne in mind that heterologous, human, LDL was used in the animal models because of its ease of preparation and known biological properties. Such an approach may produce different results than might be obtained if homologous LDL were used, but these should be of minor quantitative significance.

## 3.3 DRUG INCORPORATION INTO LDL

The LDL particle consists of an apolar core of cholesteryl esters and triglycerides surrounded by a phospholoid monolayer containing free cholesterol and apoprotein B[27,34]. This provides several sites for the insertion of drug molecules, depending on their lipid solubility. The core is by far the best site, since chemically sensitive drugs sequestered there would be well protected from serum hydrolases and the potentially damaging effects of an aqueous environment. Furthermore, the core has the capacity to store a large number of drug molecules. However, only very hydrophobic agents are suitable for inclusion in this domain. A second site for drug intercalation is the phospholipid monolayer. Agents which have both polar and lipid soluble components may partition here, between the apolar core and the aqueous environment, but in this location, they are less well protected from serum hydrolases and water and further, may be able to stimulate the immune system to remove the drug-LDL complex from the circulation. A third approach would be to attach the drug covalently to the apoprotein moiety of the LDL particle[17]. However, there are a number of immediately obvious drawbacks here since:-

1) Drug attachment to apo B may distort the protein and reduce or abolish completely its binding to LDL receptors.

2) The drug-LDL link must be successfully hydrolysed to liberate the active agent within the cell.

3) The potential of each LDL particle for storing

drug in this manner is considerably less than the capacity of the core.

4) Drug carried in this way is exposed to water and serum enzymes.

5) Distortion of apo B in linking the drug molecules may render the complex susceptible to removal by the immune system.

Several procedures have been detailed as adequate for the incorporation of trace amounts of apolar substances into the core space of the LDL particle. However, only Kreiger's method[156], which, in effect, produces "synthetic LDL", has been shown to insert significant amounts of exogenous substances into the lipoprotein. "Kreiger particles", although they react like native LDL in vitro are rapidly removed from circulation by cells of the R.E. system when injected in vivo. This method however is still potentially useful since Masquelier et al[147] have modified Kreiger's method to produce an LDL species containing a lipophilic anthracycline (AD-32) which is removed from mouse plasma at a similar rate to that of native human LDL.

Our attempts to incorporate drugs into LDL focused on using native human LDL as acceptor in order to reduce potential R.E. clearance of the drug complex in vivo. Drugs were either partitioned into the lipoprotein from concentrated drug solutions in various aqueous buffers (anthracyclines) or by exchange from HDL or micellar lipid suspensions (chlorambucil cholesteryl ester).

## 3.4 DAUNOMYCIN AND ADRIAMYCIN

Daunomycin was easily intercalated into LDL following the method described by Iwanik et al[23]. In solutions of high daunomycin concentration, an average of 190 drug molecules were able to diffuse into each LDL particle (table 21). The resulting complexes retained their specific binding ability, 99% of the apo B being precipitated on interaction with LDL antibodies. Furthermore, daunomycin-LDL uptake into P388 leukemic cells was shown by these authors to be subject to competition from native LDL but not HDL. Interaction of the daunomycin-LDL complex with DNA resulted in the quenching of only 45% of the fluorescence of the drug[23]. The size of the DNA molecule precludes its entry into the core space of the particle and it is likely that only drug molecules sequestered on or near the surface of the LDL particle are susceptible to quencing by this probe. A further 13% of the fluorescence was quenched by potassium iodide[23], since this agent has access to parts of LDL that DNA is unable to reach. Thus Iwanik et al[23] deduced that around 50% of the daunomycin is loosely held on the surface, with the remainder located in the hydrophobic core of LDL. This was further confirmed by the "leakage" of approximately 40% of the daunomycin from LDL during centrifugation at a density of 1.063 Kg/L.

Despite the structural similarities between daunomycin and adriamycin (fig.11), the latter is much more reluctant to partition into LDL. Adriamycin, when incubated with the lipoprotein under conditions similar to those used for preparing daunomycin-LDL, caused its immediate aggregation, a reaction which has been observed by others[23]. Consequently, alternative approaches were considered in the preparation of adriamycin-LDL, since this complex has a much larger

potential spectrum of clinical use than daunomycin-LDL. LDL is known to be less prone to aggregation when stored at low concentration in solutions of high ionic strength. For this reason, many of our experiments used the pooled LDL fractions obtained directly from the rate-zonal centrifugation[185]. Nevertheless, aggregation still remained a problem, possibly due to the formation of an insoluble complex between the drug and cholesterol on the surface of LDL. Brown et al[202] used a sodium chloride buffer containing 10% DMSO (v/v) in a procedure for incorporating labelled cholesteryl esters into LDL. This approach in our hands generated a complex in which a small amount of adriamycin became associated with the lipoprotein. Comparison of the drug incorporation at pH7.2 and pH 10 showed neutral conditions to be better than basic by a factor of 2 (table 20). At pH.10 there was a larger degree of drug degradation (fig.51) although only native adriamycin was incorporated into LDL to any detectable level, i.e. none of the drug's metabolites were as lipophilic as or more lipophilic than the native drug. Tucker et al[203] investigated the use of surfactants for enlarging the structure of the LDL particle. Rapid increases in the Stokes radius of the LDL particles were observed after exposure to a variety of surfactants but the incorporation of adriamycin into these surfactant-enlarged LDL particles was not significantly higher than that obtained in the absence of surfactants (table 20). It would appear therefore that steric factors are not the major cause of the low adriamycin concentration observed in adriamycin-LDL preparations. It is more likely that the hydrophobic environment provided by the LDL core is not conducive to sequestration of the drug. Since the only difference between adriamycin and daunomycin is a hydroxy substituent on C14 in the former, it is obvious that this highly hydrophilic group has a dominating effect on the lipid solubility of the drug. Better incorporation might be achieved if this hydroxy

group were selectively esterified by, for example, a fatty acid residue. Vitols et al[21] have esterified adriamycin with linoleate at this position and have coupled N-retinoyl leucine to the amino group of the sugar. 100-200 molecules of this derivative became incorporated into each LDL particle although the authors do not comment on its aggregant effect on LDL in solution.

## 3.5. CHLORAMBUCIL

The chemical structure of chlorambucil (fig.11) was suitable for esterification with cholesterol. This was achieved by a single reaction, using dicyclohexylcarbodiimide as a coupling agent. The object of this derivatisation was to produce a molecule which would be more amenable than the original chlorambucil molecule to incorporation into LDL. The benefits of synthesising a cholesteryl ester were three-fold:-

1) The polarity of the carboxylic acid group was markedly reduced, making the drug-ester more hydrophobic than the native drug

2) Cholesteryl esters are the native occupants of the core of LDL and the subsequent incorporation of chlorambucil cholesteryl ester there should produce minimal disruptions of the structure of the lipoprotein

3) There exists a cholesteryl ester transfer protein in human plasma which mediates the

exchange of cholesteryl esters between LDL and HDL and which may be utilised for incorporation of the drug-cholesteryl ester into LDL.

Stoffel et al[190] have described a method for loading labelled cholesteryl esters and fatty acids into HDL by exchange from cholic acid/lipid micelles. Taking this process a stage further, iodinated cholesterol linoleoyl ether was incorporated into LDL by exchange from HDL, utilising the cholesteryl ester transfer protein from human plasma[191]. Initially this was the method used for chlorambucil ester incorporation, but the resulting LDL complex had virtually no cytotoxic activity (fig.46). This lack of cytotoxicity had two obvious possible causes: either the cells did not have adequate receptor expression; or the drug was being inactivated during the incorporation procedure. The former explanation was ruled out since the glioma cell line used, G-CCM, had already been reported[20] to have high receptor expression under similar conditions. On examination of the buffers used in the loading process tris(hydroxymethyl) methylamine (tris) was identified as a probable culprit for the drug inactivation. This nucleophile was likely to be attacking the carbon attached to chlorine on the chloroethyl side chains of chlorambucil, releasing chloride and rendering the drug inactive. Drug activity was conserved by replacing tris buffer with phosphate buffer, although the incorporation of chlorambucil ester remained low (table 16). Later, drug ester was exchanged from the cholic acid/lipid micelles directly to LDL, with an increase in the overall drug recovery (table 16) and no obvious effects on the integrity of the lipoprotein. By following the cholesteryl chlorambucil ester incorporation by HPLC at each stage of the cholate preparation, it was clear that a substantial amount of the

drug had been degraded over the course of the experiment. Studies on the degradation reaction surprisingly showed no free chlorambucil in the solution. Consequently the process appeared not to involve simply a hydrolysis of the ester bond between chlorambucil and cholesterol. At first the degradation was attributed to enzymes present in the crude preparation of cholesteryl ester transfer protein, but the extent of disappearance of the drug ester was higher in solutions containing no enzymes (fig.49) This is in accordance with the observation by Alberts et al[167] that free chlorambucil is degraded faster in water than in human plasma in vitro.

As an alternative to the modified Stoffel procedure (Section 2.3.2), which yielded LDL of low drug content, Craig's procedure (Section 2.3.4) for making drug/lipid microemulsions was examined. This method was originally devised for making LDL of defined cholesteryl ester composition and involves preliminary preparation of drug-lipid microemulsions. The process is much shorter than the Stoffel method, thus reducing the degree of drug degradation. Up to 30% of the drug used was recovered in LDL after exchange from these microemulsions and the drug-LDL complex was easily separated from them by micro-filtration and centrifugation. It is interesting to note that, on storage at 4°C, a portion of the drug within the LDL complex was degraded rapidly over the first 24 hours and thereafter degradation was much less significant (fig.50). This biexponential drug decay points to the possibility that chlorambucil cholesteryl ester molecules are located both on the surface (the rapidly degraded component) and in the core of the LDL particle. Assuming that this deduction is correct, core-located drug molecules obtain protection from the aqueous environment when sequestered in LDL under the conditions used. Native chlorambucil decays with monoexponential kinetics[204], leaving only 10%

of the original drug after 6 hours incubation at pH7 and 25°C, compared with recovery of 50% of the drug in LDL after 24 hours of incubation under similar conditions, a further indication of the protective environment provided by LDL.

## 3.6 IN VITRO TESTING OF DRUG-LDL COMPLEXES

The sensitivity of tumours to various antineoplastic agents *in vitro* has long been studied as a possible indicator of their *in vivo* response to proposed chemotherapeutic regimens. The relative *in vitro* colony forming ability of treated and untreated cell samples is commonly used as a method of assessing drug sensitivity[207]. However, this method is not always readily applicable to primary cultures of human tumours as a result of their low cloning efficiency and slow growth rate. Furthermore, assays based on cell proliferation ability alone require extensive cell counting and are impractical for large numbers of samples. As an alternative to clone counting, cellular metabolic activity may be measured after exposure to drugs. Protein synthesis is one biosynthetic event which has been identified as a fundamental metabolic process, without which the cell cannot survive. Since leucine is an essential amino acid, the cell must acquire it from the surrounding medium and protein synthesis is most unlikely to occur without incorporation of this molecule. Thus $[^3H]$-leucine may be added to the culture medium and its extent of incorporation will be indicative of the metabolic activity of the cells. Consequently, the absence of intracellular $[^3H]$-leucine several hours after drug removal will almost certainly correlate with cell death. A microtitre drug sensitivity assay based on such an approach has been described for HeLa[208] and human glioma[207] cell lines. This procedure has several advantages over the aforementioned clonogenic assays,

particularly with regard to the large numbers of replicates which can easily be handled. The relatively long drug exposure time ensures that the majority of the cells in culture are able to pass through the entire cell cycle at least once during this period. Consequently, even phase-specific drugs may be adequately assessed by the procedure.

Intermediate between monolayer culture systems and in vivo tumour models is the spheroid model[210] of cell culture. In this system, cells are grown in multi-layer 3-dimensional arrays which are able to mimic better the arrangement of cells in the intact tumour. The ability of drugs to penetrate the microarchitecture of tumour cell layers is a variable which the monolayer system completely ignores. This distribution factor can be tested, to some degree at least, in the spheroid culture system. Drug penetration is, of course, of major importance in the treatment of tumours, since the deeper the drug is able to move into the tumour, the larger the fraction of tumour cells which will be killed.

Since relatively high concentrations of daunomycin were able to be incorporated into LDL, this complex was tested on a variety of cell culture systems. In monolayer experiments, using human glioma cells (fig.54) and HeLa cells (fig.58) in the microtitre assay and L-DAN cells (table 22) in the clonogenic assay, the targeting of daunomycin within LDL did little or nothing to increase the cytotoxicity of the drug. There are two obvious reasons for this lack of enhancement of daunomycin cytotoxicity:-

1) Drug accumulation within the cell is not increased when daunomycin is carried within LDL either because the daunomycin influx/efflux mechanism is altered by the change in the initial metabolic treatment of

the drug following internalisation via the LDL pathway or because the LDL pathway in these cells becomes saturated at relatively low daunomycin-LDL concentrations; alternatively

2) Daunomycin may be denatured during its internalisation with LDL, before it can successfully carry out its cytotoxic action.

Iwanik et al[23] in their experiments with daunomycin-LDL on human leukemic cells found that drug accumulation within the cells was higher when the drug was administered within LDL, but, unlike the native drug, which was located, as expected, mostly in the nuclear fractions of the cells, daunomycin delivered via LDL was more abundant in the microsomal-mitochondrial-lysosomal membrane fractions. This observation lends credence to the possibility that the drug had undergone a metabolic transformation before its cytotoxic action could be achieved. Of course, drug and LDL uptake and membrane fractionation experiments would have to be carried out on the cell lines used in our work to evaluate the validity of the above explanations. The results of the spheroid experiments were more favourable, daunomycin-LDL being more effective than native drug on the spheroids both when measured by spheroid growth delay and in clonogenic assay after disaggregation. Fluorescence microscopy on spheroid cross-sections after drug exposure (fig.56,57) showed that daunomycin had penetrated further into the tumour structure when carried within LDL. This improvement in drug distribution obviously had a beneficial effect on the growth delay of the spheroids treated with daunomycin-LDL.

Adriamycin was more successful as a cytotoxic agent when administered

within LDL to HeLa cells in the microtitre assay (fig.59). The action of this drug differs from daunomycin in that it has well-documented cytotoxic effects in the cytoplasm[130] and at the plasma membrane, as well as at the nucleus[172]. Consequently the change in drug distribution seen when daunomycin was carried in LDL[23], if it occurs in a similar manner with adriamycin-LDL, may not decrease the cytotoxicity of adriamycin so significantly, or may even increase it. Lysosomal degradation of adriamycin is not a problem with free or LDL-borne drug since it has already been shown that this anthracycline is resistant to lysosomal enzymes[216]. There is also the possibility that adriamycin uptake into HeLa cells is low in comparison to drug uptake via LDL.

Chlorambucil cholesteryl ester-LDL was by far the most successful of the complexes tested in the glioma microtitre assay, with the $ID_{50}$ of the drug lowered by a factor of 200 when it was targeted in LDL (fig.60). As already mentioned, encapsulation of the chlorambucil ester within LDL appears to reduce the hydrolysis rate of the drug and this may result in the availability of a comparatively higher concentration of active drug inside the cell over the period of drug exposure. Influx of nitrogen mustards has been shown[217] to be an active transport process in human leukemic and normal lymphoid tissue. Chlorambucil-resistant cells of Yoshida ascites sarcoma have been reported[218] to accumulate 50% less drug than their sensitive counterparts, under identical conditions. Therefore the cell's ability to internalise chlorambucil may have a profound effect on cellular susceptibility to this agent, although in the case of the Yoshida ascites sarcoma[218], other differences were also observed between the sensitive and resistant sublines. Targeting of chlorambucil within LDL in this way may then conceivably increase the drug's cytotoxic effects simply by increasing

drug influx, so exposing tumour to high drug concentrations for a longer period. It has also been postulated[219] that, once inside the cell, chlorambucil immediately associates with lipid-protein complexes whence it is released at a slow rate prior to carrying out its alkylating action. Thus, administration of the drug within LDL may effectively insert the drug into the cell at a stage further along its normal metabolic pathway. Conversely, the distribution of daunomycin within subcellular compartments is markedly different when it is administered within LDL, and this difference may well account for the observed decrease in cytotoxicity of daunomycin-LDL compared to the native drug.

## 3.7 IN VIVO TESTING OF DRUG-LDL COMPLEXES - THE SUBRENAL CAPSULAR ASSAY

Cell experiments are of value in predicting the likely sensitivities of tumour cells to antineoplastic drugs, but they do not take into account the effects of other organs on the distribution, metabolism and excretion of the drug. The object of using drugs within a targeting vehicle is to favourably alter the pharmacokinetic disposition of the drug so that tumour cells are harmed considerably more than normal tissues. This property can, obviously, only be tested significantly using an *in vivo* tumour system. Also, drugs requiring *in vivo* activation may only be adequately assessed using an animal model unless the active agent can be isolated. There is still considerable controversy over the best type of animal model to use for experimentation with cytotoxic drug regimens and over the relevance of the results obtained with regard to extrapolation to the human situation. In many of the models currently in use, drugs are administered very early in the growth of the tumour, often less than 24 hours after implantation, whereas in the clinical situation the neoplasm, and often metastases,

are usually long established before treatment commences. Although transplantable animal tumours are, in general, much faster growing than human tumours, the administration of drugs to the animals occurs undoubtedly at a relatively early stage in the progression of the neoplasm. Also, doses up to the $LD_{10}$ (the lethal dose which kills 10% of the animals which receive it) are often used against animal tumours while similar doses cannot be used in humans.

In order to test drug-LDL complexes in vivo the mouse model first described by Bogden et al[212] was employed. This model, the Subrenal Capsular (SRC) assay, involves implantation of tumour fragments under the renal capsule in normal immunocompetent mice. Bogden's model has several advantages over other tumour systems. The assay is rapid, giving a result in only six days, and other workers[220] have used the same procedure with a four-day timescale. Normal immunocompetent mice are used[218], so reducing the need for sterile conditions. Fragments of solid tumours are implanted thus maintaining the spatial relationship between tumour cells of the same, and different subpopulations, a relationship which may have a profound effect on drug penetration into the tumour and ultimately on overall tumour response to chemotherapy[221]. The drug administration protocol resembles that used in the treatment of humans, unlike the single drug dosage scheme employed in many animal models.

Results obtained here using daunomycin-LDL against a human primary gastric adenocarcinoma (fig. 61) appeared at first to be very promising with tumour showing an apparently lesser size increase after treatment with the complex compared to treatment with native daunomycin. However, histological examination of tumour sections revealed a major problem with the SRC assay: Bogden's original statement that the host

immune system did not invade the tumour implants to any great extent until after six days[212] was incorrect. The large, but variable degree of lymphocyte infiltration observed in all tumour groups made the interpretation of results, by measurement of tumour dimensions, very difficult. This was further complicated by the low numbers of viable tumour cells present at day 6 (fig.62), even in the control animals, and it was concluded that the apparent differences in tumour size were in fact differences in lymphocyte infiltration. It appeared therefore that daunomycin-LDL had a greater effect in suppressing the mouse immune response (and limiting immunocyte infiltration) than that observed after treatment with native daunomycin.

At only one day after tumour implantation, Cunningham et al[214] noted a considerable lymphocytic infiltrate in tumour graft sections. Others too[222,223] have observed immunocyte contributions to tumour size, although many have classed this contribution as insignificant, stating that it accounted for less than 20% of the total tumour size[223]. However, even at this relatively low level, the degree of lymphocyte infiltration may bias the results, as in the case of daunomycin-LDL above, since many of the common cytotoxic agents have marked immunosuppressive activity. Levy et al[220] have composed a scoring system whereby tumour size, lymphocyte infiltration, necrosis and fibrosis are among the factors taken into consideration. This is obviously a better system of assessment but it has the disadvantage of requiring more detailed and more laborious analysis of tumour sections before conclusions can be drawn. After consideration of the low number of viable tumour cells in the control animals and the significant degree of lymphocyte infiltration in the grafts used for testing daunomycin-LDL, and numerous other grafts of this type, the SRC assay was concluded[214] to be of no value in assessing the chemosensitivity of

human primary gastric tumours.

When animal tumours were used in the SRC assay a less extensive lymphocyte response was observed[214]. The rat Walker-256 tumour, originally a mammary adenocarcinoma, has been used in rat models for many years. When daunomycin and daunomycin-LDL were tested against this tumour, neither agent was able to limit its growth; but after treatment with chlorambucil ester-LDL or chlorambucil, Walker-256 tumour sizes were smaller than those observed in the control or native-LDL treated groups (fig.63). Microscopic evaluation of tumour sections showed large aggregates of viable tumour cells in each graft, but also present in random fashion throughout both control and test groups were variable proportions of necrotic, fibrotic and lymphocytic tissue, although lymphocytic infiltration was at a much lower level than that observed with the primary gastric tumours. The pattern of tumour necrosis, when present, was always the same. Those tumour cells in the layers furthest from the kidney surface were the first to die (fig.64). This implies that the tumour's demand for nutrients were in excess of the capabilities of the transport system within the graft, despite the concentration gradient between kidney and graft and the presence of simple blood vessels within the tumour. Perhaps this indicates another limitation of the SRC assay, although other groups [212,220] have reported high percentage increases in tumour size with no mention of necrosis.

So, interpretation of the results obtained from the SRC assay is not straightforward since factors other than tumour dimensions must be taken into account.

The object of using a vehicle for chemotherapeutic agents is not only

to deliver more drug to its site of action, thereby inducing more tumour cell kill, but also to decrease its toxic effects on normal tissues. A common dose-limiting side effect of cytotoxic therapy is immunosuppression and since the degree of lymphocyte infiltration into tumour in experiments with gastric tumours was affected by daunomycin-LDL , white cell number per mL of blood was measured following drug exposure in later studies. On comparison of the mean white blood cell counts in each treated group with the control group on day 6 of the assay (fig.65), only native chlorambucil was found to have a significant lowering effect. Thus, since chlorambucil and chlorambucil ester-LDL treated groups exhibited similar rates of tumour growth (fig.63), this drug, when carried in LDL, may be said to exert the same apparent tumour inhibition as an equivalent dose of the native drug, with less severe immunosuppression.

## 3.8 CONCLUSIONS

The consistent lack of success in designing specific drugs which will effectively destroy tumours without harming other tissues has prompted the development of new methods of delivery for the existing cytotoxic agents. Several drug-vehicle combinations[1-7] have been used with varying efficacy. Liposomes[6] fulfilled a number of the requirements of the ideal vehicle, but tumour specificity and extensive clearance of these particles by the R.E. system *in vivo*[8-11] remain serious problems. Following the observations of Ho et al[12] and later Gal and co-workers[13], a "natural liposome", low density lipoprotein, was proposed as a vehicle for cytotoxic drugs or imaging isotopes.

Numerous reports[12-22] on LDL assimilation by cultured human and animal tumour cells have been published, and the concensus of opinion is that neoplastic cells express higher LDL receptor activity than their equivalent non-neoplastic counterparts, although there have been instances of cultured cancer cells with defects in LDL metabolism[224-226]. Receptor-mediated uptake of LDL by solid tumours has been measured in several animal models (Section 2.2) and was found to be second only to liver and adrenal glands on a gram for gram basis. Thus LDL is not a totally specific carrier *in vivo*, but it may be sufficiently so to favourably increase the benefit:risk ratio of treatment. Specificity may be enhanced by reducing receptor-mediated uptake of LDL by liver and adrenal glands by administration of the appropriate agents (Section 2.2).

The efficacy of chemotherapy depends critically on effective penetration of the drug into tumour microarchitecture, although the investigation of this property *in vivo* has been largely neglected. In order to

enhance drug penetration, a number of lipophilic cytotoxic agents have been synthesised, and these are currently being evaluated[229]. As an alternative, existing drugs may be introduced further into the tumour within a lipophilic carrier. Daunomycin, encapsulated in LDL, showed more extensive penetration into tumour spheroids than did the native drug (figs. 56 & 57) or a number of lipophilic anthracyclines[228] and consequently induced a considerably longer growth delay in this model system. It would therefore be of interest to obtain a measure of comparative distributions of free and LDL-encapsulated drug in animal tumour models and correlate drug penetration with tumour regression.

A potentially useful property of this delivery system was illustrated[158] using daunomycin-LDL on anthracycline-resistant leukemic cells. Drug carried by the lipoprotein was able to penetrate the resistant cells much better than the native drug, suggesting that resistance was mediated by a change in the membrane transport of the daunomycin. Since acquired resistance is an all too common phenomenon following initial chemotherapy, the use of LDL as a vehicle for some cytotoxic agents may help to circumvent this problem. Further resistance is unlikely to be a result of blocking this route of entry since the tumour must rely heavily on LDL for the cholesterol it requires to maintain its rapid rate of membrane synthesis[16], and LDL has been cited as a necessary cofactor for the initiation of DNA excision repair in cultured human lymphocytes[227]. Thus the cells of tumours receiving, in LDL, a chemotherapeutic agent which attacks DNA, are likely to further increase their LDL receptor expression and this positive feedback may enhance the cytotoxic effects of the drug-LDL complex.

To-date, the extent of drug incorporation into LDL is probably too low for therapeutic use in humans. Perhaps additional improvements to core

replacement techniques, like Kreiger's method[156], may permit the synthesis of LDL with a high drug content, which behaves like native LDL in vivo. Alternatively the optimisation of methods of fusing drug-rich microemulsions with LDL[200], as used in producing chlorambucil-ester-LDL (Section 2.3.4) might produce drug-laden LDL particles which retain their native structure. However, it may be that the recently introduced "intensely potent anthracyclines"[228] will enhance the efficacy of existing procedures for making drug-LDL complexes for clinical use. These new drugs[228] are active at 1/10th to 1/100th of the doses commonly required for existing anthracyclines, and sufficient levels of these agents may therefore be incorporated into LDL simply by the diffusion techniques used previously (Section 2.3.5).

Another aspect of drug-LDL complexes which requires further study is the way in which the cytotoxic agents are distributed within the lipoprotein particle. For example, it appears that approximately 30% of the chlorambucil cholesteryl ester (Section 2.4.5) and 50% of the daunomycin[23] are located in the apolar core of the lipoprotein, while the remainder is held on the surface. The degree of protection afforded to core-located drug has not yet been documented, nor has the rate of leakage of drug molecules into plasma, but these properties are likely to be of importance in determining the efficacy of delivery of some cytotoxic agents.

Existing preparations of drug-LDL complexes, of course, require further testing in animal models, although unlike new cytotoxic agents, many of the toxic side effects of these drugs are already well known. The distribution of drug should, however, be dictated by the vehicle, and the major sites of toxicity may consequently be quite different from those of the free drug. Studies with radioiodinated LDL in animal

tumour models (Section 2.2) have shown that the adrenal glands and the liver are likely to be sites of dose-limiting side effects of drug-LDL therapy. Comparative dose response and tissue distribution data for free and LDL-encapsulated drug should be collected prior to testing any drug-LDL preparation in humans. Among the toxic effects which may be reduced by administering drugs within LDL is immunosuppression. Preliminary work with chlorambucil ester-LDL indicates that the complex may be significantly less injurious in this regard than the free drug.

In summary, LDL is a particle which exhibits a number of characteristics which make feasible its use as a drug vehicle in vivo. Tumours express relatively high LDL receptor activity and it is likely that serious damage to other organs can be avoided. Furthermore, LDL enhances drug penetration into the tumour and may be of use in overcoming drug resistance. It is not unrealistic to expect that, with the development of better incorporation methods for existing drugs and the synthesis of more potent or more lipophilic drugs, LDL will be successful as a targeting vehicle for the treatment of cancer in humans in the not too distant future.

## SECTION 4 : REFERENCES

## 4. REFERENCES


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