STUDIES ON THE FATE AND MODE OF ACTION OF LIPOSOMES AND ENTRAPPED AGENTS

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ABSTRACT

This work was undertaken in an attempt to assess the feasibility of using liposomes as carriers of anti-tumour agents. Entrapment of several chemotherapeutic agents (actinomycin D, bleomycin and asparaginase) in liposomes was carried out. At timed intervals after intravenous injection into rats, liposomes were found to transport their drug contents primarily to the liver and spleen.

In general, the distribution of entrapped drugs in tissues was different from that of similary injected free (unentrapped) drugs. Subcellular fractionation of the livers of injected rats showed that liposome entrapped agents mostly localised in the lysosome rich fraction, whereas free drugs localised in the nuclear fraction. Lysosomal localisation of liposomal drugs posed the question as to whether drugs retained their biological activity in the presence of lysosomal enzymes. However, the ability of entrapped agents to suppress DNA and/or RNA synthesis in the liver of partially hepatectomised rats following intravenous injection suggests that either the drugs are unaffected by their passage through the lysosomes on their way to the nucleus or that some other mode of uptake (non-lysosomotropic) by liver cells was involved.

The interaction of liposomes with plasma proteins was studied to establish whether the carrier itself was affected by this biological environment prior to its uptake by the tissues. Electrophoretic mobility studies on liposomes in plasma suggested that a negatively charged component was interacting with the liposome surface irrespective of the initial surface charge of liposomes. This plasma component was identified as alpha-$_2$-macroglobulin in human plasma (alpha-$_1$-macroglobulin in the rat).

Furthermore studies on the association of liposomal lipid constituents with plasma components were carried out in rats.

Plasma lipoprotein fractions were assayed for the presence of liposomal cholesterol and egg phosphatatidylcholine at timed intervals after intravenous injection of liposomes. Results obtained suggest that liposomal cholesterol (but not phosphatidylcholine) is transferred to low and high-density lipoproteins.

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Corrections.

Page 52. line IO/II insert "(^{III}In) Indium chloride, (IO Ci/mg)."

page I03 line I6. The additional possibility that triton X-I00 might have released radioactivity from ASPase was not controlled for.

page I38 line 32 In the rat the transformation of VLDL to LDL seems to be a pathway of minor importance.

page I64 line I3 Urea does not alter electrostatic interactions specifically and so the conclusions drawn may not be valid.

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ABBREVIATIONS.

Note: the terms "liposome" and "vesicle" are used interchangably throughout this thesis. Standard chemical symbols are not included.

alpha-1-M	: Alpha-1(2)-macroglobulin
alpha-2-M	
Ab	: Antibody
ACT-D	: Actinomycin D
AP	: Aqueous phase of the liposome
APB	: Aqueous phase buffer
Apo-	: Delipidated lipoprotein
apo-A,B,C, etc	: Individual proteins of the lipoprotein series - (see table 3:1)
Ara-C	: 1-beta-D-arabinofuranosylcytosine
ASPase	: Asparaginase (EC : 3.5.1.1)
B/H	: Bolton and Hunter method (reagent)
BLM	: Bleomycin
^{111}In-BLM	: Indium radiolabelled Bleomycin
^{125}I-BLM	: Radioiodinated (^{125}I) Bleomycin
BLM-ICL(ICL-BLM)	: Bleomycin radiolabelled by iodine monochloride method.
BLM-B/H(B/H-BLM)	: Bleomycin radiolabelled by Bolton and Hunter method (Bolton and Hunter, 1973).
BSA	: Bovine serum albumin
C_1-C_9	: Components of the "classical" complement pathway
6-CF	: 6-carboxyfluorescein
CHOL	: Cholesterol
CHYL	: Chylomicrons
CRP	: C-reactive protein
d	: Density (g/ml)
d > x < y	: Density between x and y
DMF	: Dimethylformamide
DMPC	: Dimyristoylphosphatidylcholine

DNA	: Deoxyribonucleic acid
DPH	: 1,6-diphenyl-1,3,5-hexatriene
DPPC	: Dipalminoylphosphatidylcholine
DPPG	: Dipalmitoylphosphatidylglycerol
EC	: Hepatic sinusoid endothelial cells
E-CHOL	: Cholesterol ester
EDTA	: Ethylenediaminetetraacetic acid
EPC	: Egg-yolk phosphatidylcholine
Fab, Fc	: Fragments derived from IgG (q.v.) after treatment with papain: Fragment capable of Antigen Binding and Crystallizable Fragment.
FCS	: Foetal calf serum.
g	: Gravitational constant
G_1 phase	: The period within the cell cycle between mitosis and the onset of DNA synthesis.
G_2 phase	: The period within the cell cycle between the completion of DNA synthesis and mitosis.
HDL	: High density lipoprotein
HDL_1, HDL_2, HDL_3	: Subdivisions of different densities within the HDL population reflecting maturation of HDL particle.
HRPase	: Horseradish peroxidase
HSA	: Human serum albumin
IE	: Immunoelectrophoresis
agg.Ig	: Heat aggre gated immunoglobulin
Ig	: Immunoglobulin
IgA, IgE, IgG, IgM	: Subclasses of Immunoglobulin
I/M.	: Intramuscular
INTase	: Mitochondrial enzyme (equivalent to succinate dehydrogenase); succinate-2-(p-iodophyl)-3-(p-nitrophenyl)-5-phenyltetrazolium reductase.
I/P	: Intraperitoneal
I/V	: Intravenous

KC	: Küpffer cell
LCAT	: Lecithin : cholesterol acyl transferase (EC : 2.3.1.43)
LDL	: Low density lipoprotein
LES	: Lifetime of the excited state
LP	: Lipid phase of liposomes
LPC, LPE, LPS	: Lyso derivatives of PC, PE, PS (q.v.)
LUV	: Large unilamellar vesicle.
MDP	: Muramyldipeptide.
ML	: Large granule (mitochondrial/lysosomal) subcellular fraction of the liver.
MLV	: Multilamellar vesicle.
N	: Nuclear subcellular fraction of the liver.
n.	: Number (of values in a mean)
NABGase	: Lysosomal enzyme : N-acetyl-beta-glucosaminidase.
OA	: Ovalbumin
o.d.	: Optical density
P	: Small granule (microsomal) subcellular fraction of the liver.
PA	: Phosphatidic acid.
PBS	: Phosphate buffered saline (see Materials)
PC	: Phosphatidylcholine
PCMC	: Hepatic parenchymal cells
PE	: Phosphatidylethanolamine
PI	: Phosphatidylinositol
pI	: Isoelectric point
PL	: Phospholipid
POPOP	: 1,4-bis-2(5-phenoxazolyl) benzene
PPO	: 2,6 diphenyloxazole
PS	: Phosphatidylserine
PVP	: Polyvinylpyrrolidone
RBC	: Red blood cells
rbf.	: round-bottomed flask

RES : Reticuloendothelial system

RME : Receptor-mediated endocytosis

RNA : Ribonucleic acid

m-RNA : Messenger - RNA

r-RNA : Ribosomal - RNA

t-RNA : Transfer -RNA

S : Solu ble (cytosolic) subcellular fraction of the liver.

S phase : The period within the cell cycle of DNA synthesis

SA : Stearylamine (octadecylamine)

SE : Standard error of the mean

S/G_2 : The boundry between the S and G_2 phases within the cell cycle.

St.dev. : Standard deviation

Sub.Cut : Subcutaneous

SUV : Small unilamellar vesicle.

T : Temperature

$T_{1/2}$: Plasma half-life

t_c : Thermal transition temperature

TCA : Trichloroacetic acid

TCA-ppt (pptable) : Material precipitated by TCA.

TLC : Thin layer chromotography

T-X100 : Triton-X100.

U/S : Unsonicated (handshaken) liposomes

UV : Ultraviolet light

VHDL : Very high density lipoproteins

VLDL : Very low density lipoproteins.

WH. : Whole liver homogenate prior to fractionation.

CHAPTER 1

INTRODUCTION

i) PURPOSE OF RESEARCH

The research described in this thesis was performed to assess liposomes as drug carriers. Particularly, this assessment was made in relation to the following questions :

i) where do liposome entrapped drugs go after intravenous injection?
ii) what is the subcellular location of liposome entrapped drugs ?
iii) are the liposome drugs active ?
iv) what happens to liposomes when they are in contact with the blood?
v) are liposomes themselves toxic ?

ii) DRUG CARRIERS

Seventy-five years have now passed since Erlich coined the phrase Chemotherapia specifica (Erlich, 1907). The carrier concept is only an extension of Erlich's original idea (Albert, 1965) of drug design; he envisaged the haptophore portion of the haptophore-toxophore drug molecule as the receptor binding moiety bearing the active toxophore group. The haptophore gave the toxophore the specificity for the target tissue (or in Erlich's case, a pathogenic organism) without affecting the rest. Since this is still the aim of chemotherapy, it follows that the efficient delivery of the drug to the target is as important as the molecular action of the drug itself. The discovery of toxophore moieties has progressed considerably in the last 75 years mostly as the result of empirical chemical synthesis of drug molecules and their congeners in attempts to interact these molecules with cell structures, receptors or biochemical pathways. However, the discovery of haptophore moieties is still in its teens : many of the molecules and structures used as drug carriers are no more than 15-20 years old.

Carrier molecules have now come to be regarded as macromolecular structures in or on which the active drug molecule resides. In the last ten years the literature on this subject has been steadily increasing, so that no attempt will be made here to give a comprehensive review of either drug carriers or even, more specifically, liposomes. Drug carriers in general have been the subject of two recent books (Gregoriadis, 1979; Juliano, 1980). Liposomes, as carriers of a variety of molecules, have been the subject of three books (Gregoriadis and Allison, 1980; Knight, 1981; Nicolau and Paraf, 1981); two symposia (Papahadjopoulos, 1978a; Tom and Six, 1980) and numerous reviews (Gregoriadis, 1973a; Ryman, 1975; Gregoriadis, 1976a; Poste et al., 1976; Tyrrell et al., 1976a; Fendler and Romero, 1977; Gregoriadis, 1977;

Finkelstein and Weissmann, 1978; Juliano, 1978; Kimelberg and Mayhew, 1978; Pagano and Weinstein, 1978; Papahadjopoulos, 1979; Ryman and Tyrrell, 1980; Gregoriadis, 1981).

Research involving liposomes as drug carriers will be reviewed in so far as it affects the work presented in this thesis.

iii) LIPOSOMES

The popularity of liposomes as drug carriers has been, in part, due to : i) their characteristic morphology, where a relatively impermeable lipid bilayer completely encloses an aqueous space, ii) their ability to encapsulate various solutes present in the aqueous and/or lipid phases during their formation, and iii) the simplicity of their preparation. In so far that an ideal multipurpose drug carrier exists, it may have to conform the specifications proposed by Gregoriadis (1977) . In terms of these specifications, it would seem that liposomes, whilst by no means ideal carriers, are currently best able to satisfy these requirements.

a) Structure and chemical composition.

Bangham et al. (1974) have described liposomes as : "All assemblages of phospho- and other lipids sustaining a biomolecular configuration but which by themselves do not require a mechanical support for their stability". The original work of Bangham and his co-workers (Bangham, 1968; Bangham, 1972; Bangham et al., 1974) and many others, has established the structure of the liposome which results when dried phospholipids are allowed to swell in an aqueous environment. The ordered assembly (more correctly known as a smectic mesophase) that spontaneously forms in excess water consists of multilamellar concentric bilayers; each lipid bilayer is separated from the adjacent bilayer by an aqueous space. The number of bilayers is variable but the innermost layer surrounds a large central aqueous core. During formation (rehydration of the polar head groups and the subsequent action of bulk water to form the smectic mesophase) there is unrestricted entry of water and solutes (drugs, proteins, etc) into the structure. But once the closed structure has formed the internal aqueous phase and solutes are trapped inside. If multilamellar liposomes (MLV) are subjected to ultrasonic irradiation (sonication) (Saunders et al., 1962; Hauser,1971) the closed structure is broken up and the lipid assemblies

are fragmented into smaller pieces which themselves reform small multilamellar or even smaller unilamellar vesicles (SUV). Again there is unrestricted entry of water during sonication. Thus, any water soluble compound can be entrapped within the liposome unless it interferes with liposome formation. In addition, lipid soluble substances that are not incompatible with liposome formation can be entrapped in liposomes by adding them to the lipids during liposome manufacture. Phospholipids usually form the major component of liposomes; those most commonly used (Gregoriadis, 1980a; Szoka and Papahadjopoulos, 1980) are : phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin, cardiolipin and synthetic lipids such as dipalmitoylphosphatidylcholine (DPPC). Phosphatidylethanolamine (PE) alone will not form the necessary closed structure (Papahadjopoulos and Miller, 1967) although it may be used in conjunction with the other lipids mentioned above. Lipid extracts of cell membranes (Bangham, 1968) can also be used for liposome manufacture.

The chemical composition of the lipids forming the liposome bilayers has important consequences for the physical state of the liposomes. Each phospholipid molecule contains fatty acyl chains of variable length and degree of saturation, depending on the source of lipid. Liposomes are normally formed at temperatures at which the fatty acyl chains are fluid (de Gier et at., 1968) i.e. above the reversible gel to liquid crystalline phase transition temperature (t_c). Below the t_c vesicles are "solid"; above it they are "fluid" but near or at t_c there is an enhanced release of entrapped molecules (Haest et al., 1972; Blok et al., 1976). Sonication below the transition temperature results in liposomes which "leak" through structural defects. These liposomes exhibit a pronounced tendancy to fuse near their transition temperature (Lawaczeck et al., 1975) although heating the liposomes to above the t_c anneals the defects.

The fluidity of the liposome can be modified not only by the chain length and degree of saturation of the hydrocarbon chains in the phospholipid, but also by the inclusion of sterol (most commonly cholesterol; CHOL) molecules (Chapman and Penkett, 1966). As the fluidity of the acyl chains is decreased the liposomes become more "solid" and the permeability of the bilayers to entrapped so-

lutes decreases (Scarper and de Gier, 1971). Once a drug is entrapped in the liposome it can only be liberated by leakage through the bilayers or disruption of the whole vesicle.
Solutes of high molecular weight or large size (e.g. proteins) which cannot pass through the bilayers are said to be "latent" and will remain in the liposome until the bilayers are disrupted (Sessa and Weissmann, 1970; Gregoriadis, 1976b). However, most drugs are smaller molecules and their leakage from liposomes will be dependent upon the lipid solubility and degree of ionization of the molecule at the existing pH. Consideration of these permeability factors has resulted in the introduction of a further variable into the liposome structure, namely the overall charge of the vesicle.

The net charge of liposomes can be easily altered by incorporating a charged lipid in the lipid phase. Addition of a long chain amine (usually stearylamine, n-octodecylamine : SA) results in a positively charged vesicle. Negatively charged vesicles can be prepared by the addition of PS, PA or dicetyl phosphate; whilst liposomes containing only PC, with or without CHOL, are neutral. The separation between the bilayers of pure egg phosphatidylcholine (EPC) in water is 2.75 nm (Bangham, 1968). This separation is determined by the balance of the repulsive forces between the layers, mainly electrostatic interactions between head groups and hydration forces of head groups, and attractive forces of van der Waal's type between the layers (Le Neveu et al., 1976). The introduction of a charged lipid increases the repulsive forces between the bilayers and thus increases the aqueous interspaces until a new equilibrium is established with the attractive forces. The internal volume of a charged vesicle is, therefore, capable of sequestering higher volumes of the aqueous phase than an uncharged vesicle (Bangham et al., 1967). However, there is a limit to the increase in repulsion between the bilayers and addition of more than 10 moles percent of a charged lipid has not been shown to give any further increase in the internal aqueous volume of the liposome (Bangham et al., 1967).
Moreover, the composition of the aqueous phase itself will have some effect on the internal volume. At high ionic strength, buffers will quench the repulsive effects of a charged lipid and so reduce the internal volume (Tyrrell et al., 1976a); a similar effect should also be found with drugs which are highly ionized.

A further advantage of adding a charged lipid can be seen from permeability studies of liposomes. Bangham et al., (1965) showed that liposomes were permeable to water and ions, others have shown permeability to non-electrolytes (de Gier et al., 1971). However, cationic liposomes are impermeable to cations whilst anionic liposomes are permeable. The permeability of hydrogen ions through all types of liposome bilayers is small (Scarpa and de Gier, 1971). Anion permeability is not altered by the charge on the lipid membrane. It is, therefore, possible to reduce the leakage of the ionized form of a drug by entrapping it within liposomes of similar charge; the electrostatic repulsive charges will retard the passage of the drug through the bilayers.

The total amount of the aqueous phase entrapped within a liposome preparation is a function of the amount of lipid used, since more lipid will form a larger number of aqueous spaces. In this respect multi-compartmental liposomes will entrap a higher proportion of a given volume of aqueous phase than single compartment (unilamellar) liposomes (Gregoriadis, 1973a; Fendler and Romero, 1976). However, the amount of aqueous phase entrapped per mole of lipid will be lower in multilamellar than in unilamellar liposomes (Papahadjopoulos et al., 1980a), mainly because of the favorable volume to surface area ratio. For chemotherapeutic purposes the total drug concentration is more important that the amount of drug per mole of lipid. For this reason, amongst others (ease of preparation), the multilamellar vesicle MLV has been used in this study.

In summary, MLV are suitable for the encapsulation of a variety of substances and can be made from a wide variety of lipid compositions. Of all the types of liposome preparation, they are the simplest to prepare and have been the choice for many experiments in drug delivery.

b) Preparation of liposomes.

During the course of the work described in this thesis a number of methods of liposome preparation were described and these have recently been reviewed (Szoka and Papahadjopoulos, 1980). These newer methods, whilst contributing to the versatility of the liposome as a structure, are not, in general, as easy to prepare as the "standard" method of Bangham (Bangham et al., 1974). Often further equipment is required for their preparation (Batzari and

Korn, 1973; Barenholz et al., 1979) or there is a risk that solvents or detergents will remain associated with the vesicle after manufacture (Kremer et al., 1979; Kagawa and Racker, 1971; Gerritsen et al., 1978); this would make them unsuitable for use in vivo.

The exception to this is the manufacture of liposomes of defined size either by the use of a novel permutation of the standard method (Szoka and Papahadjopoulos, 1978) or by use of filters (Olson et al., 1979) of defined pore size or other means (Van Renswoude et al., 1979) to produce homogeneous liposome preparations. Unfortunately, none of these methods was available during the research described here and the liposomes used were a heterogeneously sized population eluted from agarose gel columns (Huang, 1969).

The standard method liposome preparation (Bangham et al., 1974; Gregoriadis, 1976b).

A solution of phospholipids, cholesterol and other hydrophobic components (e.g. charged lipids, drugs) in organic solvent (usually chloroform) is rotary evaporated under reduced pressure to remove the solvent. The thin film of the lipids which is deposited on the flask walls can be removed by the addition of an aqueous solution followed by gentle shaking. During this time the lipid is rehydrated and swells, leaving the flask walls to form an aqueous suspension of large multilamellar vesicles (handshaken MLVs) of several microns in diameter. The length of time during which the lipid is allowed to swell and form the MLVs is important (Olson et al., 1979) and it is preferable to leave the suspension overnight. These large MLVs contain a high proportion of liposomes which will not absorb into agarose gel columns (but the more recent method of Fry et al., 1978, as modified by Layton and Trouet, 1980, might overcome this problem). So separation of unentrapped material from the liposomes is usually carried out by centrifugation followed by several washes. However, even centrifugation at 100,000 g for several hours will leave small unilamellar liposomes in the supernatant, and these will be lost on repeated washing (Roseman et al., 1975).

Smaller MLV preparations and small unilamellar vesicles (SUV) can be produced by sonication (Huang, 1969; Johnson et al., 1971; Mason and Huang, 1978) either with a probe or in a bath. Prolonged

sonication will eventually produce a homogeneous population of SUVs, the so-called "smallest possible" vesicles of 25 nm diameter (Huang, 1969). Sonication for shorter periods of time will produce a heterogeneous population containing MLVs and SUVs : this type of liposome has been used throughout this research. Again, it is advisable to leave the sonicated preparation for a few hours to ensure complete hydration and sealing of the liposomes.

The separation of liposomes from unentrapped material is most easily performed on agarose gel columns, pretreated with liposomes to prevent irreversible binding of liposomes to the beads (Huang, 1969; Huang and Thompson, 1974). The MLVs elute in the void volume as a turbid solution but a second peak of SUVs are optically clear and therefore more difficult to detect. MLVs can be concentrated and separated from SUVs by centrifugation (100,000 g for one or more hours) (reviewed in Tyrrell et al., 1976a).

c) Stability of liposomes.

The stability of liposomes to entrapped solutes has been considered by Kirby et al. (1980b). These authors have shown that 6-carboxyfluorescein remains at least 75 % latent (i.e. entrapped) during 53 days of storage. This latency remained even after injection into animals, suggesting that prolonged storage at 4° C has no effect on lipid bilayers. This was confirmed when 1-beta-D-arabinofuranosylcytosine (AraC) liposomes were stored at 4°C under nitrogen (> 50% latency of AraC after 1 year : as quoted in Szoka and Papahadjopoulos, 1980). In addition, it has also been reported that some liposome/drug preparations can be lyophilized and rehydrated to retain 60 - 70 % of the entrapped drug (quoted in Szoka and Papahadjopoulos, 1980).

iv) DRUGS USED IN THIS STUDY

a) Actinomycin D (ACT-D).

The actinomycins are a group of highly toxic, peptide-containing, antibiotics that were discovered in 1940 they were the first of a series of antibiotics to be isolated from soil Actinomyces sp.

Following their discovery and the elucidation of their structure they were subjected to chemical modification and later prepared by total chemical synthesis. The history and chemistry of the actinomycin molecule has been reviewed by Brockmann (1974).

Actinomycin D has been widely used as an antitumor agent (Frei, 1974; Goldin and Johnson, 1974). However, it has always been known that ACT-D was very toxic and the sites of toxicity are usually tissues undergoing renewal or growth, eg: regenerating liver (Schwarz et al., 1965), or organs involved in the constant production of cells, eg : bone marrow, lymphoid organs and germinal regions in the intestinal epithelium and testis (Schwarz et al., 1968). In dividing cells ACT-D has been shown to arrest growth primarly in the G_1 phase (i.e. the period between mitosis and the onset of DNA synthesis) although effects have also been shown at the S/G_2 boundary (at the end of DNA synthesis) and in the G_2 period (period between the end of DNA synthesis and mitosis - Hill, 1978).

Actinomycin is selectively concentrated in the nucleus of mamalian cells where it is found associated with DNA. The mode of action of the drug is to totally inhibit DNA-dependent RNA synthesis (especially that of ribosomal RNA) and this is due to the inhibition of RNA polymerase (Goldberg, 1975). Sobell (1974) has crystallized the ACT-D/DNA complex and shown, by x-ray crystallography, that the phenoxazone ring system of the drug intercalates between base pairs of the sequence deoxyguanosine-deoxycytosine in double-helical DNA. The peptide rings lie in a narrow groove of the DNA helix and interact with deoxyguanosine residues on the opposite chain through specific hydrogen bonds. (See fig.2 :1a for structure of ACT-D).

The activity of actionomycin D is highly dependent upon its chemical structure. Thus, it has been found essential to have :

i) a free amino group on position 3 of the chromophore,
ii) the unreduced quinoidal oxygen and
iii) intact, cyclic pentapeptide lactones.

Minor alternations or substitutions in the pentapeptide rings do not cause loss of activity but modifications which produce conformational changes in the ring markedly effect the activity of the drug (Goldberg 1975).

b) Bleomycin

Bleomycin (BLM) is the generic name for a group of antibiotics isolated from Steptomyces verticillus. The different bleomycins in the group have been isolated and their structure elucida-

ted. The compounds consist of sulphur containing glycopolypeptides which differ only in terminal amine groups (Umezawa, 1974). The drug is marketed as bleomycin sulphate which is predominately the BLM-A_2 glycopeptide. (See fig.2 :1b).

The clinical use and susceptibility of various tumour types has been reviewed (Blum et al., 1973; Pietsch, 1975). The drug seems to affect cells in the G_2 stage of the cycle and possibly during mitosis itself (Hill, 1978). The mode of action of BLM remains unclear although it is known to bind strongly to DNA. Early work established that BLM could cause breaks in single stranded DNA, which could be enhanced by the presence of hydrogen peroxide. Free bases (especially thymine) were released from DNA as a result of the strand breaks (Donehower et al., 1979).

The binding of BLM to DNA seems to be via the bi-thiazole rings and sulphonium groups (fig.2 : 1b). The drug/DNA complex contains iron (Fe^{2+}) (Sausville et al., 1978) which is important for the DNA strand breaking activity and which may (on oxidation to Fe^{3+}) produce free radicals that promote the breakdown of the nucleic acid (Donehower et al., 1979).

Bleomycin has been shown to inhibit DNA ligase but not DNA-dependent DNA polymerase. In addition, an enzyme has been found in animal tissues which hydrolyses part of the BLM molecule and results in reduced activity of the drug (Umezawa, 1974).

c) Asparaginase (ASPase).

L-Asparaginase (E.C. 3.5.I.I.) catalyses the hydrolysis of L-asparagine to yield L-aspartic acid and ammonia. The enzyme has been prepared from a number of different sources including bacteria (E. coli, Erwinia cartovora) and animals (chickens and guinea pig). The properties and antitumour activities of the enzyme depend upon the source. Asparaginase has a molecular weight of 133,000 and is composed of four sub-units (33,000 mol wt). Each sub-unit is probably identical and contains an active site for asparagine hydrolysis (Patterson, 1975).

The enzyme has been used as a treatment for leukaemia (Hill et al., 1969) and as an addition to other antitumour agents (Bodey et al., 1974). Asparaginase also has immunosuppressive effects (Ohno and Hersh, 1970).

The mode of action of ASPase is not clearly understood.

However, only cells in the 'S' phase are effected by the protein, suggesting that, in vivo at least, ASPase has little direct action on DNA. Most evidence supports the idea that the basis of the antitumour and immunosuppressive effect is starvation of cells by hydrolysis of L-asparagine. This has been shown to be the case for tumours which are asparagine sensitive. However, other modes of action have been suggested (Patterson, 1975) including :

i) the conversion of asparaginyl-t.RNA to aspartyl-t. RNA causing the wrong amino acid to be inserted into proteins,
ii) increase in alkaline and acid RNase activity,
iii) decrease or inhibition of RNA polymerase activity, and
iv) inhibition of synthesis of enzymes responsible for nucleic acid metabolism and repair.

v) LIPOSOME INTERACTIONS IN THE PLASMA.

a) Plasma clearance of liposomes.

It has been clear for some time that entrapment of drugs within liposomes changes the rate at which the drug is removed from the plasma if the drug and carrier remain together (Gregoriadis, 1973b). Subsequently, a large number of studies have demonstrated that liposome encapsulation changes the plasma half-life ($T_{1/2}$) of drugs.
However, the $T_{1/2}$ is dependent upon a large number of parameters :

1) Composition

a) Charge. Gregoriadis and Neerunjun (1974) and Juliano and Stamp (1975) both showed that anionic liposomes were cleared more rapidly from the plasma than cationic or neutral liposomes. But Gregoriadis and Neerunjun (1974) also showed that dicetyl phosphate anionic liposomes are cleared faster than those containing PA. This result seems to suggest that, within a particular type, effects other than charge may be operating. Although there may be clearance rate differences between neutral and cationic MLV preparations (neutral > cationic) (Gregoriadis and Neerunjun, 1974), cationic and neutral SUVs are cleared at about the same rate (Juliano and Stamp, 1975; Kirby et al., 1980b).

b) Cholesterol. The effects of cholesterol incorporation in the lipid bilayers of liposomes has been investigated because of the evidence that CHOL stabilizes the vesicle and not only pre-

vents the release of entrapped solutes but also reduces the exchange of the liposomal lipids with lipoproteins (see stability of liposomes and lipoprotein interactions and references therein). Because of the exchange of CHOL, cholesterol esters (E-CHOL) and phospholipids with lipoproteins it has not been possible to demonstrate whether cholesterol per se might change the plasma clearance rate of liposomes. However, the role of cholesterol as a liposome stabilizer makes its presence essential.

c) Lipids. Investigations of the effects of the lipid composition (i.e.: the major lipid component) on the interactions of the liposomes with the plasma have not been studied until recently because most of the vesicles contained low amounts of CHOL and were therefore unstable. Scherphof et al., (1978) demonstrated that SUVs composed solely of EPC were destroyed in the plasma, however, other phospholipids above their t_c are more stable (Scherphof et al., 1979). Recently Gregoriadis and Senior (1980) and Huang et al (1980) have investigated liposomes made from sphingomyelin and have reported very long plasma halflives (16hrs) for these stable preparations. However, these long halflives are not due to the presence of sphingomyelin alone since it has been shown that, with appropriate CHOL concentrations, a wide variety of $T_{1/2}$ values can be obtained with stable liposomes and this depends upon the phospholipid content (Senior and Gregoriadis, 1982a). These authors believe that liposomal halflife in the circulation is determined by the extent to which phospholipids, alone or in association with CHOL, prevent plasma-induced bilayer permeability (Senior and Gregoriadis 1982b).

2) Size.

As noted above, the size of the liposomes injected into an animal also determines the rate of plasma clearance.

The results of Juliano and co-workers (Juliano and Stamp, 1975; Juliano, 1976; Juliano et al., 1978) and Gregoriadis and Neerunjun (1974) have demonstrated that liposomes are cleared biphasically from the plasma. The heterogeneity of size found with MLV preparations makes analysis of the plasma pharmacokinetics very difficult but for homogeneous SUV preparations Juliano

(1976) has suggested a function of the form

$$A = A_0^1 \, e^{-0.693 \; t/T_1} + A_0^2 \, e^{-0.693 \; t/T_2}$$

where the rate of clearance, A, at time t after injection depends on a rapid initial loss A_0^1, with a $T_{1/2}$ time of T_1 and a longer period of decline A_0^2 with a $T_{1/2}$ time of T_2. When MLV preparations are used, the initial clearance rate (T_1) is much more rapid than with homogeneous SUV preparations. Kirby et al., (1980b) have shown that, when the SUVs contain enough cholesterol to stabilize the bilayers and contents of the vesicle, the initial rapid phase of clearance is almost completely lost and the log per cent plasma concentration is a straight line related to the time after injection.

Since the size of the liposome determines its plasma clearance rate, it follows that even a small size change within a homogeneous population of SUVs may have an effect on plasma clearance. Thus, a 50 % increase in the diameter of SUVs occurs when 50 mole % CHOL is incorporated in a vesicle made of EPC (Johnson, 1973) and up to 300 % increase in diameter, depending on the chain length of the acyl groups, in other phosphatidylcholine vesicles (de Kruijff et al., 1976). Since both these vesicles are SUVs it could be expected that their plasma clearance and tissue distribution would be the same but their sizes are different enough to make this unlikely.

3) Effect of entrapped solutes.

It has always been assumed that the plasma clearance of truly entrapped and non-leaky solutes will only be determined by the liposome carrier (Gregoriadis and Ryman, 1972; Juliano et al., 1978). However, in view of the data on size and charge effects on plasma $T_{1/2}$ it might be predicted that ionized solutes entrapped in liposomes could i) decrease or increase the charge separation between adjacent bilayers by charge interaction thus changing the size of the liposome; ii) affect the overall final charge of the liposome and iii) if they are amphipathic, allow hydrophilic groups to project onto the surface of the liposome which could interact with blood components (e.g. proteins) and thereby increase the size or change the charge of the vesicle in such a way

that its plasma clearance will be altered.

To date, no study of the effect of entrapped solutes on liposome plasma clearance has been published.

b) Liposome - Lipoprotein interactions.

1) Plasma lipoproteins and lipids

The major lipids of plasma are, in order of decreasing concentration by weight : cholesterol esters, phospholipids, triglycerides, free cholesterol and free fatty acids. These substances circulate as lipid-protein complexes which have been classified according to size, density and electrophoretic mobility (Scanu and Wisdom, 1972). The ratios of lipids to each other and to the protein are variable even within classes of molecules that are biologically closely related. This heterogeneity separates lipoproteins from other classes of plasma or cellular proteins which are normally quite homogeneous. Yet enough similarities within lipoprotein types do exist to allow them to be separated from other types by both physical and chemical methods (Hatch and Lees, 1968). Lipoprotein types, defined by their density following ultracentrifugation, are designated as chylomicrons (CHYL); very low-(VLDL), low-(LDL), high-(HDL) and very high (VHDL) density lipoproteins.

2) Phospholipids.

It has been known for some time that apoproteins from HDL will bind to phospholipids (PL) (Scanu, 1967); the PL used were almost certainly liposome-like (possibly MLVs) in structure. The reconstituted protein-phospholipid complex behaved like an HDL particle in centrifugation studies and also in its ability to activate plasma lipoprotein lipase. Later Forte et al. (1971) reported that HDL apoproteins recombining with phospholipids can result in the formation of phospholipid bilayer discs. which accumulate a core of cholesterol esters when exposed to plasma lecithin : cholesterol acyltransferase (LCAT) (EC : 2.3.1.43) and are thus converted into spherical mature HDL particles. When multilamellar liposomes were incubated with intact HDL, they displayed a thermal transition typical of the discoidal apoprotein-PC complexes (Tall and Small, 1977). These workers postulated that

the HDL particle dissociated in the presence of the liposomes to give up HDL-apoA to the liposomes. This new discoid PC/apoA complex was characterized by ultracentrifugation and electron microscopy (Tall et al., 1978). Liposomal lipid was reported to be incorporated into the HDL lipid coat (Chobanian et al., 1979).

Another group of workers reported the plasma-induced release of liposome entrapped solutes (Zborowski et al., 1977) was dependent on HDL-liposome interaction which resulted not only in liposome destruction but also in the transfer of protein to liposomes (Scherphof et al., 1978). This same group have also shown that the leakage and lipid transfer to HDL only occurs around the phase transition temperature of the PL and that CHOL incorporated in the liposome will abolish the susceptibility of the vesicle to attack by HDL (Scherphof et al., 1979). It should be noted that unilamellar vesicles seem to be much more susceptible to HDL attack than multilamellar or unsonicated liposomes (Scherphof et al., 1981). Recently (Kirby et al., 1980a) have also investigated the transfer of phospholipids and cholesterol esters to lipoproteins (see later).

Mechanisms of HDL transformation involving the release of apoprotein, with subsequent uptake of phospholipid or fusion of PC with HDL particles, may also occur in vivo. Infusion of EPC-containing SUVs into rats resulted in an HDL-mediated transformation of the vesicles into particles floating in the HDL density range (Krupp et al., 1976). Intravenous administration of "Intralipid" (phospholipid-triglyceride emulsion) into rats resulted in the accumulation of vesicle/protein complexes containing PL, unesterified CHOL and protein of the LDL type (according to its density) (Thompson et al., 1975). This process was accompanied by a depletion of HDL and an increase in the lipid : protein ratio in HDL. Transfer of PL to purified LDL and VLDL, unlike HDL, does not seem to occur (Chobanian et al., 1979).

3) Cholesterol.

In contrast to phospholipid-lipoprotein interactions, the interaction of liposomal cholesterol with lipoproteins has not received much attention. In a comparison of plasma clearance rates of liposomes radiolabelled in both CHOL and PC, Scherphof et al. (1975) suggested that the higher rate of CHOL disappearance might

be due to exchange of cholesterol with red-cell membranes. Later Black and Gregoriadis (1976 and this work) showed that CHOL was lost from MLVs to LDL and HDL in rats. At the same time, Krupp et al., (1976) showed that cholesteryl-oleate from SUVs was transferred to HDL in vivo. Subsequently, Kirby et al. (1980) showed that, in the absence of unesterified cholesterol in SUVs, cholesteryl-oleate was transferred in vitro to LDL and HDL. However, although SUVs will co-elute and co-sediment with LDL from serum, the amount of cholesteryl-oleate found associated with the various lioprotein fractions was constant even when cholesterol was included in the membrane of the SUVs.

From a series of papers from Gregoriadis' group (Gregoriadis and Davis, 1979; Kirby and Gregoriadis, 1980; Kirby et al., 1980a, 1980b) it has become clear that the concentration of cholesterol in the bilayer membrane is of great importance to the stability of the liposome-entrapped solutes and lipids both in vivo and in vitro. Thus, increasing amounts of CHOL in SUVs reduced the transfer of both PC and E-CHOL to lipoproteins and prevented the escape of entrapped 6-carboxyfluorescein both in vivo and in vitro. Moreover, using a larger molecule than 6-carboxyfluorescein, Gregoriadis and Davis (1979) showed that proteins like beta-fructofuranosidase remain latent in vivo and in vitro although an initial rapid leakage was more pronounced when lower CHOL concentrations were used in the liposome bilayers.

c) Interactions of Liposomes with other proteins.

Exchanges between lipoproteins and liposomes are not the only interactions which take place between proteins and vesicles. Early studies demonstrated that charge interactions between acidic phospholipids and basic proteins resulted in a very tight binding (probably not, finally, electrostatic in character) and alteration of the electrophoretic-mobility patterns of liposomes. (Kimelberg and Papahadjopoulos, 1971a). However, many other interactions studied (Tyrrell et al., 1976) have been concerned either with proteins not normally found in the blood or with those which would not be be expected to affect the in vivo handling and disposition of intravenously injected liposomes. Nevertheless, it has become clear that, in general, protein binding to liposomes often results

in the release of entrapped solutes in vitro. (Kitagawa et al., 1976 and Tyrrell et al., 1976a for earlier work). The extent to which solute release occurs in vivo following protein-liposome interaction is still the subject of controversy and where liposome constituents or physical parameters are involved this has been considered in other sections (effects of cholesterol).

The proteins of direct interest to this study are those which are known to occur in plasma or serum. There has been relatively little work concerning the interaction of liposomes with whole blood plasma; more interest has been shown in the interaction of individual, purified, plasma proteins with lipid membranes in vitro.

1) Whole Plasma/Serum.

The presence or absence of serum has been shown by several authors to influence the uptake of liposomes by cells in vitro. (Tyrrell et al., 1977; Blumenthal et al., 1977; Hoekstra and Scherphof, 1979; Mayhew et al. 1980).

Tyrrell et al. (1977) examined the fraction of whole foetal calf serum (FCS) responsible for this effect and found that, in general, the presence of serum caused an increase in the cell uptake of CHOL from anionic and neutral liposomes and a decrease in uptake from cationic liposomes. Albumin enhanced the transfer of CHOL between liposomes and cells but the alpha and beta-globulin fractions of serum decreased the transfer of both lipid and entrapped methotrexate from cationic liposomes to both cells and perfused whole rat liver. Finally, although the beta-globulin fraction caused an increased leakage of entrapped drug from all types of liposomes, both the alpha and beta-globulin fractions enhanced the capture of anionic liposomes by the perfused liver.

Finkelstein and Weissmann (1979) studied the integrity of liposomes of various compositions in biological fluids and showed that inulin and horseradish peroxidase (HRPase) were both rapidly lost from liposomes in the presence of 10 % human serum. However, if the serum was decomplemented (56° C for 30 min.) this loss was reduced. In addition, when 50 % serum was used much more entrapped solute was lost although human serum albumin alone stabilized the liposomes and prevented leakage.

Juliano and Lin (1980) incubated MLVs with 50 % fresh human

serum, citrated human plasma or blood-bank serum and found that a wide variety of proteins adsorbed onto liposomes. In general cationic liposomes adsorbed more protein than either anionic or neutral MLVs. The bound proteins were sensitive to trypsin digestion but could not be released by high salt concentrations or chelating agents. A large number of high (>2 x 10^5) and intermediate (6-15x10^4) molecular weight proteins of unknown identity were bound. Of the identifiable proteins, the most abundant was albumin followed by IgG heavy and light chains, HDL-apoA, alpha-2-macroglobulin (alpha-$_2$-M) and fibronectin. In addition, incubation of plasma with liposomes resulted in a depletion of clotting factors VII, VIII and XII by some or all of the liposomes; depending on their charge.

Finally, Kirby et al. (1980b) have shown that liposome stability is dependent on the cholesterol content of the vesicles. Thus, for the type of liposome used in this study (CHOL poor) the stability was whole blood > plasma > serum after a rapid initial loss of about 50 % of the entrapped solute. No explanation has yet been put forward to explain why there should be differences between blood, plasma and serum. It has been suggested (Kirby et al., 1980a, 1980b) that the packing of cholesterol and phosphatidylcholine prevents the loss of phosphatidylcholine by reducing their mobility within the bilayer (Demel and de Kruyff, 1976). The fact that red blood cells donate cholesterol to liposomes (Bruckdorfer et al., 1968) and that a preferential interaction of lipoproteins with red cells rather than liposomes occurs (Bruckdorfer and Graham, 1976; Demel and de Kruyff, 1976) have been suggested as mechanisms by which liposomes would be (or become) more stable in blood. The loss of clotting factors and proteins from serum might account for the differences in stability between plasma and serum; indeed Juliano and Lin (1980) have shown that loss of these elements is found when liposomes are incubation with whole blood.

It is possible that the study of liposome/protein inter actions is best done with plasma since, for example, alpha-2-M has to be prepared fresh; serum is an inadequate starting material because alpha-2-M will have already fixed thrombin, kallikrein and plasmin (Steinbuch et al., 1975).

2) Albumin, Bovine Serum Albumin (BSA) and Ovalbumin.

The original work of Sweet and Zull (1969) demonstrated that BSA would enhance glucose diffusion from anionic liposomes at acid pH. This BSA binding to the liposomes, which could not be disrupted at high salt concentrations, was considered to be apolar in nature although an initial electrostatic interaction was found. Later Sogor and Zull (1975) showed that binding of albumin also occured to cationic liposomes but their low angle x-ray diffraction studies, whilst demonstrating the lamellae structure of the bound liposome, did not show hydrophobic interactions occuring between lipid and protein. Kitagawa et al. (1976) measured BSA-liposome binding at neutral pH and showed that the protein enhanced the release of entrapped solutes when lysolecithin (LPC) was present in the liposome membrane, the release was independent of the charge of the vesicle but CHOL in the liposome reduced the leakage. Zborowski et al. (1977) showed that not only small solutes but large molecules like inulin and albumin could be released from liposomes in the presence of plasma or albumin. This same group (Scherphof et al., 1978) later showed that only the LPC of the phospholipids was associated with the albumin, but Weissmann et al.(1974) showed that BSA was not active in releasing anions and glucose from either anionic or cationic liposomes. More recently, Finkelstein and Weissmann (1979) have shown that human serum albumin acts to stabilize MLVs against solute leakage. Ovalbumin has been shown to react with PA-containing, anionic, liposomes to form aggregates which could be broken up by the addition of Ca^{2+} ions or by increasing the pH from 2.5. to 6: no aggregation occured above pH 4.0. (Oshima and Nagasawa, 1973).

3) Immunoglobins.

The work of Weissmann's group (Weissmann et al., 1974), on the introduction of enzymes from liposomes into cells, has shown that most immunoglobulins (Ig's) and heat aggregated Ig's release anions and glucose from liposomes. Only heat aggregated IgG_2, IgA_2, IgM and Fab fragments were inactive. These authors suggest that heat aggregation produces a conformational change in the Fc regions of the Ig molecules which enhances their binding to the lipid bilayers. Later this group (Weissmann et al, 1975) showed that heat aggregated IgM-coated liposomes were taken up better by

phagocytes from Mustelus canis. (the smooth dogfish) and by human leucocytes (Cohen et al, 1976) but the in vivo distribution of the coated liposomes showed no differences from uncoated liposomes (Weissmann et al, 1978).

Extensive work has shown that liposomes prepared from pure haptenated lipids undergo membrane damage and release entrapped solutes in the presence of a specific antibody and complement (Kinsky, 1972; Kinsky and Nicolotti, 1977) but liposomes composed of non-haptenated lipids do not appear to be antigenic. The entrapment of proteins or mixing of liposomes with proteins increases the immune response to those proteins when compared to proteins without liposomes (Allison and Gregoriadis, 1974; Heath et al, 1976) but this adjuvant activity is probably the result of antigenic protein exposed on the liposome surface (Manesis et al. 1978; Van Rooijen and Van Nieuwmegen, 1980). Entrapped proteins do not give hypersensitivity reactions (Allison and Gregoriadis, 1974). Other work on entrapped virus antigen and other antigens has been reviewed by Gregoriadis (1979b; 1980a; 1980b).

4) Complement.

Complement can mediate the lysis of liposomes (Hesketh et al. 1971; Kataoka et al., 1973) and the leakage of entrapped solutes was found to be inversely proportional to the cholesterol content of the bilayers (Shin et al., 1978). Richards et al. (1977, 1979) have shown that interactions between C-reactive protein (an acute phase protein) and liposomes sensitize the liposomes to complement damage. This damage involved all the components of the classical pathway but only occured with cationic liposomes containing a ceramide glycolipid.

Both Finkelstein and Weissmann (1979) and Juliano and Lin (1980) have shown that liposomes will bind complement components from serum and Cunningham et al. (1979) demonstrated that cationic, fluid, liposomes would activate the alternate pathway of human complement.

5) Alpha and beta-Globulins.

The interaction of alpha-2-M with liposomes has been demonstrated by Black and Gregoriadis (1976 and this work) and Juliano and Lin (1980). As has been noted above, Tyrrell et al. (1977)

showed that alpha and beta-globulins effect the uptake of liposomes by cells and the perfused rat liver. The alpha-globulin, alpha-2-M, not only binds to, and is degraded by, fibroblasts (Willingham and Pastan, 1980; Mosher and Vaheri, 1980) but is also the major protein taken up by these cells from serum-containing growth medium. (van Leuven et al., 1977).

6) Alpha2 Macroglobulin.

Alpha-2-macroglobulin is a glycoprotein and one of the major components of a heterogenous group of serum proteins collectively known as the alpha-globulins. Many alpha-globulins are acute phase proteins whose activity is known to increase under certain physiological and pathological conditions (e.g.pregnancy or in foetal life, malignancy, inflammation) (Gauthier and Mouray, 1975). The majority of the published work on alpha-2-M has involved its unique role as binding protein for most of the known endopeptidases (Starkey and Barrett, 1973; Barrett and Starkey 1973; Werb et al, 1974; Harpel, 1976).

The molecular weight of human alpha-$_2$-M is 725,000 but it may dissociate into 4 subunits each of 185,000. The interactions of alpha-2-M with pepidase enzymes causes a conformational shape change in the alpha-2-M which probably involves covalent bond formation between the two proteins (James 1980); it does not involve the active site of the enzyme which, therefore, retains its enzymatic activity, at least towards small molecules. (Barrett and Starkey, 1973). These interactions suggest that one of the physiological roles for alpha-2-M is to act as a defense against pancreatic, granulocyte-derived or bacterial proteinases released into the circulation. After binding of the enzyme the complex is removed from the circulation by the reticuloendothelial system (Hanna et al, 1967).

In addition to its ability to bind proteases, alpha-2-M also binds thrombin, kallikrein and plasminogen activator (Gallin and Kaplan, 1974) suggesting that the protein has a function not only in the clotting of blood but also in chemotaxis. Other functions (reviewed by James, 1980) in the immune system involve binding to lymphokines, soluble Ia antigens, immune complexes, histones, mitogenic compounds (some lectins), foreign tissue antigens, Zn^{2+}

and the control of differentiation in leucopoeisis and eythropoiesis. Other workers have shown alpha-$_2$-M bound to parasitic worms. (Kemp et al., 1976).

Finally, Allen et al. (1973) and Blumenstock et al. (1976) have shown that an alpha-$_2$-globulin protein with alpha-$_2$-M-like properties will stimulate the uptake of lipid particles by hepatic Kupffer cells in vitro. Current work in the field of reception-mediated endocytosis (reviewed by Goldstein et al., 1979; Willingham and Pastan, 1980; Willingham et al., 1981; Pastan and Willingham, 1981) has shown that specific receptors for alpha-$_2$-M exist in large numbers (200,000/cell in fibroblasts) on many types of cell.

From this discussion it is apparent that alpha-$_2$-M is a very important plasma protein with a variety of actions on many different physiological pathways. However, the exact role of alpha-$_2$-M still remains to be established.

vi) TISSUE DISTRIBUTION OF LIPOSOMES.

The clearance of blood-borne particulate matter (Saba, 1970) is an efficient but complicated mechanism influenced by:

i) particle size,
ii) surface charge,
iii) hepatic bloodflow,
iv) opsonin activity and
v) species and age.

It has long been known that the primary site of localization of colloids and particles (Wilkins and Myers, 1966) following intravenous injection are the tissues comprising the reticuloendothelial system (RES). It was not surprising, therefore to find that liposome entrapped proteins (Gregoriadis and Ryman, 1972a, 1972b) and drugs (Gregoriadis, 1973b) were deposited in the liver and spleen following intravenous (I/V) injection. Apart from the RES, there have been comparatively few studies on the distribution of liposomes and/or entrapped agents to other tissues (Kimelberg and Mayhew 1978).

a) Liver distribution of liposomes.

1) Effect of charge and lipid composition.

The original work of Gregoriadis and Ryman (1972) and Gregoriadis (1973b) demonstrated that both anionic and cationic liposomes delivered their contents preferentially to the liver. The relative uptake of "vesicles" (?small MLVs) of different lipid composition has been studied by McDougall et al (1974). The addition of either, PA, PE or PS to a standard vesicle composed of PC+gangliosides, did not greatly alter the in vivo distribution of entrapped $^{99m}Tc0_4$ radioactivity. Incorporation of cholesterol however, enhanced the hepatic uptake.

Using a similar liposome/radioactivity system, Richardson et al. (1978) studied the distribution of radioactivity in various organs of rats following I/V injection. Their results suggest that the liver uptake 24 hours after injection is not dependent on the charge of the MLV. However, Jonah et al. (1975) found that large neutral (handshaken) MLVs were taken up better by the liver than either anionic or cationic liposomes. Omission of cholesterol from the vesicle reduced the liver uptake.

2) Effect of size.

As well as charge effects both Richardson et al. (1978) and McDougall et al. (1974) studied the distribution of different sized liposomes (produced by increasing the sonication time). These authors concur that the hepatic uptake is reduced as the liposomes become smaller. Other workers (Juliano, 1976, and Kimelberg et al., 1976) agree. Finally, Sharma et al (1977) using large unilamellar vesicles (LUVs) also reported high hepatic uptake.

3) Effect of Entrapped Drug.

Rahman et al. (1975; 1978) have studied the distribution of liposomal ACT-D in animals. They have shown that if the drug is entrapped in the lipid phase of liposomes it has a different hepatic uptake than when it is entrapped within the aqueous phase of the same liposomes. Three hours after injection the liver contained 2-3 times more lipid entrapped drug than aqueous entrapped drug. These results are explained by differential leakage of the drug from the aqueous spaces.

4) Cellular uptake within the liver.

The original work of Gregoriadis and Ryman (1972a) demonstrated that ^{3}H-cholesterol-labelled liposomes were localized in both the Küpffer cells and the parenchymal cells of the liver as early as 3 minutes after I/V injection of protein-containing liposomes. These results can be criticised on the grounds that cholesterol is able to exchange between liposomes and lipoproteins (Black and Gregoriadis, 1976) and a specific uptake process for low density lipoproteins is known to exist (Goldstein et al., 1979). However, the internalization of LDL molecules takes about 10 minutes so that the kinetics of uptake may not favour this hypothesis.

Later work (Segal et al.1974) showed that, morphologically, liposomes can only be found in Küpffer cells. Gregoriadis et al. (1974a) showed that only the parenchymal cells had liposome-like structures inside them. This latter experiment performed in human cancer patients can be criticised on the grounds that "liposome like" structures are often found inside cells when viewed by electron-microscopy. (Scherphof et al., 1980). In addition, the human reticuloendothelical system has been shown to be very sensitive to distrurbances caused by infection or neoplasm (Saba,1970).

Wisse et al. (1976) suggested that horseradishperoxidase (HRPase) entrapped in liposomes localized not only in the Küpffer cells but in parenchymal and endothelial cells as well. The amount of HRPase found in the parenchymal cells increased over time after injection. In similar experiments using HRPase and ^{125}I-labelled polyvinylpyrrolidone, Scherphof et al (1981) have reported that the only cell type involved in liposome uptake is the Küpffer cell. These authors believe that previous results showing parenchymal cell involvement are due to the release of entrapped materials from the liposomes.

Other reports (Freise et al., 1980, 1981), using portacaval shunts in rats, have suggested that liposome location can be totally accounted for by parenchymal cells with no Küpffer cell involvement.

These results all suggest that the study of the particular cell type involved in liposome uptake by the liver is very difficult. Since much of the work was performed with exchangeable lipid markers and liposomes which might be leaky in the presence of plasma proteins, it seems reasonable to await further clarification of this complicated issue.

5) Subcellular distribution in the liver.

Liver fractionation studies have demonstrated that liposome entrapped agents localize mainly in the lysosomes. (Gregoriadis and Ryman, 1972b; Gregoriadis et al., 1974b; Black and Gregoriadis, 1974 and this work; Segal et al., 1976; Steger and Desnick, 1977). Drugs localized in the lysosomes seem to be able to escape and enter other cell compartments (Black and Gregoriadis, 1974). Steger and Desnick (1977) have found a proportion of enzyme from cationic liposomes associated with the small granule, "post-lysosomal-mitochondrial", fraction of the liver. These authors believe that this demonstrates either fusion of liposomes with hepatic cells or destabilization of the lysosome membrane by stearylamine.

Indirect evidence for lysosomal localization (and indeed Küpffer cell uptake) of liposomes comes from the studies of Black et al., 1977; New et al., 1978; and Alving et al., 1978, 1980) who showed that liposome entrapped compounds were many hundred-fold more active than the unentrapped drugs against Leishmania donovani parasites. These intracellular protozoa are known to parasitize

Küpffer cells and to live and multiply within parasitophorous vacuoles which are, in effect, secondary lysosomes. Contrary evidence has been presented by Rahman (1980) who found EDTA from liposomes in the cytosol of mouse livers.

Finally, the degradation of liposomes by liver cells has been reported by Huang et al., (1980) who have elegantly studied the hepatic uptake and degradation of SUVs. It was found that the time to digest the liposomes was 3.6 hours but the time for maximum release of the liposome-entrapped agent was about 8 hours. The enzymes necessary to digest liposomes have been located in liver lysosomes (Fowler and de Duve, 1969).

b) Spleen : Distribution of liposomes.

The spleen comprises part of the RES. Early work on drugs entrapped in liposomes (Gregoriadis 1973b) showed that there was an increased uptake of liposomal drug into this tissue. Segal et al., (1974) presented morphological evidence of liposome entrapped agents in the red pulp (phagocytic cells) but not the white pulp (lymphoid tissue).

In most respects the uptake of entrapped agents by the spleen is very similar to that found in the liver. Indeed, if the amount of entrapped material is calculated as per cent of dose per gram of tissue, it can be shown (Juliano, 1976; Richardson et al., 1978; Kimelberg et al., 1976) that the spleen takes up more of the entrapped agent than the liver.

Both Jonah et al., (1975) and Richardson et al., (1978) have shown that anionic liposomes are taken up into the spleen better than cationic or neutral liposomes. Moreover, Juliano (1976), Kimbelberg et al., (1976) and Richardson et al., (1978) have shown that unsonicated or large MVLs are taken up to a greater extent than SUVs.

However, using liposomes containing adriamycin, Forssen and Tokes (1979) failed to find higher drug levels in the spleens of liposome treated animals. Rahman et al., (1978) found higher drug levels in the spleen of animals treated with ACT-D in the lipid phase of liposomes.

c) Kidney and Urinary excretion of Liposomes.

A decrease in the kidney location of liposome-entrapped ACT-D compared with the free drug was reported by Gregoriadis (1973b) and this is almost certainly due to the encapsulation of the drug in the

carrier. Sonication reduces the liposome's location in the kidney (Richardson et al.,1978; Kimelberg et al., 1976).

Kimelberg and Atchison (1978) have studied the urinary excretion of methotrexate (MTX) entrapped in liposomes and found a very low rate of urinary drug output. However, Gotfredsen (1982) has identified metabolites of entrapped BSA in mouse urine following its administration in liposomes. Urinary excretion of metabolites of liposomal lipid was found to be very low: the bulk of radiolabelled PC was excreted as CO_2 in expired air (Gotfredsen, 1982).

d) Lung:

Jonah et al., (1975) and Rahman et al., (1975) have reported high levels of liposome-derived radioactivity in the lungs of animals injected I/V with unsonicated liposomes containing EDTA or ACT-D in the lipid phase. This effect seems to dependent upon the charge of the carrier (cationic >> anionic or neutral), and, perhaps more likely, the size of the liposome preparation since others using small sonicated MVLs and SUVs have found little lung localisation. (Hunt et al., 1979).

e) Muscle tissue:

The work published to date uniformly suggest that uptake of liposomes, of whatever charge or size, by skeletal or heart muscle tissue is very low. This is an unfortunate result because muscle tissue can be one of the sites of storage product in lysosomal storage diseases.

f) Brain:

Jonah et al., (1975) and Rahman et al. (1975) have reported uptake and retention of liposomally entrapped drugs in the brain. Most other workers do not agree with this finding. Indeed, it seems unlikely that whole liposomes can cross the blood-brain barrier.

g) Intestinal tissue:

Gregoriadis (1973b) showed that liposome entrapment of ACT-D reduced the amount of drug localising in the small intestine. Because liposomal drugs normally have longer plasma half lives and higher uptake by tissues, e.g: the liver and spleen, the amount of drug available to effect sensitive germinal tissues, like the intestine, is normally less than for non-entrapped drugs. Rahman et al.,(1978) have also shown that entrapping ACT-D in liposomes results in a lower intestinal uptake.

h) Bone Marrow:

Rahman et al., (1978) have studied the liposome uptake and effects on stem cells in the bone marrow. Kimelberg et al., (1976. 1978) did not find such high levels of entrapped drug in the tissue.

vii) DRUG ENTRAPMENT IN LIPOSOMES.

Gregoriadis (1980a) has reviewed more than 120 compounds which have been entrapped in liposomes of various compositions. The entrapped agents range in size (weight) from viruses and nucleic acids to ions, via enzymes, immunoglobulins, vitamins and a large number of drug molecules. This list alone emphasizes the versatility of the liposome as a carrier.

The antitumour drugs used in the research reported in this thesis have been ACT-D, BLM and ASPase. Several reports of the liposomal entrapment and distribution of these drugs have appeared during the course of the work.

a) Liposome Entrapped Actinomycin D (ACT-D)

Following the initial report of Gregoriadis (1973b) on the plasma clearance and tissue distribution of liposome entrapped ACT-D, there have been a number of reports on the distribution and use of this carrier system.

Black and Gregoriadis (1974 and this work) showed that liposome entrapped ACT-D localized in the lysosomes of rat liver from where it could be released in an active form. Segal et al., (1975) studied the distribution of ACT-D liposomes of different sizes following local injection. The work of Rahman's group (Rahman et al., 1974, 1975, 1978) has shown that the entrapment of ACT-D in liposomes reduces the toxicity of the drug (if it is in the aqueous phase) whilst its tumouricidal activity is retained. They have also studied the tissue distribution of the ACT-D liposome carrier. Gregoriadis and Neerunjun (1975) have shown that liposome entrapped ACT-D prolongs the survival of tumour bearing mice when compared to the non-entrapped ACT-D and Papahadjopoulos et al. (1976) demonstrated that SUV-ACT-D preparations could overcome the resistance to the drug of cultured tumour cells. More recently Kaye et al., (1981) have studied the effects of liposome entrapped ACT-D in the treatment of sensitive and resistant tumors in mice this will be discussed in more detail later.

The plasma and tissue distribution of ACT-D liposomes has been studied by Juliano and Stamp (1978) and Juliano et al., (1978).

b) Liposome entrapped Bleomycin.

The uptake of liposomal ^{111}In-bleomycin into mice liver and tumour tissue has been reported by Dapergolas et al., (1976) who found that smaller liposomes were taken up better by the tumour tissue. In contrast, Segal et al., (1976) could find no selective uptake by malignant tissue in a clinical study of the cellular and sub-cellular distribution of ^{111}In-bleomycin liposomes. The radioactivity within the liver was concentrated in the lysosomes.

In an attempt to improve the localization of liposomes in animal tumours, Gregoriadis et al., (1977a) studied the cellular and tumour uptake of normal BLM-liposomes and antibody to tumour cells co-entrapped in BLM-liposomes. It was found that the normal BLM-liposomes were taken up by the tumours. Antibody-containing BLM-liposomes were taken up slightly better by the tumour than the other liposomes but the presence of antibody in the liposome enhanced the liver uptake of radioactivity.

c) Liposome entrapped Asparaginase.

Asparaginase has been entrapped in liposomes by Fishman and Citry (1975) and Neerunjun and Gregoriadis (1976). The latter showed that liposome-ASPase was not as effective as the free drug against asparagine dependent tumours. However, the tumour levels of asparagine were lowest in the liposome treated mice (Gregoriadis 1980a). Allergic reactions and anaphylactic shock occur following free enzyme administration but this is prevented when the enzyme is liposome entrapped (Neerunjun and Gregoriadis 1976).

viii) ROUTES OF DRUG ADMINISTRATION.

The work presented thus far has been entirely concerned with liposomes administered by the intravenous route. Other routes of administration have been used :

a) Local injection.

Segal et al., (1975) showed that small liposomes could escape from a local injection site and localize, finally, in the liver and spleen.

The route taken was via the lymphatic system. Ryman et al., (1978) and Osborne et al., (1979) have developped this system using small neutral or cationic ^{99m}Tc-liposomes to visualize lymph nodes. Dapergolas et al. (1976) and Kirby et al., (1980 b) have shown that the intramuscular route can be used to deliver liposome entrapped agents to the plasma and liver.

b) Intraperitoneal Injection (I/P)

Kirby et al., (1980b) have shown that incorporation of cholesterol in liposomes renders them stable enough to cross several permeability barriers from the peritoneum to the plasma whilst retaining their entrapped solute. Rahman et al., (1978) have compared the I/V with the I/P route and found that the deposition of lipid phase ACT-D liposomes was the same in both spleen and bone marrow.

c) Oral Route.

The work of Patel and Ryman (1976,1977), Gregoriadis et al., (1976), Dapergolas et al., (1976), Dapergolas and Gregoriadis (1977) and Ryman et al., (1978) has shown that the oral administration of liposomal insulin will lower blood glucose levels. Other agents have also been administered by this route to test the ability of liposomes to protect molecules during transit through the stomach and to enhance their uptake in the intestine (Dapergolas and Gregoriadis, 1977; Ryman et al., 1978), but the results have proved to be variable and generally disappointing.

d) Other routes.

Kimelberg et al., (1978) have reported the distribution of methotrexate-liposomes following cerebral intraventicular injections. Juliano and McCullough (1980) have used the respiratory system to deliver drugs to the lungs. Mezei and Gulasekharm (1980) have used the topical route to deliver liposome entrapped drugs.

ix) TOXICITY OF LIPOSOMES WITHOUT ENTRAPPED DRUG.

A few studies have been carried out on the toxicity per se of liposomes and their constituent components. Thus, Gregoriadis (1978) presented the results of a toxicity test carried out by Nattermann Chemie, Cologne. FDR. No toxicity of soya bean phosphatidylcholine

liposomes was found except for transient rises in the plasma lipid and sterol levels. The rats and dogs were given lipid up to 10mg/kg over a period of several weeks. It should also be remembered that a solution of "Intralipid" used for I/V therapy in humans, and passed as a safe medicament, contains up to 20g of phospholipid in 500ml.

The charge or lipid composition may be a more important factor in the toxicity of liposomes. Adams et al., (1977) demonstrated toxicity in mice when high (250-500mg lipid/kg) amounts of liposomes containing stearylamine (cationic) or dicetyl phosphate (anionic) were injected intracerebrally but other anionic phospholipids were not toxic. However, Kimelberg and Mayhew (1978) did not find any toxicity in mice injected I/P with stearylamine containing liposomes (up to 300mg lipid/kg).

Bruni et al., (1976a, 1976b) and Bigon et al., (1979a, 1979b) have shown that phosphatidylserine liposomes induce glucose accumulation in the brain of animals injected I/V with these vesicles.

The function of platelets in contact with liposomes has been studied by Berdichevsky et al., (1979) who demonstrated an effect on their agglutinability with phosphatidylethanolamine-containing liposomes but this did not effect their clotting functions.

The interaction of liposomes with proteins and clotting factors has already been considered.

x) LIPOSOME / CELL INTERACTIONS.

The uptake of liposomes by the liver suggests that in vivo at least some of the injected vesicles are taken up by endocytosis since their contents can be found associated with lysosomes (see above). In this sense liposomes can be said to be lysosomotropic (de Duve et al., 1974, 1978).

The mechanism of interaction of lipid vesicles with cells in culture has been studied in detail during the last few years and the results have been reviewed (Pagano and Weinstein, 1978; Kimelberg and Mayhew, 1978; Poste and Papahadjopoulos, 1978; Gregoriadis, 1980a, 1980b, 1980c). The evidence has been obtained with various types of liposomes and cells under varying conditions. It is not surprising, therefore, that the results often seem contradictory. However, there is a general agreement that liposomes are taken up quite efficiently by cells in culture, that the process of uptake is temperature dependent but does not require metabolic energy in most cases, and that at least

part of the vesicle contents are incorporated along with the lipids. In cases where the uptake has been quantified, it appears that several million SUVs can be incorporated per cell within a few hours (Papahadjopoulos et al., 1974b).

The exact mechanism of uptake of vesicles by cells remains to be clarified, the evidence indicates that it could involve any of the following, either singly or in combination : fusion with the (cell) plasma membrane (Papahadjopoulos et al., 1974a; Pagano and Huang, 1975; Poste and Papahadjopoulos, 1976a; Martin and MacDonald, 1976a, 1976b, 1976c; Weissmann et al., 1977; Schroit and Pagano, 1978; Batzari and Korn, 1975), endocytosis (Poste and Papahadjopoulos, 1976b; Batzari and Korn, 1975; Cohen et al., 1976) and/or receptor mediated endocytosis (Leserman et al., 1980a), adsorption to the cell surface (Pagano and Takeichi, 1977; Szoka et al., 1978), followed by liposome destabilization and leakage (Szoka et al., 1979; 1980) or molecular exchange (Pagano and Huang, 1975; Pagano et al., 1978).

The exact role of any of these mechanisms in the uptake of liposomes in whole animals remains to be determined but it is clear that the presence of serum in the medium profoundly affects the way in which cells in vitro handle the liposomes (Tyrrell et al., 1976a; Martin and MacDonald, 1976a; Kimelberg and Mayhew, 1978; Pagano and Weinstein, 1978).

CHAPTER 2.

Materials and Methods : Basic Results.

Section 2:0 MATERIALS

i) CHEMICALS

a) General

Unless stated all chemicals were of 'ANALAR' grade from BDH Ltd., Poole, Dorset.

Cholesterol (Regent grade) : BDH Ltd.

Phospholipids (EPC and PA) : Lipid Products, Redhill, Surrey.

Stearylamine : K and K Inc., New York, U.S.A.

S epharose 6B and Sephadex G-10 : Pharmacia Ltd., Uppsala, Sweden

Argon and Nitrogen gas : British Oxygen Co, Park Royal, London.

Silicic acid and precoated Plastic Silica-gel sheets ($^{60}F_{254}$) : Merck and Co. at BDH.

p-Nitrophenyl-2-acetamido-2deoxy-beta-D-glucopyranoside, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride and Triton-X-100 : Koch-Light Labs., Ltd, Colnbrook. Bucks.

Agarose (Type A37) : L'Institut Biologique Français, S.A., Gennevilliers (Seine), France.

1,6-diphenyl 1,3,5-hexatriene (purist grade) Aldrich Chemical Co. Milwaukee, Wi.53233, U.S.A.

b) Proteins and Antisera

Bovine Serum Albumin : Sigma Chemical Co, London.

Rat Serum Albumin, Rat $alpha_1$-macroglobulin, Acylated bovine albumin (stained with Evans blue) : Generous gifts from Mr. L. Louis, Clinical Research Centre, Harrow.

Sheep anti-whole rat plasma serum and sheep anti-whole human plasma serum : DAKO-Immunoglobulins; Mercia Diagnostics Ltd., Watford, Herts.

Goat Anti-human alpha-2-macroglobulin serum; Behringwerke Antisera; Hoechst Pharmaceuticals; Hownslow, Middlesex.

c) Drugs.

Actinomycin-D : Merck, Sharp and Dohme. Hoddesdon; Herts. mixed with mannitol to aid solubility.

Bleomycin Sulphate : Lundbeeck, N.V. Amsterdam, Holland. ex. Nippon Kayaku Co., Tokyo, Japan.

Asparaginase (From Erwinia carotovora); Gift from Dr. H.E. Wade. Centre for Microbiological Research. Porton Down. Wiltshire.

d) Radiochemicals

All radiochemicals were supplied by the Radiochemical Centre. Amersham, Bucks.

(^{3}H) Actinomycin-D (5.1 - 7.7 Ci/mmol).

(4-^{14}C) Cholesterol (53 mCi/mmol).

(U-^{14}C) Cyclohexane (2.5 Ci/ml).

(^{14}C) methyl Iodide (58 mCi/mmol).

(6-^{14}C) Orotic acid monohydrate (61 mCi/mmol).

(6-^{3}H) Thymidine (5 Ci/mmol).

(^{3}H) Water (5 Ci/ml).

(^{111}In) Bleomycin (1.66 mCi/mg).

Bolton and Hunter reagent (Bolton and Hunter, 1973) : -

N-succinimidyl 3-(4-hydroxy, 5-(^{125}I) iodophenyl) propionate (> 1700 Ci/mmol of mono-iodoester).

(^{125}I) Iodine monochloride (> 1500 Ci/mmol).

e) Solutions

Double distilled (in glass) water was used throughout this work.

PBS-(phosphate buffered saline) : -

28 ml $NaHPO_4$ (2.78 g/l) + 72 ml $NaHPO_4$. $12H_2O$ (7.17 g/l) + 100 ml 0.29 M NaCl. pH 7.2

Saline : - 0.145 M NaCl.

Solutions were sterilized by autoclaving.

ii) EQUIPMENT

Unless otherwise stated, laboratory equipment was provided by Scientific Supplies Ltd. London. Rotary evaporator : Bucchi S.A. Zurich, Switzerland. Centrifuges, rotors, centrifuge tubes and caps. Ultrasonic irradiator, Ultra violet lamp : MSE Scientific Instruments Ltd. Crawley Sussex. Spectrophometers (SP.500, SP.800) and "Spectrosil" quartz cuvettes (1cm) Pye-Unicam Ltd. Cambridge. Water baths and temperature control systems. Grant Bros. Cambridge. Beta and Gamma radioactivity counters. Wallac-LKB Ltd. Stockholm, Sweden. Immunoelectrophoresis tank and power supply (MBI.260/264). Scientific Supplies Ltd. Refrigerated thermocirculator. Churchill Instrument Co. Perivale, Middx. Laboratory Jack. Gallenkamp Ltd. London. Glass Plates (5x5cms) for agarose gels. Ilford Ltd. Southern distribution centre. Basildon, Essex. Micro-burette and syringe (S1/4LT). Micro-metric Instrument Co.

Cleveland, Ohio 44122 U.S.A. Particle microelectrophoresis equipment, Platinum electrodes and cylindrical glass cell. Rank Bros. Bottisham, Cambridge. Microviscometer and control system. Elsint (G.B.) Ltd., Crawley, Sussex.

iii) ANIMALS

Male albino Sprague-Dawley rats were used throughout this work. The animals were bred and maintained until use under specific-pathogen-free conditions in the laboratory animal unit of this institute.
During use the animals were maintained on "Laboratory Diet 1" (Spratts Patent Ltd., Barking, Essex) and were given water ad libitum.

Section 2:1 Methods

A. Manufacture of liposomes

1. General proce dure

Throughout this study liposomes were prepared by a single method although during the course of the work several other methods became available (Szoka and Papahadjopoulos, 1980). Liposomes (MLV) were prepared according to the method of Bangham et al.(1974) as modified by Gregoriadis (1976b).

i) Preparation of liposomes

The lipids of the standard liposome mixture (q.v.) were mixed with 10-20 ml of chloroform in a round bottomed rotary evaporation flask(rbf: 50 or 100 ml) which was then flushed with nitogen (N_2) or argon (Ar) and stoppered. After flushing the rotary evaporator with N_2 the contents of the flask were evaporated to dryness under reduced pressure at a temperature of 37-40°C. Evaporation was continued for a further 10 minutes after the last of the solvent had been removed and the lipids deposited as a dry film on the flask wall. The pressure inside the rotary evaporator was restored to normal with a stream of N_2 and the flask was removed. The aqueous solution was immediately added to the dried lipid along with a few clean and dry glass beads. After flushing the flask again with N_2 and tightly stoppering the neck, the lipids were removed from the flask walls by gentle hand shaking. The flask, containing the turbid solution, was wrapped in aluminium foil and kept at room temperature for 30 minutes before being further treated. Large MLV preparations (handshaken) were left overnight at room temperature and then centrifuged at 100,000 g for 15 minutes. The pellet was washed twice with aqueous phase buffer and the final pellet (hereinafter called unsonicated MLVs) was suspended in an appropriate volume of buffer and stored in a stoppered rbf under N_2 at 4°C until use.

ii) Sonication

Smaller MLVs were prepared by sonication of the turbid solution (Huang, 1969). After an elapse of at least 30 minutes following preparation, the flask was cooled in a beaker of melting ice whilst the solution was subjected to ultrasonic irradiation (60 watts) at maximum amplitude (about 8 um peak to peak) using a titanium vibrator probe of end diameter 19 mm (end ratio 3.5:1) at the frequency of 20-22 Kc.sec^{-1}. During sonication, which was carried out for 2 1/2 minutes, in 30

seconds bursts followed by 15 second rests, the liposome solution was kept under an inert atmosphere of N_2 or Ar continuously supplied to the liquid surface. Following sonication the liposome solution appeared optically clear at the edges. This solution was again flushed with N_2 and then left overnight at room temperature protected from light by aluminium foil.

iii) Column chromatography

The separation of sonicated liposomes from unentrapped material and other debris was performed by molecular sieve column chromatography using Sepharose 6B (Huang, 1969). New columns were prepared by packing Sepharose into a glass column plugged with a small quantity of glass wool. The final size of the column was 50 x 1 1/2 cms. Columns were eluted from an overhead reservoir of buffer (50-60 cms above the upper surface of the Sepharose). The buffer flow was controlled by a teflon stop cock. Most liposome separations were carried out at room temperature. Before use for the separation of liposomes from unentrapped material, columns of new Sepharose were exhaustively eluted with saline to remove the sodium azide which is used as a bacteriostatic by the manufacturers. In addition, each column was pretreated with a solution of empty liposomes (buffer entrapped) to prevent the adsorption of lipids onto the surface of Sepharose beads (Huang, 1969). Following these pretreatments, columns could be used several times without any noticeable effect in liposome separation. Between uses all columns were stored at 4° C.

Before use, pretreated columns were eluted for 1-2 hrs with the same buffer used to prepare the liposomes (aqueous phase buffer; APB). Sonicated liposome solutions (usually 5 ml) were added to the top of the column and allowed to sink into the Sepharose. The separated liposomes were eluted from column in the void fluid (20-25 ml) where they appeared as a milky suspension. The whole of this turbid fraction was collected in every case. Sonicated liposomes eluted from the column were stored at 4° C under N_2 in the dark until use. After removal of liposomes from the columns, the elution was continued for at least 4 hrs to remove all the unentrapped material.

iv) Concentration of liposomes

In cases where highly concentrated liposome suspensions were required, the liposomes eluted from the column were centrifuged at 100,000 g for 1 hr in a high speed centrifuge (Tyrrell et al., 1976a).

2. Standard liposome preparation

Unless otherwise stated a standard mixture (or multiples thereof) of lipids and cholesterol were used throughout. Before use, a new vial of EPC or PA was checked for purity by thin layer chromatography (TLC) on plastic sheets precoated with silica gel $60F_{254}$ using a chloroform: methanol:water (65:25:4) solvent (Bangham et al., 1974). The dried sheets were sprayed with Dittmer spray (Dittmer and Lester, 1964) to develop the phospholipid spots. Only vials giving single spots on TCL were used.

The standard lipid mixture consisted of

i) 15.0 mg egg lecithin (EPC) (20 umoles)
ii) 2.17 mg cholesterol (CHOL) (5.7 umoles)
iii) either a) 2.5 mg phosphatidic acid (PA) for negatively charged MLVs (2.85 umoles)
or b) 0.81 mg stearylamine (SA) for positively charged MLVs (2.85 umoles).

Neutrally charged MLVs were made by omitting (iii) all together. These constituents give molar ratios of 7 mols lecithin: 2 mols cholesterol: 1 mol charged lipid. The aqueous phase buffer (APB) used to rehydrate the lipids was usually 5 mM phosphate buffered saline pH 7.4 unless otherwise stated. Normally 2.5 ml of buffer was added for each unit (15.0 mg lecithin) of standard lipid mixture used.

3. Drug entrapment in liposomes

Additions to the standard mixture were made when drugs were to be entrapped in the liposomes.

i) Drugs to be entrapped in the lipid phase (LP) of the liposomes were mixed with the lipids in chloroform before rotary evaporation.
ii) Drugs be entrapped in the aqueous phase (AP) were dissolved in APB before its addition to the dried lipid film.

B. Preparation of drugs and phospholipids used in liposome manufacture

1. Drugs

i) Actinomycin D (ACT-D)

The manufacturers of actinomycin D supply this drug in sterile ampoules, containing 500ug powdered drug/vial with mannitol as an aid to water solubility.

The active drug was removed from the mannitol powder by the addition of 5 ml chloroform to the vial. The resulting orange solution was filtered through Whatman no.1 paper. The insoluble mannitol residue was washed repeatedly with chloroform and similarly filtered. Finally the filter paper itself was washed in a large volume of chloroform. All the washings were pooled and the drug solution in chloroform was rotary evaporated to a constant volume (4 ml). Standard volumes of ACT-D in chloroform : methanol (1:1) were prepared. The absorbance at 443 nm was measured following an initial estimation of the absorbance maximum. A standard dilution curve proved to be linear when read against a chloroform : methanol blank and, assuming 100 % extraction of the drug, the extinction coefficient (E_{443} = 23480) was in good agreement with published work (E_{443} = 24800 $\pm$ 200) (Katz and Weissbach, 1963). The amount of ACT-D extracted (assuming each vial contains 500 ug ACT-D) was between 85 and 93 %. Each ACT-D solution was made up to 100 ug drug/ml in chloroform and then stored in the dark at -20° C until use.

Radiolabelled ^{3}H-ACT-D was mixed with the stock solution of the drug to give 25uCi/mg cold ACT-D in the chloroform in which the lipids were to be dissolved. ACT-D entrapped in the AP of liposomes was carried out using the manufacturer's preparation of ACT-D (including mannitol) which had been dissolved in APB to give a stock solution of 100 ug/ml. Radioactive ACT-D was added to the stock solution in APB, just before use, to give a final concentration of 10 uCi/mg cold drug. This stock solution was used as free (unentrapped) drug for animal experiments.

ii) Bleomycin (BLM)

For indium (^{111}In) labelled bleomycin, see Materials.

Iodination of bleomycin

Two methods were used to iodinate bleomycin with ^{125}I :

i) directly in the imidazole ring with iodine monochloride (Meyers et al., 1975);

ii) indirectly by reaction with the Bolton and Hunter reagent (N-succinimidyl 3- (4-hydroxy 5-^{125} Iodophenyl) propionate) (Eckelman et al., 1976; Bolton and Hunter, 1973).

i) Direct iodination of bleomycin

Iodine monochloride labelling was performed exactly according to the method described by Meyers et al., (1975). Briefly, 140 ug (0.1 umol) of bleomycin sulphate was dissolved in 200 ul saline/citrate buffer (0.15 M NaCl, 0.02 M trisodium citrate pH 7.0). Radioiodine (2mCi ^{125}I) in 250 ul buffer was added to the BLM along with 30 ul of iodine monochloride (3.3 mM). The reaction mixture was shaken on a flask shaker for 5 minutes and then left for 2 hrs at room temperature in the dark. Iodinated BLM was separated from I' IO_3^- and other reactants by preparative chromatography on a Sephadex G-10 column (50 ml bed volume) eluted with sterile 0.9%NaCl. Fractions (1ml) of the eluant were assayed spectroscopically (280nm) and for ^{125}I in a gamma-counter. Fractions with the highest specific activities were pooled and stored in sterile containers at 4° C until use.

ii) Indirect iodination

The Bolton and Hunter reagent (1.1 mCi : 2.0 mCi/ml in benzene and 0.2 % dimethylformamide) was placed in a small test tube kept at 4° C. The reagent was blown to dryness with a stream of N_2. The walls of the tube were thoroughly washed with more benzene which was also evaporated with N_2. An ice-cold solution of bleomycin sulphate (75 mg/ml in water) was prepared and 100 ul very gently added to the dry reagent. After gentle vortex mixing for two minutes the reaction was allowed to proceed at 4° C for a further 15 minutes. The reaction was stopped by the addition of 0.5 ml glycine/borate buffer (0.2 M glycine in 0.1 M sodium borate buffer, pH 8.5) at 0° C. After 5 minutes the reaction mixture was chromatographed on a Sephadex G-10 column using sterile 0.9 % NaCl as eluent. The fractions (1ml) were assayed for BLM and ^{125}I content as before and fractions with the highest specific activity were pooled and stored at 4° C for use.

The specific activities of the radiolabelled bleomycin solutions were

i) iodine monochloride (^{125}I-ICL-BLM) method 643.13 uCi/umol
ii) Bolton and Hunter (^{125}I-BH-BLM) method 354.66 uCi/umol.

Neat aliquots of these iodinated BLMs were used in the preparation of liposomes for liver subcellular fraction and tissue distribution studies. Free (unentrapped) drug solutions for these studies were diluted in 0.9 % NaCl to give the same amount of radioactivity.

iii) Preparation of ^{125}I-labelled asparaginase

Erwinia asparaginase (EC 3.5.1.1) (860 units/mg protein) was supplied as a lyophilized powder (12,000 units/vial). Sterile distilled water (1ml) was added to one vial of enzyme and the solution gently mixed before being allowed to dissolve for 30 minutes at room temperature. Undissolved material was removed by centrifugation in a bench centrifuge at 3,000 rpm for 10 minutes. The concentration of the enzyme was calculated following an estimation of the protein content of the solution (Lowry et al., 1951). Asparaginase was radioiodinated by the iodine monochloride method (McFarlane, 1958; 1963). Briefly, 10,000 units of asparaginase in water were pre-oxidized with iodine in sodium acetate buffer (pH 4.5) before being mixed with 0.55 mCi ^{125}Iodine. After the reaction had finished the labelled enzyme was eluted from an Sephadex G-10 column with sterile 0.9 % NaCl. Fractions (1ml) were collected and those with high radioactivity were pooled and estimated for protein content (Lowry et al., 1951). The specific activity of the final preparation was 72.4 uCi/mg protein (0.084 uCi/unit). Enzyme activity was not assayed.

Before entrapment in liposomes, a small amount (usually 20 uCi) of ^{125}I-asparaginase was added to an aqueous solution of cold enzyme to give a final concentration of 3 mg enzyme/ml. For use as a drug (i.e. in partial hepatectomy studies) 10 ml of this solution was used with the standard mixture to make the liposomes. For subcellular fractionation and tissue distribution studies the radiolabelled preparation was used without any addition of cold enzyme. Free drug solutions were prepared by dilution with 0.9 % NaCl to give similar radioactive counts to the liposome solutions.

2. Preparation of ^{14}C-labelled lecithin

Pure egg phosphatidylcholine was radiolabelled on the choline moiety with ^{14}C-methyl groups derived from ^{14}C-methyl iodide (Stoffel, 1975). Some modifications to the published methods were used since preliminary experiments showed that dimethyl formamide (DMF) continued to contaminate the phosphatidyl-N, N dimethylethanolamine after the initial demethylation step. This was overcome by subjecting the demethylated lipid to the Bligh and Dyer (1959) extraction technique.

One mmole EPC (750 mgs) and 6 mmoles of 1,4-diazobicyclo (2,2,2) octane were dissolved in 20 ml DMF and then refluxed under a stream of N_2 for 5 hours. The progress of the reaction was followed by removing

20 ul samples from the refluxing mixture at 30 minutes intervals and subjecting them to TLC (see Standard liposome mixture). Unreacted samples of EPC in DMF at 36 mgs/ml served as controls. The reaction was stopped once the treated PC ran as a single spot on the TLC plate. The refluxing solution was cooled to 4° C in ice and 40 ml of ice cold 1 N HCl was added. This mixture was then shaken well for 10 minutes on a flask shaker at 4° C. Finally, 20 mls chloroform was added to the mixture and the shaking were continued for a further 10 minutes. The contents of the reflux flask was then centrifuged at 3,000 rpm for 10 minutes at 4° C to ensure total separation of the chloroform layer from the upper DMF layer. The upper layer was extracted twice more with chloroform and then these extracts were pooled and rotary evaporated to near dryness.

The residue from this evaporation was then extracted with 10 ml of a chloroform : methanol (1:1) solvent before water (9ml) was added. The mixture was again shaken well and after the addition of a further 2 ml of chloroform the mixture was centrifuged, the chloroform phase removed and the aqueous phase extracted twice more with chloroform. After pooling the chloroform phase and washes, a sample of the mixture was spotted onto a filter paper and assayed for contaminating DMF which could be seen as a deep red spot when viewed in green light. Samples still contaminated with DMF were rotary evaporated to near dryness and re-extracted as above.

In initial experiments the demethylated EPC was further purified on a silicic acid column (25 x 1 1/2 cm, 150 g SiO_2) by applying increasing concentrations of methanol in chloroform (Bangham et al,1974). No further increase in yield, as measured by lipid phosphorus(Baginski et al., 1967) or purity, as measured by TLC, was gained by this column chromatography; it was omitted in later preparations.

Remethylation of the phosphatidyl-N, N-dimethylethanolamine was performed at 4° C with 25 umols $^{14}C\text{-}CH_3I$ by the published method (Stoffel, 1975). The ^{14}C-EPC was purified by silicic acid chromatography and the final fractions were pooled and concentrated. The remethylated lipid was compared with pure EPC by TLC. The concentration of EPC was assayed by measuring lipid phosphorus (Baginsky et al., 1976) and the radioactivity of the sample was counted by liquid scintillation spectroscopy (q.v). The total recovery of ^{14}C-EPC was 0.21 mmol and the specific activity was 16.5 uCi/mmol.

C. Estimation of drug distribution in rats following intravenous injection of either liposome entrapped or unentrapped drugs

i) Intravenous injection

Rats were injected I/V into a tail vein which had previously been dilated by warming the tail under running water (40-50° C) for about one minute. The volume of injection was usually 1 ml unless otherwise stated. No immediate toxicity or death was ever seen in animals following injection in this way, either with liposome or free drug solutions. Animals which accidentally received the injection into the tissues, i.e. other than I/V, were not used in the experiments.

ii) Plasma concentration of the drugs

Blood was collected from the rats at timed intervals after injection by rapid exsanguination of the animals into a heparinized (20 ul heparin(5,000 units/ml)) and graduated test tube (15ml). Exsanguination was achieved by decapitation of the animals. The blood volume was measured and the blood was then mixed on a vortex mixer for 10 seconds. It was stored at 4° C and then later centrifuged at 3,000 rpm for 15 minutes in a bench centrigue. The plasma layer was removed and a sample taken for scintillation or gamma-counting. The remaining sample was stored frozen at -20° C for future reference.

iii) Tissue concentration of drugs

a) Removal

Apart from the liver which was treated separately, tissues used for distribution studies were removed from the animal as soon as possible after decapitation (usually between 2 and 4 minutes). The tissues were washed twice in ice cold saline to remove contaminating blood and then blotted dry on filter paper. The small intestine was flushed out twice with saline to remove all the faeces. Tissues were then individually weighed before being frozen at -20° C (table 2:1).

b) Homogenization

In animals injected with gamma-emitter-labelled drugs, the tissues were thawed at room temperature by the addition of an appropriate volume of water for each tissue type (Table 2:1). They were then chopped into small pieces with scissors and made up to a final concentration with more water before being counted in a gamma-counter.

Table 2:1 Organ weights in rats *used in tissue distribution studies and volumes used for homogenization.

Tissue	Weight gms (mean ± St.dev.)	(n)	Volume of water added per whole tissue for thawing and/or homogenization (ml)	Final volume of chopped or homogenized tissue prior to counting for radioactivity (ml)
Liver	4.890 ± 0.423	(225)	2g/8ml sucrose	8
Spleen	0.565 ± 0.129	(150)	2.5	5
Kidney (2)	1.532 ± 0.272	(150)	5	10
Small Gut	5.752 ± 1.801	(150)	10	15
Lung	0.979 ± 0.186	(150)	4	10
Heart	0.696 ± 0.101	(125)	4	10
Brain	1.746 ± 0.126	(125)	4	10
Muscle (thigh)	0.591 ± 0.116	(125)	4	10
Skin (1 cm^2)	0.698 ± 0.253	(125)	2.5	5
Thymus	0.512 ± 0.121	(36)	2	5
Testes (2)	1.244 ± 0.177	(36)	5	10
Bone (femur)	0.751 ± 0.134	(36)	5	5

* Male Sprague Dawley rats (95 - 135 g body weight). n = number of values.

In experiments with ^{125}Iodine-labelled compounds the tissues were homogenized as described below for beta-emitting compounds. Half of the final volume of the homogenate was counted for gamma-activity. The other half was treated with ice cold trichloroacetic acid (TCA) to a final concentration of 10 %. The resulting protein precipitate was then centrifuged at 3,000 rpm on a bench centrifuge for 10 min and the pellet washed once with 10 % TCA. Finally the pellet was suspended in water to its original volume and then counted for gamma-activity.

Tissues from animals injected with beta-emitting drugs were homogenized in a fixed volume of water (Table 2:1) with a Potter-Elvehjem homogenizer (tight pestle). During homogenization of usually 2 - 3 strokes (but 6 - 10 strokes for skeletal muscle, heart and lung tissues) the mortar was cooled in a beaker of melting ice. After homogenization the solution was decanted into a graduated measuring cylinder, and the mortar was washed twice with water. Finally the homogenate was made up to a fixed volume with water. After rapid mixing on a vortex mixer, a sample of the solution was removed and counted for radioactivity.

iv) Liver concentration of drugs

The rat livers used for drug distribution studies were removed as quickly as possible following exsanguination of the animal. The whole excised liver was washed twice in ice cold saline, blotted dry with filter paper and then weighed. Between 1 and 4g of the liver were chopped with scissors into ice cold 0.3 M sucrose and homogenized at once using a Potter-Elvehjem homogenizer (3 strokes - tight teflon pestle) cooled in a beaker of melting ice. All subsequent steps were carried out at 4°C.

For control studies a sample of the injected material (usually 0.5 ml) was added to the chopped liver from a normal (i.e. untreated) rat before homogenization. The livers were then treated exactly as before.

Subcellular fractionation of the liver was carried out by differential centrifugation according to the method of Gregoriadis and Sourkes (1967) which has previously been used for liposome distribution studies (Gregoriadis and Ryman, 1972a). In brief, the liver homogenate was centrifuged at 6000 g.min to sediment the cell debris and nuclei. The supernatant was saved and the pellet washed once with 0.3M sucrose.

These supernatants were pooled and recentrifuged at 4.2×10^5 g.mins to pellet the lysosomal/mitochondrial fraction. Finally, supernatant from this centrifugation was separated into a post-lysosomal/mitochondrial pellet (microbodies) and a clear supernatant (cytosol) at 100,000 g for 1 hour.

The purity of the fractions and latency of the enzymes in the lysosomes and mitochondria was assayed by studying N-acetyl-beta-glucosaminidase (NABGase) (lysosomes) and succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium-reductase (INTase) (mitochondria). For the lysosomal marker, the activity in the liver subcellular fractions of NABGase was measured by the method of Borooah et al.(1961) in the presence of Triton X-100 (TX-100 : 0.1 % final concentration) using p-nitrophenyl-2-acetamido-2-deoxy-beta-D-glucopyranoside as substrate and measuring the release of p-nitrophenol following incubation at 37°C. Similarly, INTase activity, in the presence of TX-100 (0.1%), was assayed by the methods of Pennington (1961) and Hinton et al.(1969) using sodium succinate as substrate and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium as the electron acceptor.

D. BIOLOGICAL EFFECTS OF DRUGS

i) Partial hepatectomy of rats.

Partial hepatectomy refers to the removal of the median and left lateral lobes of the liver (about 67 % by weight). Male Sprague-Dawley rats weighing between 100 and 150 g were starved for 24 hours and then subjected to partial hepatectomy under sterile conditions using ether anaesthesia (Higgins and Anderson, 1931). Sham-hepatectomy was performed in the same way but no liver was excised. The peritoneal cavity was closed with a nylon suture on an atramatic needle and the skin was sealed using Micheli clips. The animals were allowed to recover in single cages before being transferred to larger ones. This method resulted in more than 95 % recovery of the animals after the operation.

ii) Drug treatment

Unless otherwise stated, drug treatment of the hepatectomised animals was performed immediately after the operation by a single I/V dose of either free or liposome entrapped drug into a tail vein.

iii) Measurement of nucleic acid synthesis in the regenerating livers .

The synthesis of DNA and RNA was determined at timed intervals after partial hepatectomy. The animals were injected I/P. with either 1.5 uCi of (6-^{14}C) orotic acid (61 mCi/mmol) diluted in 0.5 ml 0.9 % NaCl (for RNA) or 10 uCi of (6-^{3}H) thymidine (5 Ci/mmol) diluted in 0.5 ml 0.9 % NaCl (for DNA). The radiolabel injections were all made 1 hour before the animal was killed.

The liver was removed from the animal as quickly as possible and washed. A 20 % homogenate was made in 0.25 M sucrose. An aliquot (1 ml) of this homogenate was treated according to the method of Schneider (1957) to extract the nucleic acids according to their differential solubility in trichloroacetic acid (TCA). The DNA and RNA were separated by the Schmidt and Thannhauser (1945) method, for solubilizing RNA in KOH, as modified by Munro and Fleck (1966).

The RNA was assayed by the orcinol reaction (Mejbaum, 1939) using yeast RNA as a standard. The assay of DNA was carried out by the diphenylamine method of Burton (1956) as modified by Giles and Myers (1965) using calf thymus DNA as a standard. The uptake of radioactivity into DNA and RNA was calculated following liquid scintillation counting of the DNA or RNA isolated from the livers of rats. In experiments to assess the effects of drug treatment on RNA synthesis using ACT-D liposomes or free ACT-D, the livers of the treated rats were not only assayed for DNA and RNA synthesis, but also subjected to subcellular factionation as described previously.

E. INTERACTION OF LIPOSOMES WITH PROTEINS

i) Isolation of rat plasma lipoproteins

Rat plasma lipoproteins were prepared by the standard method of flo tation ultracentrifugation using KBr to adjust the density of the plasma (Hatch and Lees, 1968). The plasma was centrifuged sequentially at densities of 1.006, 1.063 and 1.21 g/ml (Hatch and Lees, 1968). According to Windmueller and Levy (1967) rat plasma contains the following lipoproteins fractions : first fraction ($d < 1.006$) contains chylomicrons and particles, the second fraction ($d \pm 1.006$) contains VLDL. A third fraction (d : 1.006 - 1.035) contains pure low density lipoproteins although not the total amount. The fourth fraction (d : 1.035 - 1.063) contains a mixture of low and high density lipoproteins;

the next fraction (d : 1.063 - 1.21) contains most of the high density lipoproteins and the final fractions (d > 1.21) contain VHDL floating above a viscous plasma protein pellet.

Although it is agreed that rat low and high density lipoproteins overlap in their densities (Koga et al., 1969; Lasser et al., 1973), the range of densities required to separate these two lipoproteins fractions has not been established. For this reason the mixed fraction (d : 1.035 - 1.063) was treated as if it contained only low density lipoproteins (range 1.040 - 1.050 : Lasser et al., 1973) which were not isolated separately. The preparation of density solutions for lipoprotein separations is shown in Table 2:2 and the centrifugation scheme is described in Table 2:3. Fractions were removed by gentle pipetting of the upper layers using an Eppendorf pipette (500 ul). After removal the isolated lipoproteins were dialysed overnight at 4°C. against 10 liters of phosphate buffer, pH 7.4 containing 0.01 M. Na_2HPO_4, 0.124 M NaCl and 0.3 mM Na_2EDTA. Samples of the isolated fractions were then counted for beta and gamma radioactivity. Fractions were stored at 4°C or used for Laurell double immunoelectrophoresis (q.v.).

ii) Preparation of rat blood for lipoprotein analysis

Only rat plasma was used for lipoprotein fractionation, since serum is not as good as plasma for the study of some lipoprotein fractions (eg : chylomicrons) (Hatch and Lees, 1968).

At timed intervals following injection of radiolabelled liposomes containing either ^{14}C-cholesterol (LP)+^{111}In-BML (AP) or ^{14}C-EPC(LP)+ ^{111}In-BLM (AP) rats were decapitated and bled into graduated glass test tubes containing 1 mg Na_2EDTA and 20 ul of thimerosal (sodium ethylmercurithiosalicylate) (15 mg/ml in water, pH 8.0) per millilitre of blood. This anticoagulant system has been reported to be the best for obtaining plasma whilst inhibiting enzymatic activity and improving the reproducibility of ultracentrifugal patterns of lipoproteins (Hatch and Lees, 1968).

The blood samples were kept at room temperature and processed as quickly as possible. The plasma was obtained by a quick centrifugation (3,000 rpm, 5 min in bench centrifuge). An aliquot (4 ml) of plasma from each rat was used for analysis of lipoproteins.

Table 2:2

Preparation of density solutions for lipoprotein analysis

Density solutions were prepared according to the methods of Hatch and Lees (1968). Using NaCl solutions adjusted with NaBr.

i) Density = 1.006 gm/ml

Sodium chloride (11.40 gm), disodium EDTA (100 mgs) and 1 ml 1 N NaOH were dissolved in 500 ml water. A further 503 ml of water was added to this solution.
(0.195 moles NaCl/1000 gm H_2O : 0.195 molal NaCl)
(stock solution)

ii) Density = 1.182 gm/ml

Sodium bromide (24.98 gm) was added to 100 ml of the above stock solution (0.195 molal NaCl 2.44 molal NaBr).

iii) Density = 1.478 gm/ml

Sodium bromide (78.32 gm) was added to 100 ml of the above stock solution (0.195 molal NaCl; 7.65 molal NaBr).

The densities of these solutions were checked by weighing standard volumes (10 ml) at 20°C.

iii) Association of Plasma Proteins with liposomes

Empty liposomes were prepared in the usual way using four times the standard quantities (i.e. 80 umol EPC etc) in 10 ml 0.145 M NaCl pH 7.4. The liposome suspensions were divided into two equal volumes; one half was sonicated (2.5 min), the other was treated as for hand-shaken MLVs (see liposome preparation methods).

A sample of each of these preparations (8 umols EPC : 2.28 umols Chol:1.14 umols charged lipid) was mixed with an equal volume of freshly drawn rat or human plasma. This mixture (usually 2 ml) was incubated

Table 2:3. Isolation of rat plasma lipoproteins by density-flo tation ultracentrifugation

Centrifugation				Pre spin contents (final volume 6ml)	Post spin		
Step N°	Speed $x10^{-3}$ g	Time hr	Temp. °C		Supernatant (upper 2 ml)	Infranatant (lower 4ml)	Contents
1	26	1/2	16	4ml plasma + 2ml.d = 1.006 mixed on vortex	To step 1 wash	To step 2	-
1 (wash)	26	1	16	2ml step 1 supernat. + 2ml.d = 1.006 mixed on vortex + 2ml water layered on top	Saved d = < 1.006	Discarded	Chylomicrons and particles
2	114	16	16	4ml step 1 infra-natant + 2ml.d = 1.006 layered on top	Saved d = 1.006	Used to re-suspend pellet. To step 3	VLDL
3	114	16	10	4ml step 2 infra-natant + 2ml.d = 1.182 mixed on vortex	Saved d = 1.006-1.063	Used to re-suspend pellet. To step 4	LDL + some HDL
4	114	40	10	4ml step 3 infranatant + 2ml.d = 1.478 mixed on vortex	Saved d = 1.063-1.20		HDL
						Saved d = > 1.20	VHDL
						Pellet resus-pended in 4ml 0.145 M NaCl	other plasma proteins

for 1 hr in a shaking water bath at 37°C. After incubation the mixture was centrifuged at 100,000 g for 1 hour six times with a pellet-wash in 0.9 % NaCl between spins. The final pellet was resuspended to 1 ml in 0.9 % NaCl. Samples were then used for immunoelectrophoresis.

In a further series of experiments, both the liposomes and the plasma samples were precentrifuged at 100,000 g for 1 hr before mixing and incubation. In addition, liposomes were mixed with complement destroyed human plasma (heated at 56° C for 1 hr).

The protein associated with the liposome pellet was estimated by the method of Lowry et al. (1951) using BSA as a standard. Lipid phosphorus was measured by the method of Baginski et al. (1967).

iv) Two-dimensional immunoelectrophoresis

Antigen-antibody crossed electrophoresis (Laurell electrophoresis) was performed according to the method of Clarke and Freeman (1968). This method was used for i) the determination of proteins associated with liposomes following their incubation with either rat or human plasma (qv) and ii) checking the purity of rat lipoprotein fractions following density flo tation.

The first dimension was run on 5 x 5 cm glass plates covered with 1% agarose in 0.03 M veronal buffer pH 8.6. Samples (2-15 ul) were added with a microburette to 20 ul holes punched into the solidified gel. Acetylated albumin, stained with Evans blue, was used as a standard.The electrophoresis was performed in a M.B.I. electrophoresis tank using 10 volt/cm (at 500 m. Amps) between wicks dipping into 0.03 M veronal buffer kept at 4° C (Churchill thermocirculator). The electrophoresis was continued until the stained acetylated albumin had reached the anode wick (70-90 minutes) and then stopped.

The second dimension was run using strips (0.5 cm wide) from the first dimension containing the sample proteins. The 5 x 5 cm slab gel contained 1% agarose in 0.03 M veronal buffer pH 8.6 with the addition of an appropriate antiserum or specific antibody (1-5 ul/cm^2). A similar electrophoresis system was used as for the first dimension except that the voltage between the wicks was adjusted to 2 1/2 volts/cm. The gels were electrophoresed at room temperature overnight (> 18 hrs). The agarose gels were washed for 3 hours in 0.9% NaCl and for 30 minutes in distilled water. After drying, the plates were stained in 0.1% amidoblack (in methanol: water:glacial acetic acid, 5:5:1) for 2 hours and then destained in this solvent for 5 minutes until the background

was transparent. Finally, the plates were rinsed twice in 70% methanol and left to dry. Lipoprotein fractions were stained with Sudan black (saturated solution in 60% ethanol) (Segal et al., 1973).

The antibodies used were either anti-whole human plasma or anti-whole rat plasma antiserum (Dako) or, in the case of human alpha2-macroglobulin, specific anti-human alpha-2-macroglobulin antiserum (Behringwerke).

For identification of rat alpha-macroglobulin, samples of pure rat alpha-macroglobulin (6 mg/ml) and rat albumin (3 mg/ml) were added to washed liposome pellets. These rat plasma proteins, which were immunologically pure, were a generous gift from Mr L. Louis of this institute.

A typical example of immunoelectrophoresis is shown in plate 3.1 (chapter 3) where 5 ul of whole rat plasma has been immunoelectrophoresed in two directions. The agarose gel of the second dimension contained 1 ul/cm^2 whole rat plasma antiserum.

F) Measurement of Radioactivity in samples.

i) Gamma Radioactivity.

Radioactivity from ^{125}I and ^{111}In was counted using a well type sodium iodide crystal detector constructed in this institute. In addition, these isotopes were counted in a Wallac-LKB gamma counter programmed for maximum counting efficiency for each isotope (between 73 and 79%). Samples were normally made up to a fixed volume (2 or 5 ml depending on the sample) in a stoppered plastic tube. Counting was continued for 100s with a cut-off at 100000 counts.

ii) Beta Radioactivity.

Beta-emitting isotopes (^{3}H and ^{14}C) were counted in a Wallac-LKB beta counter with discriminators set to give a maximum number of ^{3}H counts without any cross-over into the ^{14}C channel and minimal cross-over of ^{14}C into the ^{3}H channel.

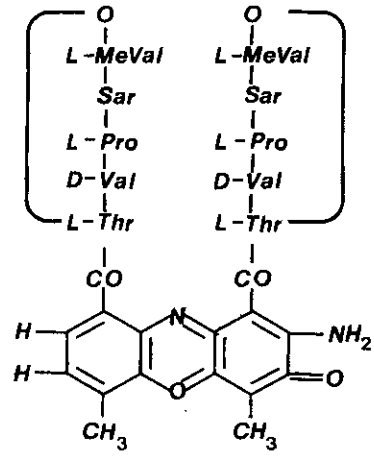
Aqueous samples (final volume 1ml) were added to 10ml of a liquid scintillation emulsion containing 2,5 diphenyloxazole (PPO) 0.6% and 1,4.bis-2(5-phenoxyazolyl)benzene (POPOP) 0.05% in toluene which was then further mixed with triton X-100 (2:1 v/v) before use (Patterson and Greene; 1965). Samples were dispensed into low potassium glass vials, well mixed with scintillant on a vortex mixer and then left

overnight at 4°C in the dark to dissolve the tissue homogenates or fractions. Counting was carried out for 10 minutes. This system gave counting efficiencies in unquenched samples of 41.78 $\pm$ 7.1% ^{3}H and 69.37 $\pm$ 8.07% ^{14}C (mean $\pm$ st dev. n=4).

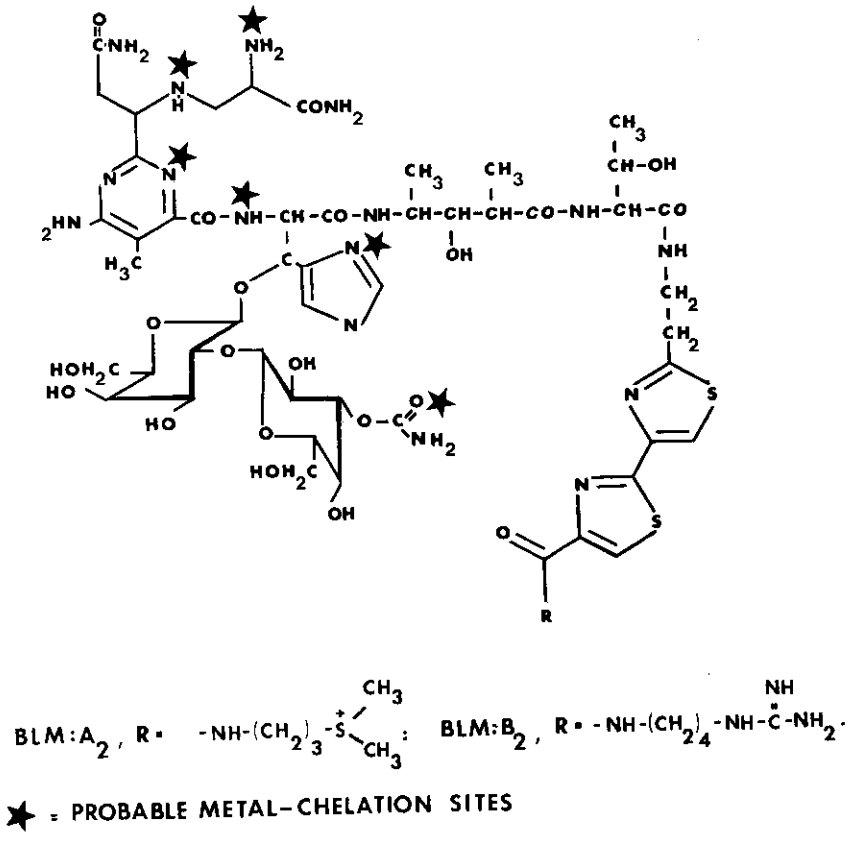
The activities in each sample (dpm) was calculated from the counts obtained following the addition of an aliquot (10ul) of a standard solution of either ^{3}H (^{3}H-water - 25 nCi/sample) or ^{14}C (U-^{14}C cyclohexane-20 nCi/sample) to precounted vials. Where both isotopes were present samples of both standards were added to each vial at different times. The method of internal standardization was adopted because of the variable protein and colour content of the individual tissue samples.

Figure 2:1.

A) Structure of Actinomycin D (Brockman, 1974)



B) Structure of Bleomycin (after Roy et al., 1981).



Section 2:2

Basic studies on drug entrapment in liposomes.

The drugs used in this study were used in initial investigations of some of the parameters of drug entrapment. These parameters include: lipid composition, aqueous phase buffer (APB) composition and pH, length of sonication time and the amount of lipid peroxidation. In addition, some other studies were carried out into the leakage of drugs from liposomes as well as the microelectrophoresis and microviscosity of drug-containing liposomes. The major part of the entrapment studies were performed with ^{3}H-actinomycinD entrapped in the lipid phase (LP) or ^{111}In-bleomycin entrapped in the aqueous phase (AP) of liposomes. The additional studies were done using iodinated bleomycin and asparaginase as well. A brief summary and a short discussion of these results is presented here since this data does not conveniently fit with the in vivo results of the following chapters.

For both types of drug the term entrapment, in its strictest sense, means drug sequestered within the vesicle. However, as others have pointed out (Tyrrell et al., 1976a; Weissmann et al., 1978) in practice entrapment can be considered as the amount of drug associated with the liposomes (ie: not necessarily inside) ; the measurement of "real" entrapment and latency of a compound requires the strict application of certain criteria. In the case of the drugs entrapped here these criteria have not been applied so that the drugs used should more reasonably be termed "liposome-associated" drugs. In addition, without some degree of care is even possible to find drugs which will coelute with liposomes from a column or cosediment in a centrifuge and these drugs will not be even "associated" with the liposome (in the sense of being, however loosely, "bound" to the lipid structure), this point has also been discussed by others (Szoka and Papahadjopoulos, 1980).

i) Actinomycin D.

Liposomes were made from egg phosphatidylcholine which was supplemented by increasing amounts of cholesterol and/or charged lipid. Overall the amount of ^{3}H-ACT-D associated with the vesicles did not appear to be very dependent upon the lipid composition. Slightly higher entrapments were found in stearylamine-containing liposomes than in anionic or neutral liposomes but only when the concentration of this

cationic lipid was high (EPC: CHOL : SA molar ratio 7:2:5). Similarly, slight increases in drug entrapment were found when the concentration of cholesterol was increased irrespective of the presence or the species of charged lipid used. Values for entrapment were in the range 7.6-14.05 ug ^{3}H-ACT-D/umol EPC used (7.6-14.05% of the original material) depending upon the lipid composition. However, since these experiments were not repeated it is possible that the differences are not significant. During experiments on the entrapment of ACT-D into anionic liposomes, it was noticed that initial concentrations of between 1.0 and 1.5 mg ACT-D/umol EPC resulted in the formation of aggregates when the buffer was added to the dried lipid. These aggregates were not broken up by sonication (5 min) and remained at the top of a Sepharose column which eluted liposomes and free drug normally. Analysis of the aggregates on silicic acid columns (see methods) did not reveal any phospholipid or cholesterol associated with these structures which remained at the top of these columns also. For this reason liposomes were made in the future at concentrations of 500 ug ACT-D/umol EPC or less.

Small changes in the ionic strength of the aqueous phase buffer, from distilled water up to 15mM phosphate buffered saline, did not affect the amount of ACT-D entrapped irrespective of the lipid composition of the liposomes. Similarly, in an experiment which changed the pH of the buffer, in one or two unit steps, from 2.5 to 10.0 did not produce any substantial changes in entrapment of ACT-D.

Increases in sonication time of the liposomes from zero up to 60 minutes also failed to change the quantity of ACT-D entrapped in liposomes. The peroxidation of liposomal lipids in the presence and absence of added ACT-D (LP) was measured by the method of Klein (1970). The presence of ACT-D slightly increased the initial oxidation of lipids when compared to empty liposomes but, whilst these empty liposomes continued to be slightly oxidized during the course of sonication (even in the presence of nitrogen), the extent of this oxidization was reduced in the ACT-D liposomes although the differences might not be significant with more values.

ii) Bleomycin

The entrapment of ^{111}In-bleomycin into the aqueous phase of liposomes was also investigated. The quantities of the lipid constituents (i.e.: the ratio of CHOL and/or charged lipid to EPC) were changed

Table 2:4. Entrapment of BLM and $^{111}InCl_3$ in liposomes

DRUG (initial conc^{n}/ umol EPC)	RADIOLABEL ENTRAPMENT (per umol EPC) (% initial concentration)		
	Liposome composition*		
	Negative (PA)	Positive (SA)	Neutral
^{111}In-BLM (41.25 ug)	28.18 ug (68.32)	2.22 ug (5.382)	1.55 ug (3.76)
^{125}I-BLM (B/H) (0.375 ug)	44.51 ng (11.87)	16.42 ng (4.38)	15.07 ng (4.02)
^{125}I-BLM (ICL) (0.266 ug)	22.57 ng (8.47)	13.67 ng (5.13)	12.74 ng (4.78)
^{125}I-BLM (ICL) (0.266 ug) + $^{111}InCl_3$ (37.76 pg)	^{125}I-58.46 ng (8.77) ^{111}In-20.29 pg (53.75)	^{125}I-42.13 ng (6.23) ^{111}In-2.28 pg (6.04)	^{125}I-35.4 ng (5.31) ^{111}In-1.47 pg (3.91)
$^{111}InCl_3$ (37.76 pg)	22.019 pg (58.35)	1.933 pg (5.12)	0.827 pg (2.19)

* Composition of liposomes : 8 umol EPC: 2.28 umol CHOL: 1.14 umol charged lipid/ml PBS
Drugs added to aqueous phase.

whilst the concentration of the drug/mol EPC was kept constant (41.25 ug BLM/umol EPC; 66ug BLM/ml APB). The results uniformly showed that anionic liposomes (containing phosphatidic acid; PA) entrapped 12-20 fold more drug/mol EPC than cationic or neutral liposomes irrespective of their cholesterol content. However, the entrapment of ^{125}I-BLMs in anionic liposomes was very similar to that in cationic and neutral liposomes and was also similar to the entrapment of ^{111}In-BLM in cationic and neutral liposomes.(Table 2:4). On the other hand the entrapment of $^{111}InCl_3$ in the presence or absence of ^{125}I-BLM in anionic liposomes was similar to that found for ^{111}In-BLM and substantially higher than that found in cationic or neutral liposomes. (Table 2:4).

Changes in the ionic strength of the buffer did not change the entrapment of ^{111}In-BLM neither did changes in pH except that at pH 2.5 the entrapment in all liposome types was substantially reduced.

Increases in sonication time reduced the entrapment of ^{111}In-BLM in anionic liposomes so that the quantity (per umol EPC) entrapped after 60 minutes sonication was only 60% that found after 2 minutes sonication. Similar reductions were found with cationic and neutral liposomes. The presence of ^{111}In-BLM (AP) in liposomes did not appear to affect the oxidation of liposomal lipids during sonication.

iii) Leakage of drugs from liposomes

Three preparations of anionic liposomes containing either i) ^{3}H-ACT-D (0.125 mg/umol EPC) or ii) ^{111}In-BLM (0.4125 mg/umol EPC) or iii) ^{125}I-BLM (B/H) (0.5 ug/umol EPC) were made according to the standard method. Following sonication and column chromatography the preparations were centrifuged at 100,000g for 1 hour to eliminate SUVs. The pellets of small MLVs were resuspended in 5 ml PBS to give concentrations of 170.2 ug ^{3}H-ACT-D/ml, 1.13 mg ^{111}In-BLM/ml and 0.112 ug ^{125}I-BLM/ml. These solutions were incubated in stoppered glass tubes under N_2 for 5 hours in a shaking water bath at 37°C. At timed intervals, samples (250 ul and 50 ul) were removed from the tubes which were then flushed with N_2 and restoppered. The larger sample (250 ul) was immediately added to a Sepharose column (5 ml bed volume eluted with PBS) at 4°C. The 50 ul sample was counted for radioactivity. The MLVs eluted from the columns were resuspended into 2.5 ml PBS and 1.0 ml samples were counted for radioactivity.

After 5 hours of incubation, the tubes of liposome-drugs were

flushed well with N_2, wrapped in aluminium foil and stored at 4°C. Additional samples were taken from these stock solutions in the same way over periods up to 6 days following that start of the incubation. The results (Fig. 2.2) were calculated as % of the total (precolumn sample) found in the column elutants corrected for volumes.

iv) Microelectrophoresis of liposomes

The microelectrophoresis of liposomes and drug containing liposomes was performed according to the method of Bangham et al., (1958a, 1958b) in a cylindrical microelectrophoresis cell at 25°C using platinum electrodes.

The electrophoretic mobilities of unsonicated liposomes containing either buffer or drugs was measured in phosphate buffered saline (Table 2:5). Similarly electrophoretic mobilities were determined for empty liposomes in the presence of plasma. In this case unsonicated liposomes were incubated in fresh rat plasma (heparinzed) for 1 hour at 37° C in a water bath (final concentration 17.2 mg lipid ml^{-1} in 50% plasma). Before electrophoresis the liposome suspensions were diluted 1:5 with 0.145 M NaCl and the pH adjusted to 7.2 if necessary with 0.1 M NaOH or 0.1 M HCL (see chapter 3 for these results).

Table 2:5 Electrophoretic mobilities of drug-liposomes

Preparations (*)	Direction towards	Mobility** $um.s^{-1}.v^{-1}.cm^{-1}$
Empty (16 umol EPC/ml)	Anode	1.27 ± 0.13
ACT-D (14.55 g/mol EPC)	Anode	1.19 ± 0.06
BLM (5.636 g/mol EPC)	Anode	1.31 ± 0.095
ASPase (53.80mg/mol EPC)	Anode	1.75 ± 0.13

(*) Each preparation contains the same quantities of lipids : (16 umol EPC; 4.56 umol CHOL; 2.28 umol PA)/ml phosphate buffered saline. Drug concentrations are given as quantity of drug/mol lecithin in the preparation. All preparations were diluted 1:10 in PBS before use.

(**) Electrophoresis conditions : 50 volts; 2 mAmps; 16 cm cylindrical cell (1 mm radius); polarity reversed after every reading. Values are the mean electrophoretic mobility (± S.E.) from at least 10 readings.

Legend Fig. 2:2. Leakage of drugs from liposomes.

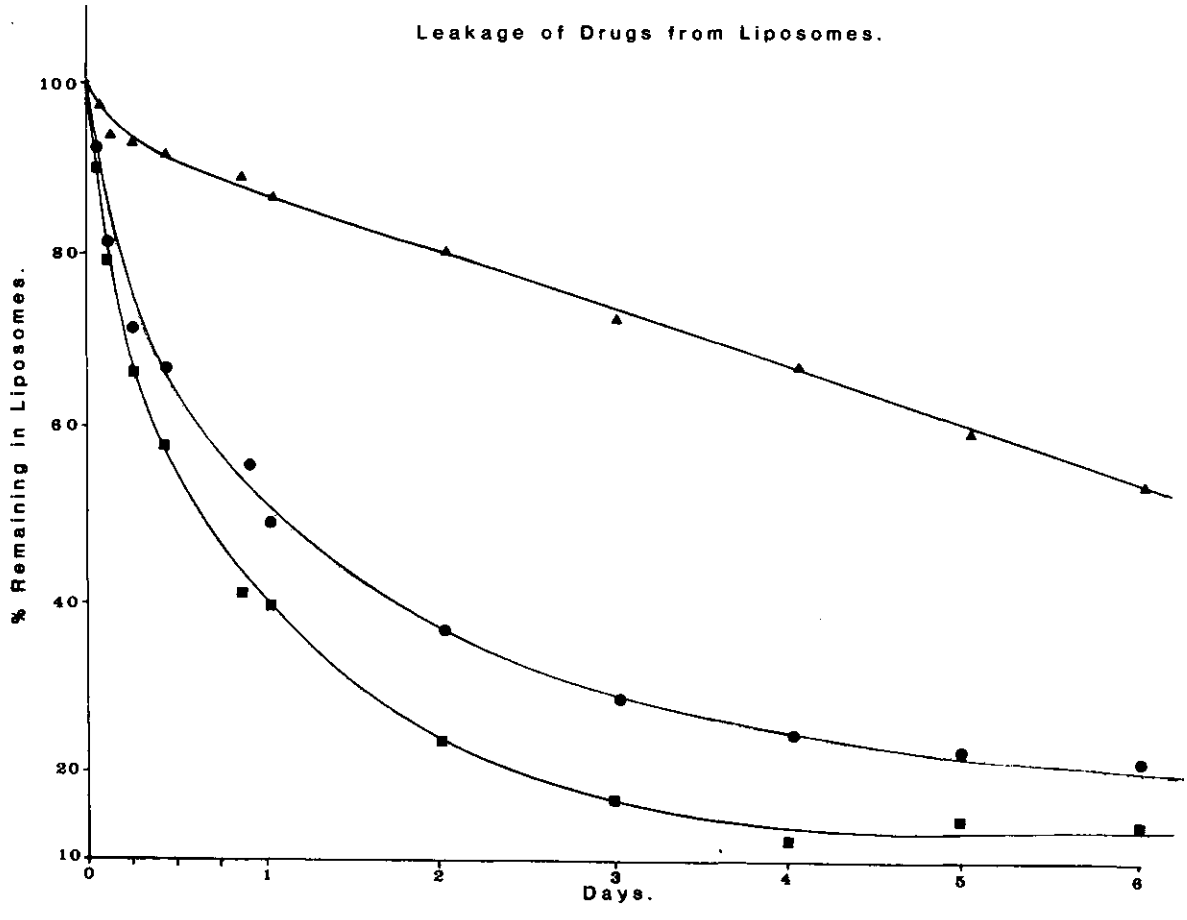
Liposomes containing ^{3}H-Act-D (LP), ^{111}In-BLM (AP) or ^{125}I-BLM (AP) were prepared by the standard method. After column chromatography the solutions were centrifuged to remove SUVs and resuspended in 5 ml PBS at concentrations of 170.2 ug ^{3}H-ACT-D/ml, 1.13 mg ^{111}In-BLM/ml and 0.112 ug ^{125}I-BLM/ml. These solutions were incubated for 5 hours at 37° C and then stored at 4° C for 6 days. Samples of each drug solution were removed at timed intervals and rechromatographed.

The graph shows % of the initial drug concentrations still liposome-associated during a 6 day period.

Circles : ^{3}H-Act-D (LP) liposomes

Squares : ^{125}I-BLM(B/H)(AP)liposomes

Triangles : ^{111}In-BLM (AP) liposomes



Some preliminary experiments using washed red blood cells were carried out in order to establish the stationary phase of the cylindrical capillary tube in the presence of increasing amounts of plasma using the method outlined by Bangham et al., (1958a). The results demonstrated that up to 25% (V/V) plasma in PBS there is little difference in the position of the stationary phase. However, at 50% plasma there were increases in the stationary phase value which are perhaps due to protein buffering of the capillary walls resulting in very slow movements of the red cells which, in turn, made calculation of the stationary phase very difficult. For this reason electrophoresis of liposomes in plasma was restricted to 10 % plasma solutions.

v) Microviscosity of liposomes

The fluidity of the lipid bilayers of liposomes containing entrapped drugs was compared with empty liposomes using an Elscint MV-1 microviscosimeter according to the method of Cogan et al., (1973) using the fluorescent probe 1,6diphenyl 1,3,5hexatriene (DPH) (Shinitzky and Inbar, 1974). Sonicated standard liposome preparations (containing actinomycin D, bleomycin, asparaginase or buffer) were diluted 1:1 with 2×10^{-6} M DPH in PBS to give a final optical density (od) of about 1.0 at 450 nm. This solution was incubated at 37°C for 1 hour in a water bath to ensure that the maximum fluorescence intensity (excited at 366nm) was obtained. The liposome preparations were then diluted with PBS repeatedly to give a range (6) of absorbances at 450nm between 0.1-1.0 od.units. Samples (5.0 ml) of each of this diluted range were then cooled or warmed to temperatures between 5 and 50°C. Silica cuvettes were filled with 2.5 ml of the samples and put into the temperature controlled chamber of the microviscosimeter.The temperature of each sample was checked just before measurement of fluorescence polarization with a thermocouple thermometer.

The degree of fluorescence polarization (460 nm) was quickly measured (<10 sec) for each dilution of each sample at the different temperatures. Graphs of the degree of polarization at each temperature against optical density were linear over the range 0.1-1.0 od.units. Extrapolation of these lines to intercept the ordinate (o.d. = 0) gave a range of fluorescence polarization values for each temperature. In this way light scattering and turbidity of the liposome preparations was eliminated from the polarization readings.

Thereafter, the method of Cogan et al., (1973) was followed. The

values of the microviscosity were deduced from the fluorescence polarization readings (at $O.D._{450} = 0$) by the use of tables, to give the function $\frac{n}{T.\ t}$ where n= microviscosity (poise), T= absolute temperature and t= excited state lifetime of the inbedded DPH.

The excited state lifetime of the DPH was determined by the indirect method of Schinitzky and Barenholz (1974) which involves the temperature profile of the fluorescence intensity. These measurements were carried out using the same equipment with empty liposomes and DPH diluted as before at temperatures between 5 and 50°C. A graph of t against temperature was used to find the appropriate value of t for the above equation. The derived values of n (microviscosity) were plotted as log n versus $\frac{1}{T}$. The graphs demonstrated that the fluidity of the lipid bilayers increased linearly over the range 5-50°C and in no case did the presence of entrapped drug cause the induction of a phase transition when compared to similarly prepared empty liposomes. The values of the microviscosity and for the fusion activation energies of the preparations are given in Table 2:6.

Table 2:6 Microviscosity of liposomes at different temperatures.

Temperature	Liposomes* containing			
	Empty	ACT-D	BLM	ASPase
°C	Microviscosity (poise)			
5	6.21	8.33	7.03	8.09
25	1.85	2.17	1.85	2.02
37	0.88	1.02	0.83	0.98
45	0.62	0.63	0.60	0.69
	Fusion activation energy (delta E) (Kcal. $mole^{-1}$) 5 - 48° C			
	10.28	10.09	10.19	10.35

* Liposomes contained 7:2:1 molar ratios of EPC:Chol:PA prepared according to the standard method. All preparations were sonicated for 2.5 minutes.

Entrapment : - ACT-D (LP); 14.50 ug/umol EPC
- BLM (AP); 25.61 ug/umol EPC
- ASPase (AP); 50.32 ug/umol EPC

DISCUSSION

i) Effect of lipid composition on drug entrapment.

a) Actinomycin-D

The entrapment of ACT-D in the lipid phase of liposomes did not, overall, appear to be very dependent upon the lipid composition. There were differences between charged liposomes on the one hand and neutral liposomes on the other, but the differences are not large and they may not be significant. The slightly higher drug entrapment in charged liposomes is probably a reflection of the greater size and internal volume of these vesicles when compared to neutral ones (Bangham, et al., 1974) and this arises from the charge repulsion between the bilayers. Since ACT-D is soluble both in water and in lipid, the entrapment differences between liposomes of the same composition, but differing in their charge, may be attributed to varying amount of drug in the aqueous spaces. Lipid phase entrapment does not imply that the drug is immobilized within the lipid. Indeed, the drug will leak from the liposomes quite readily (e.g.: fig.2:2). The highest concentration of entrapped drug was found in liposomes containing the highest amount of charged lipid. In the cationic liposomes the increase appears to be directly related to the quantity of SA in the preparation whilst this does not hold for the PA concentration of anionic liposomes. There is a suggestion that some interaction may be taking place between the charge or with stearylamine itself and ACT-D. The nature of this interaction has not been investigated but it is probably due less to lipid-drug interactions than a decrease in leakage of cationic ACT-D from the aqueous spaces through the cationic bilayers.

The liposome entrapment of ACT-D has been studied by Gregoriadis (1973b) who was the first to entrap ACT-D in liposomes (LP) and he reported that, in general, entrapment in the lipid phase was higher than in the aqueous phase. Moreover, cationic liposomes entrapped more drug (LP) (8.8 %) than anionic liposomes (5.2 %); this was attributed to a homologous charge effect retarding drug leakage. In addition, it was noted that EPC-containing liposomes entrapped less drug than DPPC-containing liposomes. The values obtained in the present study are slightly higher than those obtained by Gregoriadis (1973b) using the same liposomes; however, his liposomes did not leak whereas in this study liposomes release ACT-D over a period of several days (fig. 2.2).

Juliano and Stamp (1978) have also studied the effect of the incorporation of cholesterol and charged lipids on the entrapment of ACT-D in liposomes. In large MVLs the incorporation of ACT-D (LP) was in the order cationic > anionic > neutral; cholesterol almost doubled the amount of drug entrapped irrespective of charge. However, sonication of these liposomes released about 30 % of the captured material (probably, therefore, from the aqueous phase). Nevertheless, Juliano and Stamp (1978) reported up to 50 % capture in cationic, cholesterol-containing, liposomes and 20 % of the drug in similar anionic liposomes. These values must be compared with the average ($\pm$ st.Dev.) % entrapped figures of 8.12 $\pm$ 0.6 (neutral), 10.82 $\pm$ 1.33 (anionic) and 10.53 $\pm$ 1.93 (cationic) found in this study. The most important differences between these two investigations are that a greater proportion of both cholesterol and charged lipid (SA but not PA) was used by Juliano and Stamp; the liposomes were not sonicated and only trace amounts of drug were used. This last difference will mean that there might be less "competition" for sites of interaction with the bilayer than if an excess of drug is present (as in the current study and that of Gregoriadis (1973)). Juliano et al., (1978) concluded that ACT-D intercalates into the hydrocarbon layer.

Sudies by Kaye et al., (1981) on ACT-D (LP) capture by small cationic MLVs gave a value of 15.3 $\pm$ 1.4 % (mean $\pm$ S.E) for the entrapment. The ratio of drug weight to lipid weight was found to be important (1:100) and is twice as high as that used in this study (1:225). Nevertheless, the capture reported by Kaye et al., (1981) is similar to the values found in this study and that of Gregoriadis (1973b) (10.53 % and 8.8 % respectively) and markedly different from the 50 % reported by Juliano and Stamp (1978).

Rahman et al., (1975; 1978) and Papahadjopoulos et al.(1976) have also entrapped ACT-D in liposomes. The first group of workers used non-sonicated neutral MLVs with the ACT-D in both AP and the LP, although no quantification of entrapment has been given. Papahadjopoulos et al., (1976) entrapped ACT-D (AP) in negative (PS) SUVs to obtain up to 60% entrapment and this was accounted for by electrostatic interaction between the weakly cationic ACT-D and the anionic PS. Such a high aqueous phase ACT-D entrapment was not found during the course of this work (not shown) using the anionic PA in place of PS; nor did Gregoriadis (1973b) find such an association. Perhaps the serine in PS is more important than the charge in determining the amount of drug entrapped.

Explanations for these data are primarily concerned with the effects of cholesterol and charge on the reduction of leakage of entrapped solutes (Scarpa and de Gier, 1971) although, in SUVs at least, the inclusion of greater than 30 mol % cholesterol in the vesicle produces a sharp and progressive increase in vesicle diameter (Johnson, 1973), which might also entrap a larger volume. Finally there is the possibility that ACT-D interacts with cholesterol; such an interaction has been shown with other cyclic-peptide-containing antibiotics (e.g.: Valinomycin) (de Gier et al., 1978) although these are ionophores with a known specificity for cholesterol. However, in the studies on ACT-D/lipid aggregate formation, cholesterol could not be found associated with the aggregate after column chromatography.

b) Bleomycin

The high drug association between ^{111}In-BLM and anionic liposomes was investigated further by the use of ^{125}I-iodinated BLM in place of the ^{111}In-label. Radioiodination of BLM was carried out by two separate methods: direct iodination by the iodine monochloride method (ICL-BLM) and indirect iodination by the acylating agent N-succinimidyl 3-(4-hydroxyphenyl) proprionate (B/H-BLM).

Iodination with iodine monochloride is thought to occur in the modified histidine portion of the BLM molecule (fig. 2.2B) (Meyers, et al., 1975) since the drug does not contain tyrosine. Neither the A or B components were preferentially labelled and so Meyers, et al., (1975) assumed that the terminal amine groups do not influence the reaction. Similarly, the indirect iodination procedure, which is specific for amino groups, does not appear to iodinate the terminal amines, but more probably labels these groups in the beta amino-alanine and amino-beta-(4-amino-6-carboxy-5-methylpyrimidine-2yl) propironic acid residues of BLM (Eckelman, et al., 1976). Finally, iodination does not appear to effect the ability of BLM to chelate di-and trivalent cations (Eckelman et al., 1976). These results suggest that iodination does not effect the overall charge of BLM when compared to the ^{111}In-chelated label. Indeed, Eckelman et al., (1976) found no differences in the profiles of unlabelled and iodinated BLMs eluted from anion exchange columns.

Yet, the results (Table 2.4) show that iodinated BLM has lost its ability to interact with anionic liposomes whilst the entrapment into other liposomes is unchanged and that $^{111}InCl_3$ retains a very high

anionic vesicle entrapment even in the presence of iodinated BLM. Hwang and Mauk (1977) have also reported that $^{111}In^{3+}$ interacts with phospholipid polar head groups. The data suggest , therefore, that either BLM is binding to the anionic liposomes via its chelated cation radiolabel ($^{111}In^{3+}$: which may or may not be, finally, still bound to the BLM) or that the other (i.e.: the noniodinated) amine groups are responsible for the drug/liposome interaction found with the ^{111}In-labelled species. Umezawa (1973) has titrated BLM and shown 3 pKa values in addition to the terminal guanido groups. The 4-amino group on the beta-(4-amino-6-carboxy-5-methyl-pyrimidine-2yl) proprionate has a pKa of 2.9, the pKa of the imidazole ring of histidine is 4.7 and that of the amino group of beta-aminoalanine is 7.3. Coupled with the guanido group, this ensures that BLM is strongly cationic at around neutral pHs. However, if any of these groups are "lost" following iodination, then the charge interaction may not be strong enough either to support the high entrapment found with ^{111}In-BLM or to participate in other interactions such as insertion of the peptide into the lipid layer.

ii) Influence of sonication on drug entrapment and lipid oxidation in liposomes.

In MLVs the aqueous volume entrapped per mole phospholipid ranges between 1 and 4 litres (Szoka and Papahadjopoulos, 1980) but this will be increased when up to 10 mol % of charged lipid and/or cholesterol are included in the bilayer (Bangham et al., 1974). For SUVs the capture volume ranges from 0.2-1.5 L/mole PL depending upon the lipid composition (Szoka and Papahadjopoulos, 1980). Obviously the sonication of MLVs to produce SUVs reduces the capture volume because of a reduction in the surface area to volume ratio and a reduction in the number of bilayers; it is not totally compensated for by the increase number of vesicles.

Damage to liposome lipids during sonication has been previously described (Klein, 1970; Hauser, 1971); two forms of chemical change occur: i) oxidation of unsaturated hydrocarbon chains and ii) the cleavage of covalent bonds. Although sonication in an inert atmosphere (e.g.: N_2 or Ar) reduces i) (Klein, 1970) it has little effect on ii) (Hauser, 1971).

a) Actinomycin D

Since the length of sonication time did not substially affect the amount of ACT-D associated with EPC, it may be concluded that much of the drug was entrapped in the lipid phase because size reduction leads to lower aqueous phase volumes.

The slight protective effect of ACT-D entrapment on the oxidation of liposomal lipids was unexpected and may not be significant. However, this effect suggests that ACT-D may have the ability to "mop up" the free radicals, generated during sonication, which are thought to cause lipid oxidation (Leibowitz and Johnson, 1971; Seligman and Demopoulos, 1973). The portion of the ACT-D molecule responsible for the action is not known but it has been suggested that macromolecules, such as lipophilic proteins and DNA can inhibit free radical reactions (Pietronigro et al., 1976 and references therein). This latter observation might mean that parts of the ACT-D molecule can insert into, and perhaps span, the hydrophobic midzone between the lipid leaflets.

b) Bleomycin

The entrapment of ^{111}In-BLM was reduced by increasing the sonication time of anionic liposomes. It can be expected that at later times (30, 60 minutes) the majority of liposomes are in the form of SUVs and that most of the drug is entrapped in the aqueous phase. However, the amount of reduction in entrapment was not so great as could be expected from the entrapped volumes of MLVs and SUVs. This fact also argues in favour of interactions between the drug and the anionic phosphatidic acid. The oxidation of liposomal lipids was not apparently affected by the entrapment of ^{111}In-BLM into the aqueous phase of liposomes. This may mean that portions of the BLM molecule cannot interact with free radicals (as suggest for ACT-D) and/or are not in close contact with the lipid chains of the phospholipids.

iii) Leakage of drugs from liposomes

Obviously one important aspect of drug entrapment in liposomes is the ability of the carrier to maintain the drug in its encapsuled form over a prolonged period of time. Figure 2.2 shows the leakage of drugs from anionic liposomes over a period of 6 days (time points during the first few hours are not shown).

The best liposome preparation can be seen to be those containing ^{111}In-BLM (triangles). The leakage of the drug was less than 5 % during the initial incubation in PBS at 37° C for 5 hours. Following storage at 4°C for 6 days the liposome-associated drug concentration fell slowly but was never less than 50 % of the starting concentration. However, in view of the high entrapment of $^{111}InCl_3$ (Table 2:4) and the inability of these liposomes to retain equivalent amounts of ^{125}I-BLM (Fig. 2:2 squares) a doubt must remain as to whether the high incorporation and retention represents actual drug or merely entrapped radiolabel.

The leakage of ^{125}I-BLM (squares fig. 2:2) from liposomes does not follow the same pattern as ^{111}In-BLM. The amount of radiolabel decreases rapidly; only 40 % remains liposome-associated 24 hours after manufacture and 12 % remains after 6 days. Thin layer chromatography of the iodinated drug did not show any significant loss of radiolabel from the BLM during prolonged storage at 4° C. It may be assumed therefore that the ^{125}I-BLM leaks out of the liposome during storage.

A similar picture, although with less overall loss, can be seen in ACT-D-containing liposomes despite the fact that the drug is in the lipid phase. One day after manufacture about 50 % of the drug remains liposome-associated. Since ACT-D is soluble in both lipid and water, the drug should pass from the interior of the liposome to the outside as long as there is equilibrium between the two. The lipid solubility of ACT-D will be determined by the degree of ionization of the molecule at the pH and ionic strength of the buffer used to make the liposomes.

Gregoriadis and Neerunjun (1975a) did not find any leakage from ACT-D (LP)-containing liposomes in a bacterial assay for the drug's effectiveness. The reasons for this difference between this work and theirs on drug leakage are unclear, although the incubation time (3 hrs) with the bacteria was quite short ; a leakage rate of only 10 % of the entrapped material would have shown a significant effect. But if the liposomes were older than 36 hours it can be calculated from Fig. 2:2 that the 3 hrs leakage rate would be 1.5 % and this reduces as the age of the vesicle increases. Gregoriadis (1973b), however, reported no leakage from ACT-D (LP) liposomes even on storage for 45 days.

Juliano et al. (1978) have shown leakage of ACT-D from anionic SUV preparations of about 1 % per hour over a three hour period. The addition of serum to the vesicles did not alter the efflux rates. On the

other hand, Rahman et al. (1975) found no significant release of ACT-D (LP) from neutral hand-shaken MLVs even in the presence of serum this was in contrast to upto 50 % release of the ACT-D (AP) from the same vesicles in 1 hour; in this latter case the presence of 15 % serum retarded the release of ACT-D.

It seems therefore that the leakage of ACT-D from liposomes depends on whether the drug is in the LP of AP and this argues strongly for some interaction between the drug and the bilayer. It is tempting to suggest that the initial rapid leakage is due to loss of ACT-D from the aqueous phase of the liposome and thereafter the slower leakage (day 2 onwards) is from the lipid phase. The presence of other molecules co-entrapped with ACT-D in AP liposomes can also effect the drug's leakage. Rahman et al. (1978) suggested that the higher leakage of ACT-D (AP) as compared with Act-D (LP) was due to the presence of mannitol in the aqueous solution.

In an experiment to test leakage of ^{111}In-BLM from liposomes, Gregoriadis and Neerunjun (1975b) re-chromatographed ^{111}In-BLM liposomes after incubating them at 37° C for 5 hours in the absence or presence of culture medium (containing 10 % FCS). More than 92 % of the radioactivity was found to be liposome-associated. This result, therefore, confirms the results shown in Fig. 2:2 (triangles) but is at variance with the quite considerable leakage of ^{111}I-BLM from liposomes which appears to occur in vivo (see Chapter 3).

These results (Fig. 2:2) demonstrate the need to use freshly prepared (or freshly separated) liposomes for drug studies. The leakage of drugs is now considered better studied in the presence of serum or other biological fluids since various recent reports have emphasized the enhanced leakage of drugs from vesicles in their presence (see introduction and Chapter 3).

iv) Microelectrophoresis (Table 2:5)

The movement of particles under an applied electric fields is a phenomenon which has become of increasing importance following the findings of mobility differences between normal and tumor cells ; these findings and the general electrophoretic behaviour of cells has been reviewed (Ambrose, 1966). The surface charge of cells is not due to charged phospholipid molecules, since proteins also play a part. In the case of liposomes (Bangham et al., 1958b; Bangham, 1972; Kimelberg and Papahadjopoulos, 1971a, 1971b) the electrophoretic mobility is

due to the charged head groups in the plane of sheer. The incorporation of cationic molecules (ACT-D, BLM) in liposomes might be expected to alter the electrophoretic mobility, since they could interact with the exposed anionic phospholipid head groups (Bangham et al., 1958b). (The entrapped drug, within the liposome, will not effect the mobility, since this depends upon only the exposed, external groups). In view of the interactions of anionic liposomes with basic proteins (Kimelberg and Papahadjopoulos, 1971a, 1971b; Bangham, 1971, Papahadjopoulos et al., 1975) it was important to establish the overall charge on the drug-containing liposomes before any vesicle-protein interactions (Chapter 3) were considered.

The values obtained (Table 2:5) for the electrophoretic mobilities of empty liposomes are similar to those found by Bangham (1972) for vesicles containing 10 mol % anionic species and may be contrasted with values 7-8 fold higher found in vesicles composed of pure phosphatidylserine (Kimelberg and Papahadjopoulos, 1971a). There were apparent differences in electrophoretic mobility of liposomes containing drugs when compared with empty liposomes suggesting that drugs may be located on the liposome surface after preparation. However, these differences are not statistically significant (Student's t-test).

In preparation for experiments on the electrophoresis of liposomes in protein solutions, an investigation was made of the mobility of washed rat erythrocytes in plasma. Ambrose (1966) has pointed out the advantages of using erythrocytes to standardise microelectrophoresis apparatus. The original work by Abramson and co-workers (quoted in Ambrose, 1966) showed that the presence of non-specific proteins (eg : albumin, fibrinogen and haemoglobin) did not effect the electrophoretic mobility of enthrocytes so long as the medium was of high ionic strength (> 0.1 M). Other workers (Sachterleben et al., 1961) have used a rectangular electrophoresis cell containing semipermeable membranes to protect the electrodes from the presence of the suspending medium. In this way the electrophoresis of blood cells has been carried out in plasma.

Protection of the platinum electrodes was attempted in the Bangham cylindrical electrophoresis cell by the use of dialysis tubing, but undue leakage and the need for much higher voltages caused its abandonment. Instead, liposomes were preincubated in 50 % plasma which was then diluted to 10 % with 0.145 M NaCl. Electrophoretic conditions were chosen so that not only was the polarity reversed after every

measurement, but the potential was applied for no more than 30 seconds at a time.

Increasing the concentration of plasma did not make any appreciable difference to the position of the stationary phase up to a concentration of 25 % plasma in 0.145 M NaCl, nor were the electrophoretic mobilities (not shown) very different from those found in saline alone or 10 % plasma. However, at a concentration of 50 % plasma there was an apparent change in the position of the stationary phase which was almost certainly due to the very slow mobilities which occur in 50 % plasma solutions even under high voltage (up to 100 V). The slow movement made calculation of the electrophoretic mobilities subject to wide error (50 % in some case) ; in addition, longer times were needed so that protein could be seen to be depositing on the electrodes (mostly anode). Since, according to Lamb (1888) and Bangham et al. (1958a), the velocity of a particle is proportional to the square of the distance from the axis of the tube and is independent of the bulk viscosity of the liquid or the hydrostatic pressure along the tube, it seems likely that these properties of the plasma are not responsible for this anormalous result.

V) Microviscosity of liposomes (Table 2:6)

The temperature dependence of the lifetime of the excited state (LES) of 1,6-diphenyl-1, 3, 5-hexatriene (DPH) in anionic liposomes (7:2:1 molar ratio) was measured and at 0°C it was calculated to be 11.2 n.sec. which is within the normal range found by Shinitzky and Barenholz (1974) of 11.4 ± 0.2 n.sec. in both liposomes and paraffin oil. Other values found throughout the temperature range (5 - 50° C) were also similar despite the fact that liposomes of a different composition were used in this study. The mathematical justification for the calculations of LES and the viscosity have been presented by Shinitzky et al. (1971), here we are only concerned with the fact that measurements of the LES at temperatures approaching 0° C will give a plateau value equivalent to its maximum lifetime. Thereafter a graph of LES vs. temperature is approximately linear until a lower limit is reached at higher temperatures (Shinitzky and Barenholz, 1974 ; Shinitzky and Inbar, 1974).

The microviscosity of liposomes can be calculated by studying the fluorescence polarization of DPH which simulates a rotating rod (1.3 nm long) embedded in the hydrocarbon region of the bilayer. Thus, in their fluid state, the acyl chains are free to rotate and this is characte-

ristic of the liquid crystalline state, indeed the rotation of probes in liposome hydrocarbon layers give viscosity values very close to isotropic oils (Cogan et al., 1973). This fluidity can be decreased (higher microviscosity) by the presence of cholesterol (Cogan et al., 1973), but it is not greatly affected by the presence of anionic polar headgroups (Shinitzky and Barenholz, 1974). However, charged liposomes which have slightly less tightly packed acyl chains because of the charge repulsion between their polar head groups, might be made less fluid by the presence of oppositely charged molecules (eg : drugs or high ionic strength buffers) interacting with polar heads and quenching the charges (de Gier et al., 1978). In addition, when the acyl chains are highly disordered (isotropic) the insertion of molecules (eg : cholesterol or drugs) into the hydrocarbon layers will cause an ordering (anisotropic state) and so a reduction in the fluidity. It should therefore be possible to detect the presence of molecules in the bilayer during the isotropic state by the increased microviscosity of the vesicle preparation. Finally, the presence of lysolecithin, either as the result of lipid impurities or as the result of drug action on the acyl chains, can be detected in vesicle preparations because, in the fluid state, these liposomes will have a lower microviscosity than an uncontaminated preparation (Cogan et al., 1973).

Liposomes composed of EPC and cholesterol (up to 500 mol %) (Cogan et al., 1973) or anionic lipids (Shinitzky and Barenholz, 1974) are known to be above their t_c at all temperatures between 0 and 60° C. During the phase transition from liquid crystalline to gel state the rotation of the probe in the hydrocarbon layer decreases rapidly as the acyl chains become more ordered. Finally a new viscosity is established with a slower probe rotation and the new state can be shown to have lost several kilo calories of activation energy (Shinitzky and Barenholz, 1974).

The change of microviscosity with temperature has been shown (Shinitzky et al., 1971) to be a function in a simple exponential form

$$n = Ae^{\text{delta}_a E/RT}$$

ie : similar to the Arrhenius equation. Here n is the microviscosity, A is the Arrhenius constant, Delta E is the fusion activation energy, T

is the temperature in °K and R is the gas constant. Thus in its logarithmic form :

$$\log \; n \quad = \quad \log A + \frac{\text{Delta E}}{2.303R} + \quad \frac{1}{T}$$

The slope of the line or a graph of log n vs 1/T will equal Delta E/2.303R; since 2.303R = 4.576 cal.mole^{-1} degree^{-1} the value of Delta E can be determined from the Arrhenius plot.

Arrhenius plots of the microviscosity - at temperatures between 5 and 48° C - of empty liposomes and liposomes containing the three drugs (ACT-D, BLM and ASPase) used in the study were made. The lines did not show any points of departure from linearity (correlation coefficients were all > 0.98) which suggests that the liposome preparations are in the liquid crystalline state (Cogan et al., 1973; Shinitzky and Barenholz, 1974; Shinitzky and Inbar, 1974) and the fusion activation energies (Table 2:6) confirm this.

Although all the lines converged at higher temperatures, the ACT-D preparation was less fluid at lower temperatures. The slight decrease in fluidity may be consistant with the insertion of ACT-D into the hydrocarbon layer, or it may be a reflection of the protective effect of ACT-D on lipid oxidation noted earlier. The graphs for BLM and ASPase were very similar to the empty preparation. From these results, therefore, it would seem that BLM, although it interacts strongly with anionic PA in the aqueous phase, may not be inserting its peptide portion into the hydrocarbon layer; nor is the charge interaction enough to alter the fluidity of the bilayer.

The values calculated for the Delta E's were very similar (Table 2:6) although that for ACT-D vesicles was a little higher than the others. Shinitzky and Barenholz (1974) have shown that the Delta E at 25° C for empty EPC liposomes is 8 $\pm$ 2 Kcal/mole, but this increases to 10.1 when an 10 % anionic lipid is present (as is the case in these preparations) and a similar increase is seen when cholesterol was incorporated (Cogan et al., 1973). The values for the microviscosities demonstrate the lower fluidity of the ACT-D-containing liposomes at 5° C. At 25° C and 37° C the microviscosity measurements are similar to those found by others (references above) taking into account the presence of different amount of cholesterol and PA in these preparations.

vi) General Discussion

It must be asked if the drugs used in this study are really entrapped within the liposomes. The question is difficult to resolve. Calculation reveals that approximately 1 mol ACT-D reacts with 125 mols of phospholipids. Further calculation suggests that all the ACT-D could be accomodated on the surface of a 350 nm, 10 bilayer, MLV but without a certain knowledge of the size distribution and number of liposomes, this cannot be confirmed. However, the electrophoretic mobility of ACT-D liposome tends to suggest that the surface charge of these liposomes is similar to that of empty liposomes and this would not be the case if cationic ACT-D was all localized on the outermost bilayers. Gregoriadis and Neerunjun (1975a) found that ACT-D (LP), associated with exactly the same liposomes, was not capable of inhibiting bacterial growth even in the presence of 20 % foetal calf serum. This result suggests that the drug remains latent when entrapped and that there is no facilitated leakage in the presence of serum (but c.f. Juliano et al., 1978; and Fig. 2:2).

The insertion of ACT-D into the hydrocarbon region of the liposomal bilayers, which is suggested by the microviscosity data, might be due to the chomophore group of the drug which is more lipid soluble than the pentapeptide chains. The exact position of the two pentapeptide rings is uncertain, if they remain in the aqueous phase they might be amenable to enzymatic or chemical hydrolysis but they might also interact with lipids which constitute the liposomes as has been suggested for other cyclic peptides (eg : ionophores Bangham, 1968; de Gier et al., 1978; Chapman et al., 1978).

The entrapment of ^{111}In-BLM in anionic liposomes, which is not associated with changes in liposome electrophoretic mobility or microviscosity, shows that one BLM molecule is captured for every 5 mols of PA if the ^{111}In radiolable remains attached to the drug. Since PA only constitutes 10 mol % of the liposome it seems unlikely that all the drug could be on the outside of the liposome but theoretically this might be possible in a 350 nm diameter MLV at concentrations approaching one BLM per PA molecule. The size of this glycopeptide drug might hinder such a higher concentration but, if the ^{111}In radiolabel became detached, the hydrated volume of the $^{111}In^{3+}$ ion could be small enough to be accomodated at this high ratio. Again, the relatively unchanged electrophoretic mobilities of ^{111}In-BLM liposomes suggest that surface charge quenching by either the drug or its radiolabel does not occur.

However, iodinated BLMs, which have much lower entrapments into anionic liposomes, could be located on the surface of the carrier.

The capture of ASPase in liposomes was not investigated in such detail as the other drugs since it was found that anionic liposomes of the standard preparation gave an adequate entrapment and almost no leakage on storage (not shown). Fishman and Citri (1975) reported that liposome-entrapped ASPase was much more thermostable than the unentrapped enzyme ; the entrapped ASPase was not subject to attack by proteolytic enzymes, nor would it react with an asparaginase-specific antiserum. Neerunjun and Gregoriadis (1976) demonstrated that in vivo ASPase in liposomes protected mice against anaphylactic shock and serum sickness. All these studies suggest that ASPase is largely entrapped inside the liposome. Confirmation of this can be seen in the unchanged electrophoretic mobilities of ASPase liposomes presented here. In view of the large size of ASPase (Mol wt; 130,000 sedimentation coefficient; 7.43; Patterson, 1975) which suggests a globular protein, possibly in an alpha-helix-like form (Frank et al., 1970; Miller et al., 1971) it seems unlikely that this protein is entering fully into the bilayer although other helical peptides, eg. the MN glycoprotein of erythrocytes and gramicidin A (Chapman et al., 1978) are known to span the membrane. Such a large molecule would be expected to alter the microviscosity of the hydrocarbon chains and this was not found.

CHAPTER 3.

Plasma clearance of liposomes and their interactions with proteins.

INTRODUCTION.

The ultimate goal of chemotherapy is the understanding of the molecular interaction between a drug and so-called "targets" of a cell that will eventually bring about alterations in cellular function or finally the suppression or death of the cell involved. However, the target is the final destination of the drug and the interval between administration and effect allows drugs to interact with normal constituents of the body other than the target. With most drugs only a small proportion of the total amount in the body is at any time in direct contact with the target whilst the higher proportion is localized in parts of the body remote from the target. Yet it is usually this bulk of the drug that governs the kinetics of the movement of the drug through the tissues and so determines its ultimate distribution, time course and intensity of effect. Such considerations also determine important matters such as toxicity and dosage schedules.

The data presented in this chapter concern the interactions which occur between intravenously injected liposomes and biological environment of the blood stream. Specifically, the rate of disappearance of drug-containing liposomes from the circulation will be considered and this will be followed by considerations of the stability of the vesicle in the presence of plasma. Finally, the interactions of plasma proteins with the vesicles and their effects on the surface charge of the carrier will be studied.

SECTION 3:1

PLASMA CLEARANCE OF LIPOSOME ENTRAPPED DRUGS

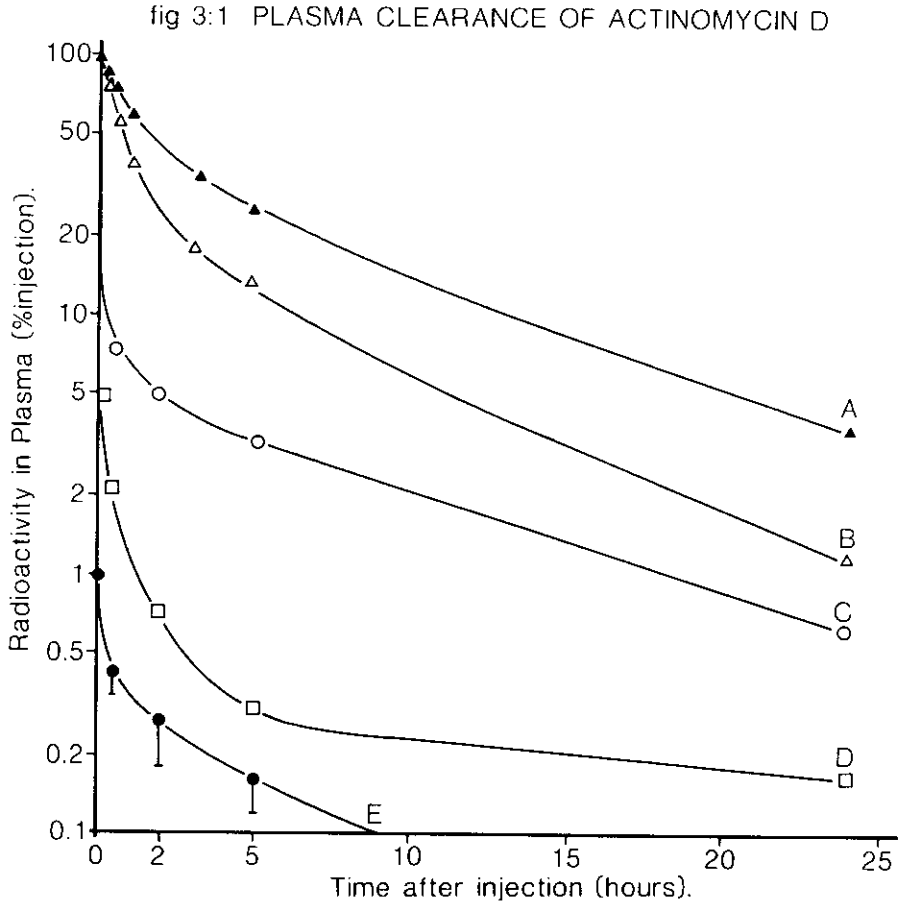
This section considers the clearance of the liposome entrapped drugs actiomycin D, bleomycin and asparaginase following their intravenous injection into rats. The results are given in figures 3:1 (ACT-D) 3:3 (BLM) and 3:4 (ASPase) in most cases the liposome entrapped drug has a longer plasma half life ($T_{1/2}$) than similar injections of untrapped drug.

A) Actinomycin D.

The plasma concentration of either free or entrapped ACT-D remaining in the rat plasma following a single intravenous injection is shown in figure 3:1. The animals (100-120 g body wt) were killed at timed intervals after each injection and cuves A and B show the concentrations of ^{14}C-cholesterol (A) and ^{3}H-ACT-D (B) which remain following an injection of a liposome preparation prepared from twice the standard quantities of lipids. The two cuves A and B show that the two radiolabels are not cleared from the plasma at the same rate and this fact is suggestive of leakage of the drug from the liposome and/or removal of cholesterol from the liposome followed by a slower plasma clearance of the removed cholesterol ($T_{1/2}$ values are approximately A) 75 min., B) 35 min.). Figure 3:2 shows the ratio ^{3}H/^{14}C in this experiment where the ratio ^{3}H:^{14}C (dpms) in the injected preparation was arbitrarily given the value of one. At timed intervals after injection the ratio was calculated from the percents of radioactivity due to each isotope which remained in the plasma. As can be seen, the ratio rapidly declines during the first 3 hours indicating either leakage of ^{3}H or loss of ^{14}C to a plasma componant which is cleared more slowly, or perhaps both. The slower decline after 3 hours coincides with the more linear portion of the plasma clearance curve and is indicative of a more "stable" liposome population (probably SUVs).

Curves C (open circles) and D (squares) (fig.3:1) represent the I/V plasma clearance rates for two different preparations of ACT-D liposomes. Preparation C was similar to B (ACT-D (LP)) except that each rat received a 1 ml injection containing 10.0 ug ACT-D and 1.5umol. EPC. Preparation D, on the other hand, contained ^{3}H- ACT-D entrapped in the aqueous phase.

Figure 3:1.


fig 3:1 PLASMA CLEARANCE OF ACTINOMYCIN D
Radioactivity in Plasma (%injection).
100
50
20
10
5
2
1
0.5
0.2
0.1
A
B
C
D
E
0
2
5
10
15
20
25
Time after injection (hours).

Legend Fig.3:1.

PLASMA CLEARANCE OF ACTINOMYCIN-D.

The plasma concentration (% of injected dose remaining) of ACT-D, either free or entrapped in anionic liposomes, was measured at timed intervals following a single I/V injection into rats.

Triangles; (A) ^{14}C-cholesterol and (B) ^{3}H-ACT-D entrapped in the lipid phase of liposomes. Each animal received :
2 umol EPC : 0.91 umol CHOL (0.088 uCi ^{14}C) : 0.18 umol PA : 10.5 ug ACT-D (25uCi ^{3}H-ACT-D/mg) in 1 ml PBS.

Open circles ; (C) ^{3}H-ACT-D entrapped in the lipid phase of liposomes; quantities as in B except ^{14}C-CHOL omitted and each animal received 10.0 ug ACT-D/ml PBS.

Squares; (D) ^{3}H-ACT-D entrapped in the aqueous phase of liposomes. Each animal received 7.7 umol. EPC : 2.2 umol CHOL : 1.1 umol PA and 25.8 ug ACT-D (10 uCi ^{3}H-ACT-D/mg) in 1 ml PBS.

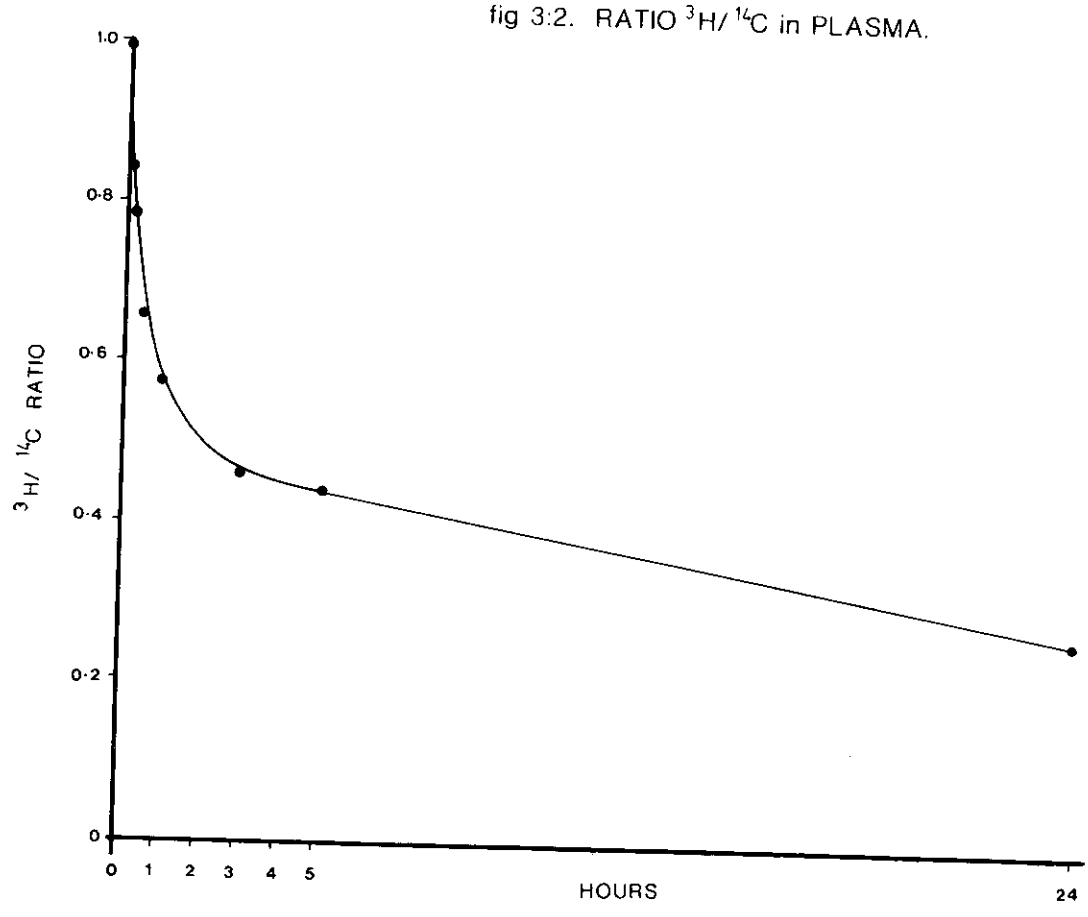
Closed circles; (E) ^{3}H-ACT-D free. Each animal received either 10 ug or 25 ug ACT-D (10 uCi ^{3}H-ACT-D/mg) in 1 ml PBS. Each point is mean (±S.E.) from 3 rats.

Legend Fig.3:2.

RATIO OF ^{3}H: ^{14}C IN THE PLASMA

These values were calculated from curves A and B, figure 3:1. The ratio ^{3}H: ^{14}C in the injected preparation was arbitrary assigned the value 1.

fig 3:2. RATIO $^{3}H/^{14}C$ in PLASMA.



The clearance curves C and D both show an initial rapid plasma loss which paralleled the clearance of the free drug (curve E). Between 3 and 5 hours post-injection both preparations reached plateau concentration although the LP-ACT-D had a 10-fold higher plasma concentration than the aqueous-phase-entrapped drug. Thereafter the plasma values declined more slowly with the LP drug remaining 5 - 8 fold higher throughout the 20 hr. period. The plasma half-lives ($T_{1/2}$) are both less than 5 minutes.

The clearance of free ACT-D (curve E) dissolved in PBS, was plotted from the plasma concentration values of rats which received either 10 or 25 ug of drug. The very rapid initial clearance ($T_{1/2}$ < 5 min.) was followed by a slight slowing of the clearance rate but by 24 hours post-injection there was no detectable ^{3}H-radioactivity left in the plasma.

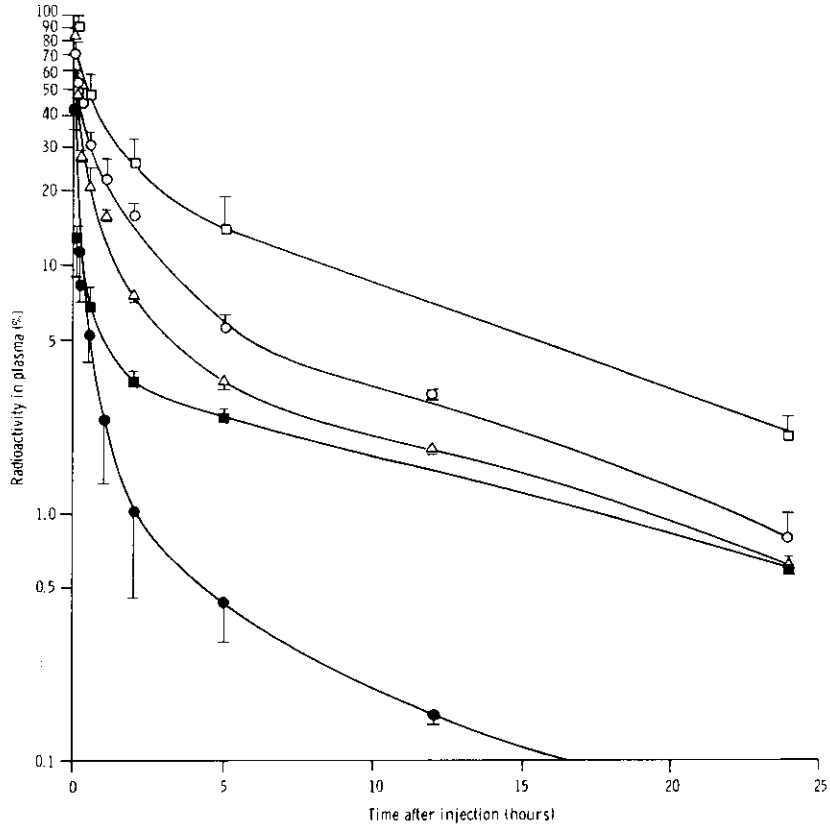
At 72 hours post-injection (not shown) all the curves for liposomal ACT-D converged and gave values between 0.015 and 0.02 % of the injected dose remaining in the plasma whilst at this time the value for ^{14}C- cholesterol (curve A) was 5-fold higher at 0.11 %.

B) Bleomycins.

The disappearance of BLMs from the blood of rats is shown in figure 3:3, where the % of the injected dose remaining in the plasma was measured at timed intervals after I/V injection (1 ml). ^{111}In-BLM liposomes (open squares) were made from four times the standard anionic liposome mixture by the addition of 2 mls of ^{111}In-BLM solution (0.66 mg BLM/ml and 5.5 uci ^{111}In/ml) and 3 ml 0.9 % NaCl. The final liposome suspension contained 55.70 ug BLM/ml and 4 umoles EPC/ml (83.5 % ^{111}In-entrapped). Free ^{111}In-BLM was diluted with 0.9 % NaCl to give approximately the same amount of radioactivity/ml (64.00 ug BLM/ml).

Radioiodinated BLMs were also entrapped in anionic liposomes for injection. Iodine monochloride labelled bleomycin at concentration of 48.57 ug ICL-BLM/ml was entrapped in anionic liposomes (2.5 ml drug + 4 times the standard liposome mixture) to give final concentrations of 0.517 ug BLM/ml and 3.68 umoles EPC/ml (9.26 % ^{125}I entrapped). Free ICL-BLM was diluted with 0.9 % NaCl to give a similar number of ^{125}I radioactive counts/ml (0.53 ug ICL-BLM/ml). Bleomycin radioiodinated by the Bolton and Hunter method at a concentration of 76.69 ug B/H-BLM/ml was entrapped in anionic liposomes (2.5 ml drug + 4 times the standard liposome mixture) to give a final liposome solution containing 1.0 ug

Figure 3:3. Plasma Clearance of Bleomycins



Legend Fig.3:3.

PLASMA CLEARANCE OF BLEOMYCINS.

The plasma concentration (% of injected dose remaining) of various bleomycins, either free or entrapped in anionic liposomes (7:2:1, EPC : CHOL : PA. molar ratio), was measured at timed intervals following a single I/V injection (1 ml) into rats.
Each point represents mean (± S.E.) of 3 animals except closed circles which are 6 animals.

Open squares : ^{111}In-BLM entrapped (AP); 32 umol EPC and 0.44 mg BLM/kg (1.66 mCi ^{111}In/mg BLM).

Open circles : ^{125}I-BLM(B/H) entrapped (AP); 28.6 umol EPC and 8 ug B/H-BLM/kg. (236 uCi ^{125}I/mg BLM).

Open triangles : ^{125}I-BLM(ICL) entrapped (AP); 29.4 umol EPC and 4.14 ug ICL-BLM/kg. (429 uCi ^{125}I/mg BLM).

Closed squares : ^{111}In-BLM free; 0.51 mg BLM/kg (1.66 mCi ^{111}In/mg BLM).

Closed circles : ^{125}I-BLMs(B/H and ICL) free; either 7 ug B/H-BLM/kg (236 uCi ^{125}I/mg BLM) or 4.25 ug ICL-BLM/kg (429 uCi ^{125}I/mg BLM).

B/H-BLM/ml and 3.58 umoles EPC/ml (11.64 % ^{125}I entrapped). Free B/H-BLM was diluted with 0.9 % NaCl to give a similar number of ^{125}I radioactive counts/ml (0.875 ug B/H-BLM/ml).

The mean of the plasma clearance values of free ICL/BLM and B/H-BLM were compared by Students 't' test and found not to be significately different so these values were pooled. The combined plasma clearance curve for free iodinated BLM is therefore the mean (± SE) from six animals at each point.

The clearance characteristics of the liposome entrapped BLMs are similar to that already described for ACT-D liposomes i.e.: a rapid initial clearance followed by a more prolonged slower fall in plasma radioactivity over the period between 5 and 24 hours. The differences between the ^{111}In-containing liposomes and those containing ^{125}I were significant ($p < 0.005$) between 5 hours and 24 hours. The differences between ICL-BLM liposomes and B/H-BLM liposomes were significant ($p < 0.005$) between 2 hours and 12 hours. At all times the concentration of the liposome drug remaining in the plasma was significantly higher ($p < 0.002$) than its appropriate free drug level. The differences between free ^{111}In-BLM and ^{125}I-BLM were found to be significant ($p < 0.002$) between 5 minutes and 24 hours post injection.

The approximate values for the plasma half-lives of the BLM preparations in fig. 3:3 were calculated to be : - i) entrapped ^{111}In-BLM, 31 minutes, ii) free ^{111}In-BLM, 1.5 minutes, iii) entrapped ICL-BLM, 5 minutes, iv) entrapped B/H-BLM, 5 minutes and v) free iodinated BLMs (ICL and B/H) < 1 minute.

C) Asparaginase.

The plasma clearance of ^{125}I radioactivity from ^{125}I-labelled ASPase entrapped in liposomes or free is shown in figure 3 : 4. Liposomes containing ^{125}I-ASPase were prepared by the standard method from freshly labelled ASPase using 0.3 ml ^{125}I-ASPase (0.847 ug protein/ml : 61.36 uCi/ml) and 2.2 ml 0.9 % NaCl added to twice the standard anionic liposome mixture. The final sonicated liposome suspension contained 1.349 ng protein/ml and 1.6 umole EPC/ml. Free ASPase was diluted in NaCl to give an equivalent amount of radioactivity/ml as the entrapped preparation (1.44 ng protein/ml).

The plasma clearance of ^{125}I-ASPase (figure 3:4) revealed that the

liposome entrapped protein was removed from the plasma in the same biphasic manner to that found in the cases of ACT-D liposomes and BLM liposomes. However, the free ^{125}I-ASPase was cleared very differently from the other free drugs. A rapid initial fall in the plasma concentration of the free ASPase was followed by a very much slower, almost linear, plasma decay over the following 24 hours.

Precipitation of plasma proteins with trichloroacetic acid (TCA) revealed that most (> 75 % in all cases) of the ^{125}I counts were precipitated by TCA i.e. : the majority of ^{125}I remained bound to ASPase. The injected material was also subjected to TCA precipitation. In the case of the free ASPase, the protein was 95.04 % precipitatable. Liposomes containing entrapped ASPase were first dissolved by the addition of 0.5 % Triton X-100 (final concentration) before addition of 10 % TCA. The precipitated material contained 71.63 % of the starting material. This loss of radiolabel was probably due to sonication during liposome manufacture.* Storage of sterile ^{125}I-ASPase (free) over a period of two months at 4° C, followed by TCA precipitation, caused the loss of 9.6 % of radiolabel. No tests were made on the enzymatic activity of the ASPase.

The plasma concentrations of TCA ^{125}I counts (solid lines : fig. 3:4) were calculated as the percentage of the TCA pptable injected material. In this way the means of the % remaining in the plasma were compared and significant differences represent additional loss of radiolabel from either the vesicles or the free drug. In general, the TCA material parallels the plasma concentration of the non-precipitated material for both free and entrapped ASPase. Liposomal ASPase was not significantly different ($p > 0.05$) from its TCA.ppt. until 2 hours post-injection, at which time loss of radiolabel from the vesicle occured. However, by 24 and 48 hours (not shown) the liposome preparation had "stabilized" and no further differences were found. Similarly, the differences between the free ASPase and its TCA.ppt. were different at one hour ($p < 0.02$) and 5 hours ($p < 0.001$) but by 24 and 48 hours (again not shown) the differences were no longer significant.

At all times, except 5 minutes post-injection, the liposome-entrapped ASPase was significantly lower ($p < 0.002$) than the free ASPase material whether TCA precipitated or not. Calculation of the $T_{1/2}$ values gave times of 18 minutes for liposomal ASPase and 60 minutes for free ASPase (non-precipitated materials).

* See Corrections page 10, 11.

Legend Fig. 3:4.

PLASMA CLEARANCE OF ASPARAGINASE.

The plasma concentration (% of injected dose remaining) of ASPase, either free or entrapped in anionic liposomes (7:2:1 EPC:CHOL:PA molar ratio) was measured at timed intervals following a single I/V injection (1 ml) into rats. Each point represents the mean (± S.E.) of 3 animals.

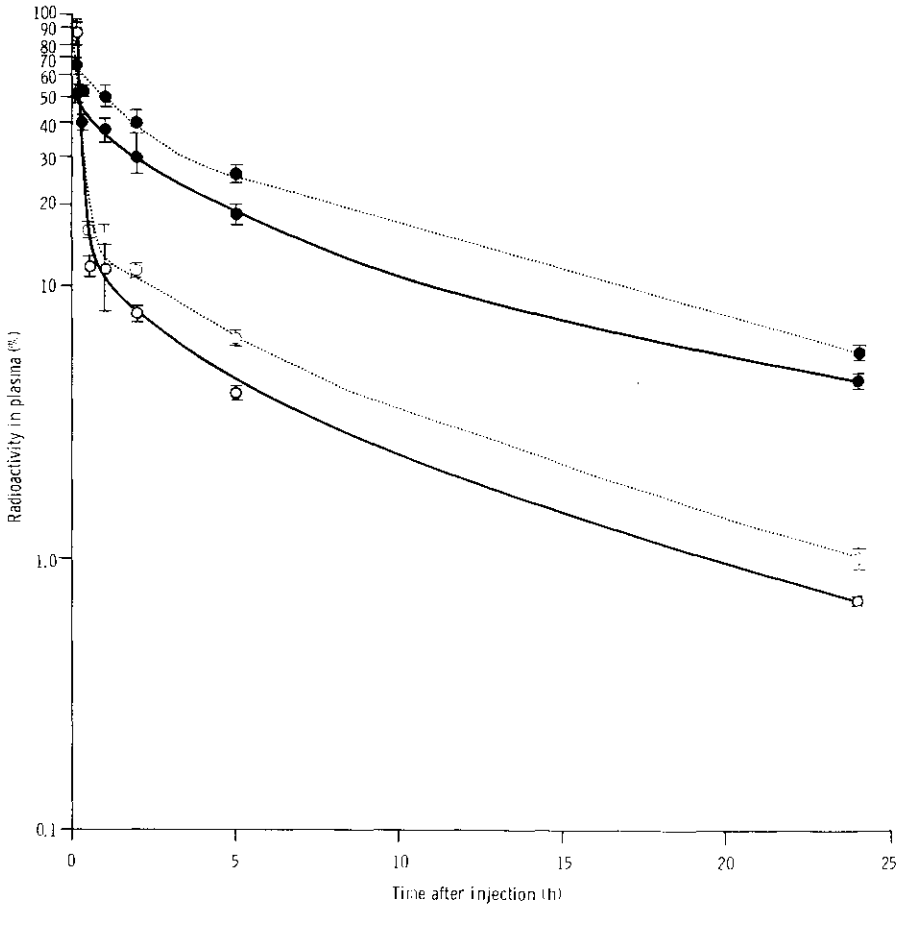
Open circles : ^{125}I-ASPase entrapped (AP); 13.3 umol EPC and 11.24 ug ASPase/kg (72.4 uCi/mg)

Closed circles : ^{125}I-ASPase free; 12.02 ug ASPase/kg (72.4 uCi/mg)

Dotted lines : Total ^{125}I radioactivity.

Solid lines : Radioactivity precipitatable with 10 % trichloroacetic acid.

fig3:4 PLASMA CLEARANCE ASPARAGINASE



D) Discussion of plasma clearance.

The mean diameter, surface charge and chemical composition appear to control the rate of liposome elimination from the blood (see introduction).

The composition of the vesicle has been shown to have the most pronounced effects on liposome plasma clearance. This will be discussed in more detail later in relation to the interactions of liposomes with plasma proteins but these data emphasize the need for caution when attempting to interpret the plasma clearance rates of liposomes especially when, i) the liposome contains low (< 30 mol. %) cholesterol, ii) either phospholipid or cholesterol is radiolabelled as a marker for the bilayer and iii) the entrapped solute is liable to leak from vesicles. A reinterpretation should be made of the earlier work of many authors (Gregoriadis and Ryman, 1972a; Juliano and Stamp, 1975; Gregoriadis et al, 1977a; Kimelberg et al, 1976, 1977) which showed parallel plasma clearances of both lipid and entrapped drug and which claimed changes in clearance rates by the alteration of liposome charge or composition.

The plasma clearance of ACT-D has been studied by several other investigators. Gregoriadis (1973b) showed that ACT-D(LP)-containing cationic liposomes were cleared from the plasma at the same rate as cationic albumin-containing liposomes. The half-life ($T_{1/2}$) of clearance of these liposomes was approximately 1 hour, whereas the free drug had a $T_{1/2}$ of less than 5 minutes. This latter value is in agreement with the values found by other workers (Schwartz et al., 1968b; Rahman et al, 1975; Juliano et al., 1978) and with this work. Kaye and Ryman (1980) have suggested that free ACT-D is bound, at least partially, to plasma albumin and that, following destruction of liposomes in the plasma (q.v.), some phosphatidylcholine is also albumin bound. If the drug (or lipid) binding does not affect the albumin then the plasma clearance should, finally, reflect the $T_{1/2}$ of albumin. In the present study the free ACT-D did not level off after several hours but continued to fall until there was no detectable radioactivity in the plasma. Presumably, the drug dissociated from the albumin quite quickly once the unbound drug was cleared from the circulation.

The $T_{1/2}$ for liposome-entrapped ACT-D, calculated from Gregoriadis (1973b) (1 hour) is close to that found by Juliano et al., (1978) (100 mins) using cationic SUVs. It seems probable, therefore, that the

majority of Gregoriadis' preparation were also SUVs. Using handshaken, neutral, MLVs Rahman et al (1975) reported very low $T_{1/2}$ values for entrapped ACT-D (AP and LP) injected I/V into mice. After 30 minutes less than 2 % of the injected dose (AP) remained in the plasma and less than 0.5 % (LP) remained after the same time. The ACT-D (AP) result is in agreement with this work where a similar value (2.2 %) was found after 30 minutes. However, curves B and C (fig. 3:1) show that, unlike the report of Rahman and co-workers, ACT-D (LP) in anionic sonicated MLVs is cleared much slower than the same liposomes containing the drug in the aqueous phase. It is possible that these differences can be explained by a variation in the clearance rates between mouse and rat (this study) although a comparison of the data of Gregoriadis (1973b) (rat) with that of Kaye and Rayman (1980) (mouse) does not suggest a large difference in the $T_{1/2}$ of small cationic MLVs containing ACT-D (LP). Moreover, in the only published comparative study (Gregoriadis et al., 1977a), the plasma clearance and drug disposition of anionic MLVs was found to be similar in both rats and mice.

A more likely explanation of these differences is to be found in the size heterogeneity of the liposome populations and therefore in the rates of their plasma clearance. Rahman et al., (1978) have shown that, even in the absence of serum, aqueous phase ACT-D will leak faster from liposomes than the lipid-phase-entrapped drug and this is the explanation for the differences between curves C and D (fig. 3:1).

In addition to the size heterogeneity of the liposome populations, a further factor influencing the clearance rates of liposomes is the dose of lipid administered. The animals of curve B received 30 % more lipid than those of curve C. Gregoriadis and Ryman (1972a) reported that liposome clearance was dose dependent and that, for 10 minutes post-injection at least, the relationship between dose and % liposomes remaining the plasma was linear (about 5.7 % increase for each mg total lipid : calculated from Gregoriadis and Ryman). Later Gregoriadis and Neerunjun (1974) reported that excess (empty) liposomes substantially slowed the clearance characteristics of injected liposomes and this was explained by saturation of the sites responsible for the uptake of liposomes. This result was confirmed by Tanaka et al (1976) using high amounts of phospholipid and cholesterol. Mauk and Gamble (1979) and Souhami et al (1981) have also shown that the liver concentration of entrapped drug was dependent upon the injected lipid dose.

The values for $T_{1/2}$ from curves B and A (Fig. 3:1) are close to

that found by Gregoriadis (1973b) and Kaye and Ryman (1980) who both used soniated cationic MLVs. The cholesterol value emphasis the difference in clearance rate between the vesicle bilayer and its contents (figure 3:2). Leakage of ACT-D from the liposome would account for the lower drug : cholesterol ratios during the first three hours. But this result can also be explained by the loss of ^{14}C-cholesterol label to blood components which are cleared more slowly than the vesicle. These two explanations are not mutually exclusive so that cholesterol loss might facilitate the leakage of the drug.

The plasma clearance of BLM-liposomes is very similar to that found for ACT-D. In a study by Gregoriadis et al (1977a) a very similar plasma clearance of ^{111}In-BLM liposomes was found to that of this study. Using liposomes only sonicated for 0.5 min they reported a $T_{1/2}$ of about 10 minutes in rats and mice whereas the value found in this study was approximately 30 minutes. The values calculated from Gregoriadis et al (1977a) for 5 hours and 24 hours are 6.0 and 1.4 % respectively the corresponding values in this study are 14.6 $\pm$ 2.8 and 2.0 $\pm$ 0.4 %. These differences can be accounted for by the longer liposome sonication time used in this study. Using SUVs Mauk and Gamble (1979) have shown that about 72 % of the injected dose of ^{111}In chelated into liposomes remains in the blood 3 hours after injection, this falls to about 6 % in 24 hours.

The clearance of free ^{111}In-BLM presents a different picture from entrapped drug but it was also significantly different from ^{125}I-BLMs. The $T_{1/2}$ was very short, however, unlike the ^{125}I-BLMs, the plasma levels stay elevated so that 0.62 $\pm$ 0.03 % remained in the circulation after 24 hours. Gregoriadis et al (1977a) report that only 1.0 % of the injected dose remains plasma associated after 10 minutes. The differences between these data and that of Gregoriadis et al., (1977a) are difficult to explain since the ^{111}In-BLM was from the same source.

The plasma clearance of free ^{111}In-BLM has been studied by several other groups. There is a general agreement that the levels (% injected dose) of ^{111}In label in the whole blood of mice are of the order 10 % (1 hour) 2.5 % (6 hours) falling to 1 % (24 hours) (Thakur et al., 1973; Robbins et al., 1974; Konikowski et al., 1975; Krohn et al., 1977) and these are similar to the values of this study in rats. However, the levels of other BLM radiolabels (cobalt and technecium; Krohn et al., 1977 or copper; Ryo et al., 1975) are lower than the ^{111}In levels when injected into mice or rats. Also in rats,

Ryo et al., (1975) demonstrated that ^{3}H-labelled BLM plasma levels were very different from the ^{111}In labelled material and quite similar to that of the free iodinated BLMs in this study. Eckelman et al., (1976) and Krohn et al., (1977) have reported on the plasma clearance of iodinated BLMs. In a comparative study, the former authors found that 10 fold more B/H-labelled BLM remained in rat blood 2 hours after injection than the ICL-BLM material but these differences were minimal at 24 hours. The levels of ICL-BLM found by these authors were similar to those found in these present studies but the B/H-BLM result is inexplicably different. Krohn et al., (1977) reported very similar free ICL-BLM levels in mouse blood to those found in this study and to those of Eckelman et al., (1976) in rats.

Native bleomycin is a copper-containing antibiotic (normally Cu^{2+} but occasionally Cu^{3+}) (Umezawa, 1974) and it contains natural chelating sites for metals (Nunn, 1976; Dabrowiak et al., 1978; Takita et al., 1978; fig 2:1b). The metal can be removed by chelating agents and replaced by other metals (Renault et al., 1972) although some metals (e.g.: cobalt) bind so tightly that they are difficult to remove in vitro (Eckelman et al., 1975). Removal of copper from natural BLM results in an increase in potency (Takita, 1959) and a reduction in toxicity (Ischizuka et al., 1967). However, there have been reports of instability of BLM-cation chelates. Cobalt-BLM seems to be more stable in vivo than most of the chelates (Poulose et al., 1975, Rasker et al., 1975) but ^{99m}Tc-BLM dissociates in vivo and the radiolabel binds to serum albumin (Lin et al., 1974). The ^{111}In-BLM is also unstable, Robbins et al., (1974) reported that Cu^{2+} will exchange for $^{111}In^{3+}$ in vitro because the copper chelate is more stable; in vivo they showed dissociation of the complex following I/V injection into mice. Thakur et al., (1973) reported that the ^{111}In was removed from BLM in vivo and could be found bound to transferrin which probably gave up iron for the indium; other serum cations (e.g.: zinc and magnesium) might also displace indium. From the work of Robbins et al., (1974) it is clear that unbound $^{111}InCl_3$ has a very different $T_{1/2}$ and kinetics from the ^{111}In-BLM complex.

From the foregoing it is clear that the plasma clearance of ^{111}In-BLM entrapped in liposomes will depend upon the quantity of radiolabel which is removed by competing chelators and the natural exchange of cations. This probably explains the differences found by various authors : in the present case the amount of radioactive ^{111}In-BLM in-

jected was almost twice that used by Gregoriadis et al., (1977a) so presumably the chelation/exchange of the radiolabel resulted in a higher protein labelling and thus a longer $T_{1/2}$.

The discussion on ^{111}In-BLM clearance raises a further important question. In chapter 2 it was suggested that the interaction of ^{111}In-BLM with anionic liposomes could have been due to binding of ^{111}In by the polar head groups of the lipid but there is no guarantee that the radiolabel stays attached to BLM. Some free $^{111}In^{3+}$ could escape from the vesicle (the cation permiability of anionic liposomes in high : Papahadjopoulos and Watkins, 1967). In addition, Hwang and Mauk (1977) and Mauk and Gamble (1979) have shown that serum will enhance the release of an ^{111}In-chelator complex from SUVs containing 33 mol. % cholesterol although the presence of a cationic charge on the vesicle membrane reduces this leakage. The plasma clearance rates of ^{111}In-BLM either free or entrapped should, therefore, be treated with caution since it is very possible that the prolonged clearance rates are due to ^{111}In bound to plasma proteins.

The plasma clearance of ^{125}I-BLMs entrapped in liposomes has not been previously reported. As with other liposome entrapped compounds the initial clearance rate is rapid but after two hours the ICL-BLM liposomes are cleared slower that the B/H-BLM liposomes which suggests that there are size differences between the two preparations because although the two preparations are similar in all respects, apart from the entrapped species, it is impossible to control the sonication to produce homogeneously sized populations. The differences are unlikely to be due to the quantity of lipid injected since these are very similar. There remains the possibility that the entrapped drug determines the clearance rate of its liposome. This cannot be ruled out in this experiment but it seems unlikely since there was no difference in the clearance rates of the unentrapped iodinated BLMs. Eckelman et al., (1976) did find a difference between directly and indirectly iodinated free BLMs but in that case the B/H-BLM was cleared more slowly than the ICL-BLM i.e.: the reverse of the results for the ^{125}I-BLM liposomes. Interestingly, the addition of cold polycations (cobalt) to BLM (Eckelman et al., 1976) did not change the extent of iodination of the drug but it did retard the plasma clearance. The preparation of a double labeled BLM (^{111}In chelated + ^{125}I indirect iodination) might provide some clarification of the extent of indium and drug leakage from liposomes.

The stability of the radioiodinated BLMs in the presence of plasma was not investigated in detail but an initial experiment in vivo (not shown) suggested that the plasma radioactivity at 5 hours was > 95% precipitatable with trichloroacetic acid (TCA ppt). Eckerman et al., (1976) found that ICL-BLM injected into rats had lost 7 % of its radiolabel after 2 hours but almost 50 % was lost in 24 hours in the rabbit; more than 90 % of the ^{125}I-BLM excreated in rat urine was radiolabeled. It is possible, therefore, that after 5 hours post-injection there is some dissociation of radiolabel from the drug.

Studies on the entrapment of asparaginase in liposomes (Fishman and Citri, 1975; Neerunjun and Gregoriadis, 1976) or in microcapsules (Chang, 1971, 1973; Mori et al., 1973) have shown that entrapment stabilizes the enzyme and protects it from heat denaturation (Fishman and Citri, 1975; Chang, 1973; Mori et al., 1973) although encapsulation in microcapsules restricts the pH optimum, changes the temperature optimum and results in a loss of activity (Mori et al., 1973). Both methods protect the entrapped enzyme from attack by proteolytic enzymes; moreover liposome entrapment prevents the enzyme from interacting with specific antisera both in vitro (Fishman and Citri, 1975) and in vivo (Neerunjun and Gregoriadis, 1976). Furthermore, the entrapped enzyme is capable of lowering asparagine levels in the plasma and tumors (Chang, 1971; Neerunjun and Gregoriadis, 1976).

There has been no report to-date on the plasma clearance of ASPase entrapped in liposomes. The data presented here show that the now-familiar, plasma clearance curve of other liposome entrapped drugs is also found when ASPase is entrapped. The loss of radiolabel from the liposomes after 2 and 5 hours may represent either loss of non-bound ^{125}I or leakage of the whole protein followed by breakdown or rapid removal of the leaked protein from the plasma. Gregoriadis and Ryman (1972a) failed to find evidence for loss of structural integrity of ^{131}I-albumin-containing liposomes but protein leakage from liposomes in contact with plasma has been shown by others, especially when the cholesterol content of the vesicle is low (see discussion below).

The rate of plasma clearance of free ASPase appears to be a very complicated phenomenon and depends upon a large number of different factors. Patterson (1975) has summarized the difference in $T_{1/2}$ values obtained in various species of animal; in rodents these values range between 0.5 and 26 hours (mouse) and 1.5-5 hours (rat). In addition, the method used to isolate the enzyme will effect the rate of plasma

removal. Frank et al. (1970) found that the enzymes isolated from different strains of E. Coli had different isoelectric points; Mashburn and Landin (1970) and Rutter and Wade (1971) demonstrated that isoenzymes from the same source but with lower isoelectric points (pI) were cleared from the plasma more slowly than the others. The source of the enzyme is also an important factor (Hall, 1970). The E. Coli enzyme (pI; 5.0) is cleared from the plasma of mice more slowly ($T_{1/2}$: 4 hrs Mashburn and Landin, 1970) than ASPase from Fusarium tricinctum (pI; 5.2 : $T_{1/2}$ 0.5 hours) (Scheetz et al., 1971), interestingly this latter enzyme has no antitumour effect. The clearance of ASPase from Erwina carotovora (pI,8.79. Miller et al., 1971), which was used in this study, appears to be an exception since its $T_{1/2}$ is about 1.5 hours in rats (Hall, 1970) and it has potent antitumour activity (Wade et al., 1968). A further complication arises if the animals used are virus infected since this markedly impairs the clearance of the enzyme (Riley et al., 1970). Finally, the storage of the enzyme also changes the plasma clearance, Hall (1970) reported that the E. carotovora enzyme will spontaneously aggregate (for this reason the enzyme preparation was centrifuged after rehydration; see methods) and Rauenbusch et al., (1970) showed that the enzyme could be slowly inactivated in glass and plastic containers but that this could be prevented by the addition of non-ionic detergents which probably retarded the aggregation and inactivation.

The very rapid loss (40 %) of free ASPase from the plasma in the first five minutes following injection could be due to the fast clearance of aggregated material and equilibration with body fluids. This also accounts for the fact that the $T_{1/2}$ values are different from those of Hall (1970) and, more recently, those of Uren and Ragin (1979) ($T_{1/2}$. 1.5h). The plasma clearance reported by Pütter (1970) and Uren and Ragin (1979) follow first order kinetics, at least for the first 8 hours post injection. However, Hall (1970) and Schwartz (1970) reported both linear and biphasic plasma clearance curves in animals and in man.

In general, foreign protein clearance is determined by such factors as the state of denaturation and aggregation (Thorbecke et al., 1960), the sialic acid content (Morell et al., 1971) and the carbohydrate content (Sly, 1980; Stahl and Schlesinger, 1980). Pütter (1970) has presented evidence that the clearance of ASPase is due to "immune-elimination" since clearance rates were increased when animals received a second injection of the enzyme one week after the first although no

serum antibodies could be detected until after a third or forth injection. Other protein-protein interactions may also play a role in the removal of ASPase from the circulation. Soru (1979) found that the enzyme would interact with alpha-2-macroglobulin which prevented the subunits of the enzyme from dissociating even in the presence of urea or detergents. The complex retained its asparaginase activity.

Despite the many reports that liposomal lipids and liposome contents are removed from the plasma together it remains true that liposomes of certain compositions become permeable to their entrapped solutes when in contact with plasma proteins (Gregoriadis, 1973b; Kimelberg et al., 1975; Tyrrell et al., 1977; Zborowski et al., 1977; Finkelstein and Weissmann, 1979; Gregoriadis and Davis, 1979; Kirby et al., 1980b; Scherphof et al., 1980; Allen, 1981) in vivo and in vitro. Initial observations suggested that the leakage was determined by the molecular size of the solute, so that proteins (Gregoriadis and Ryman, 1972a; 1972b; Gregoriadis and Allison, 1974; Gregoriadis and Neerunjun, 1974; Neerunjun and Gregoriadis, 1976) and drugs of a certain size or lipid solubility (Gregoriadis 1973; Gregoriadis and Neerunjun, 1975a; Colley and Ryman, 1975; Dapergolas et al., 1976; Juliano and Stamp, 1978) remained inside the liposome whilst other drugs (Ryman, 1975; Juliano and Stamp, 1978) and ions (Kimelberg et al., 1975), leaked out. Co-entrapment of the drug with other agents (Gregoriadis et al., 1977b) has been proposed to overcome these problems. However, an ion-chelator complex inside cholesterol-rich vesicles will still leak ions in the presence of serum (Hwang and Mauk, 1977; Mauk and Gamble, 1979). More recently, it has been shown that even proteins (Scherphof et al., 1980) can be released from liposomes in the presence of plasma or serum.
Kirby and Gregoriadis (1981) have suggested that the liposome (SUV) membrane remains intact during the protein/vesicle interaction but that pores are opened in the bilayer and the pore-size defines the leakage of the drug.

It seems probable, therefore, that the plasma clearance of the liposomal drugs in this study is a complex compounded from the individual characteristics of MLVs, SUVs and leaked free drug.

SECTION 3 : 2

INTERACTIONS OF LIPOSOMES WITH PLASMA LIPOPROTEINS.

Introduction.

The plasma clearance of ^{14}C-cholesterol from ^{14}C-cholesterol / ^{3}H-ACT-D liposomes, shown in the preceeding section (fig 3:1, 3:2), demonstrated that these two radiolabels were not removed concurrently from the plasma. The suggestion was made that this might be due to either leakage of ACT-D from the vesicle or transfer of ^{14}C-cholesterol to a slower moving plasma component or perhaps both. In this section data will be presented from studies made on the interaction of radiolabelled (4-^{14}C) cholesterol or (methyl ^{14}C-choline) egg phosphatadylcholine, incorporated in liposomal bilayer, with rat plasma lipoproteins in vivo. Lipoproteins were chosen as the most likely plasma constituents involved in interactions with liposomes since they play a predominant role in the transport and metabolism of lipids in the body (Scanu and Wisdom, 1972). Following publication of part of this work (Black and Gregoriadis, 1976) there have been many reports of liposomes interacting with the plasma proteins involved in lipid metabolism.

Plasma lipoproteins function to transport esssentially insoluble lipids in a water solu ble form. The majority of triglycerides, either endogenously synthesisted or from the diet, are transported by chlomicrons (CHYL) and very low density lipoproteins (VLDL). Low density (LDL) and high density (HDL) lipoproteins are the primary vehicles for the transport of cholesterol usually in the form of cholesterol esters. It is generally accepted that the neutral (apolar) lipids, triglycerides and cholesterol esters (E-CHOL) occupy a central core of all lipoprotein particles whereas the more polar lipids, phospholipids (PL) and cholesterol (CHOL) are in a surface film (unilamellar membrane). In addition to lipids, lipoprotein class contains specific apoproteins which also occupy the surface (Table 3:1).

A) Cholesterol exchange with lipoproteins.

The analysis of plasma lipoproteins from rats injected with liposomes containing ^{14}C-cholesterol(LP) (circles) and ^{111}In-BLM(AP) (squares) is shown in figure 3:5. The animals were killed at timed in-

Table 3:1.

Apoprotein	LIPOPROTEIN		Function
	Major Component	Minor Component	
A-I	CHYL,HDL VHDL	VLDL	LCAT Activator
A-II	HDL,VHDL	VLDL CHYL	LCAT inhibitor? Regulator of ApoE binding.
B	LDL,VLDL, CHYL.		Lipoprotein biosynthesis/secre tion; binds to cell surface receptors
C-I	VLDL,CHYL	LDL, HDL	LCAT Activator ?
C-II	VLDL,CHYL	HDL	Lipoprotein lipase activator
C-III	VLDL,CHYL	HDL	Lipoprotein lipase inhibitor; inhibitor of CHYL remnant uptake by liver.
D(A-III)		HDL,VLDL	Cholesterol ester transfer protein ?
E	VLDL	CHYL,HDL	Binds to hepatocyte surface receptors.

Modified after Owen and McIntyre (1982) and Sparrow and Gotto (1980).

tervals after injection. Blood was centrifuged to remove the cells. Washed blood cells, equivalent to 1 ml of whole blood, were lysed with an equal volume of 10 % NaCl and the centrifuged to produce a pellet. The pellet was washed with 5 % NaCl and precipitated with 5 % TCA; the precipitate was transfered to scintillation fluid for radioactive counting.

The plasma was taken and an aliquot (4 ml) was subjected to lipoprotein analysis by density gradient ultracentrifugation. A pre-centrifugation sample was stored, frozen at -20°C, for later measurement of radioactivity.

The quantity of each radiolabel in the lipoprotein fractions (fig. 3:5) was expressed as a percentage of the total plasma radioactivity measured at the same time. Control samples of liposomes and liposome constituents were also fractionated under various conditions (table 3:3).

Results

No radioactivity was found associated with erythrocytes at any time. Table 3:2 (a) shows the total plasma radioactivities of both labels. For comparison the leakage of ^{111}In from ^{111}In-BLM liposomes (table 3:2b), incubated in PBS at 37°C for similar time intervals, is also shown (taken from data in section 2:2 (iii)). The ratio of ^{111}In-BLM : ^{14}C-cholesterol (ratio A) is assumed to be 1 just before injection . In the case of in vitro incubation, the fraction of the initial ^{111}In-BLM remaining liposome-associated (i.e. % remaining in liposomes/100) is also presented (ratio B). A comparison of ratio A with ratio B (table 3:2c) gives a measure of the spontaneous leakage of the drugs from the liposomes in the presence of serum (i.e. a value of < 1.0 for Ratio A/Ratio B indicates the increased drug leakage as a result of injection into the rat).

These results show that the plasma clearance of entrapped ^{111}In-BLM was similar to that shown previously in other experiments (fig. 3:3) which implies that the incorporation of ^{14}C-CHOL has no effect on the BLM clearance. Table 3:2 (a) might be taken to imply that these liposomes were stable in the plasma (i.e. ratio A and Ratio A/Ratio B are maintained at unity). This cannot, however, be assumed from these data since it is known that ^{111}In radiolabel will bind to plasma proteins (see previous section and table 3:3.i) and it was not cleared at

Table 3:2.

^{111}In release from ^{14}C-CHOL Liposomes in vivo and in vitro.

			Time (mins) post-injection or incubation.		
PREPARATION			5'	30'	60'
			Mean % Radiolabel in Plasma.		
a)	^{111}In-BLM	(I)	90.58	84.53	57.73
	^{14}C-CHOL	(C)	85.05	84.24	57.70
	RATIO A	(I/C)	1.06	1.0	1.0
			% drug remaining/100		
b)	in vitro	(saline).			
	RATIO B	(I)	0.997	0.985	0.97
c)	RATIO A / RATIO B		1.06	1.01	1.03

a) in vivo :

Fasted (12 hr) rats (300-350 g. body weight) were injected I/V with liposomes containing ^{14}C-cholesterol (LP) and ^{111}In-BLM(AP). The liposome solution was prepared from four times the standard anionic lipid quantities (containing a trace of ^{14}C-cholesterol) dispersed with 3 ml of ^{111}In-BLM in PBS. Each animal received 2 ml of liposomes containing 17.7 umols EPC: 4:4 umols CHOL (+ 1.68 uCi 4-^{14}C-cholesterol; 87.45 % entrapped): 2.5 mol PA, with ^{111}In-BLM (0.22 uCi ^{111}In and 0.135 ug BLM; 75.63 % entrapped) in the AP. The animals were killed at timed intervals after injection and the quantity of each radiolabel (% of injection) remaining in the plasma was calculated.

b) in vitro (data taken from page 76.) shows the amount of the initial ^{111}In radiolabel associated with liposomes at timed intervals after the start of incubation in saline.

c) Ratio A/Ratio B gives a measure of the addition leakage due to the presence of serum ie: a value < 1.0 indicates increased drug leakage following I/V injection.

the same rate as the bleomycin (see fig.3:3 and discussion). For the BLM-containing liposomes the Ratio A/Ratio B calculation (table 3:2c), therefore, cannot be used to estimate the increased drug leakage in the presence of serum.

Similar calculations of the leakage of ^{3}H-ACT-D(LP) from ^{14}C-CHOL liposomes (not shown), taken from data in figs. 2:2 and 3:2, showed a large increase in the release of the drug when the liposomes were injected into animals (i.e. : Ratio A/Ratio B << 1.0).

Lipoprotein analysis

In view of the leakage of ^{111}In-BLM from liposomes it was important to establish the distribution of free ^{111}In-BLM in rat lipoproteins before using liposomes. A control experiment was performed (Table 3:3 i) which showed that 30 minutes after the I/V injection of ^{111}In-BLM most of the radioactivity in the plasma was associated with the protein pellet (d > 1.25) and VHDL or with the CHYL fraction (d < 1.006). A similar distribution was found following the incubation of the same amount of free ^{111}In-BLM (0.26 ug BLM; 0.425 uCi ^{111}In) with either 5.0 ml fresh rat plasma (table 3:3 ii) or 5.0 ml bovine serum albumin (BSA : 50 mgs/ml) (table 3:3 iii) at 37° C for 1 hour.

These results are similar to those found by others (see previous section) and suggest that BLM, or more likely the ^{111}In radiolabel, is able to bind to plasma proteins in vivo and in vitro. Alternatively, the drug might be large enough (mol wt 1500) to sediment with the proteins but this seems unlikely. The protein involved in binding the drug/radiolabel could be albumin (table 3:3 iii) or transferrin which contaminates the BSA. In addition, there was a low density (d < 1.006) component which concentrated either the whole drug or ^{111}In (CHYL. Table 3:3 i, ii and iii). In view of the interaction of ^{111}In-BLM with anionic phospholipids (section 2:2), it seems possible that the low density component might also be an anionic phospholipid(s) found at this density. In the case of BSA (table 3:3 iii), the lipid may have been associated with the protein before its lyophillisation by the manufacturers. Two control lipoprotein separations were performed with ^{14}C-CHOL/^{111}In-BLM liposomes (for doses used see table 3:2) : i) liposomes were incubated with BSA (table 3:3 iv) for intervals up to 60 minutes, and ii) liposomes were mixed with PBS before centrifugation (table 3:3 v). These experiments gave similar results and show the distribution of the liposomes on the density gradients in the absence of

Table 3:3. Control values for lipoprotein analyses.

N°	Preparation (time post-injection or of incubation)		% Total Radioactivity in each Lipoprotein fraction						
			CHYL	VLDL	LDL	HDL	VHDL	Pellet	Total Recovery
i)	In vivo. ^{111}In-BLM (30 min).	^{111}In	22.13	3.29	2.57	3.17	25.48	45.61	102.25
	In vitro. (37°C)								
ii)	^{111}In-BLM + rat plasma (60 min)	^{111}In	19.51	1.88	2.05	1.34	20.51	58.96	104.25
iii)	^{111}In-BLM + BSA (60 min)	^{111}In	26.23	8.58	9.59	10.21	9.5	30.29	94.4
iv)	^{14}C-CHOL (LP) + ^{111}In-BLM(AP) Liposomes + BSA (60 min)	^{14}C	29.62	21.89	20.23	11.58	4.13	3.98	91.43
		^{111}In	30.52	20.83	21.19	12.31	2.14	4.15	91.14
v)	^{14}C-CHOL(LP) + ^{111}In-BLM(AP) Liposomes + PBS (o)	^{14}C	31.94	21.22	21.31	12.53	5.38	1.16	93.54
		^{111}In	32.53	22.42	19.16	8.51	5.73	1.95	90.3
vi)	^{14}C-CHOL(LP) + ^{111}In-BLM(AP) Liposomes + rat plasma (o)	^{14}C	16.72	24.17	21.52	16.33	1.40	17.63	97.77
		^{111}In	20.93	20.17	15.93	10.76	1.79	23.75	93.33

Legend table 3:3.

The table shows the percentage of radioactivity found in fractions corresponding to lipoprotein densities following . centrifugation of :

i) 4 ml plasma from a rat injected 30 min. previously with 0.25 ug ^{111}In-BLM (1.66 mCi ^{111}In/mg BLM).

ii) 4 ml rat plasma incubated with free ^{111}In-BLM (5 ml plasma + 0.5ml ^{111}In-BLM (0.25 ug/ml)) for 60 minutes at 37°C.

iii) 4 ml BSA incubated with free ^{111}In-BLM (5 ml BSA (50 mg/ml) + 0.5 ml ^{111}In-BLM (0.25 ug/ml) for 60 minutes.

iv) 4 ml BSA incubated with ^{14}C-CHOL/^{111}In-BLM liposomes (5 ml BSA (50 mg/ml) + 0.5 ml liposomes (prepared as in table 3:2)).

v) 4 ml PBS mixed with ^{14}C-CHOL/^{111}In-BLM liposomes (table 3:2) (5 ml PBS + 0.5 ml liposomes).

vi) 4 ml rat plasma mixed with ^{14}C-CHOL/^{111}In-BLM liposomes(table 3:2) (5 ml plasma + 0.5 ml liposomes).

plasma lipoproteins. From these data it may be calculated that on average more than 85 % of the recoverable liposomes were found at d < 1.21; of these floating vesicles 37 % have d < 1.006, 25 % have d = 1.006, 24 % have d > 1.006 < 1.063 and 13 % have d > 1.063 < 1.21. Since others have shown that SUVs concentrate in the d > 1.006 < 1.063 density range (see discussion) the quantities of SUVs in the preparations used in the in vitro experiments (table 3:3 iv, v, vi) and for injection into rats (same preparation) contained about 24 % of the total lipids as SUVs.

In addition, table 3:3 (iv and v) shows that the liposomes remained intact during the fractionation proce dure and did not leak since little ^{111}In was found associated with the BSA pellet (cf. table 3:3 i, ii, iii) and the ratio of the radioactive markers (^{14}C/^{111}In) was close to unity in all the fractions. The distribution of the two radiolabels on the BSA gradients after 5 and 30 minutes incubation was almost identical to that found at 60 minutes (table 3:3 iv).

The extent of liposome interaction with lipoproteins during the course of the analysis is shown in table 3:3 vi. Comparison of this data with table 3:3 iv or v shows that the pellet fraction has gained radioactivity at the expence of CHYL. It is not clear if this result is due to whole liposomes which sediment into the pellet following interaction with plasma proteins or if it is due to leakage and/or lysis of MLVs (normally floating with CHYL (table 3:3 v)) followed by binding of ^{111}In and ^{14}C-CHOL by proteins of the pellet. Whatever the reason, these results (table 3:3 vi) were used as a control for the in vivo experiments (figs. 3:5 and 3:6).

Laurell double immunoelectrophoresis of isolated rat plasma lipoprotein fractions from the in vivo studies was performed; samples (20 ul) were run against 5 ul/cm^2 whole rat-plasma-protein antiserum and stained with Sudan black. Very faint immunoprecipitate lines were seen (not shown) in the lipoprotein fractions but it was clear that the chylomicron and VLDL fractions, although contaminating each other, were clearly separated from LDL. However, LDL was partially contaminated (amount not measured) with some protein which had the characteristics of HDL. The HDL fraction only contained a single protein peak whilst the VHDL fraction, despite careful pipetting, always gave protein peaks similar to those found in the pellet at the tube bottom.

The data in fig. 3:5 showing the in vivo results of ^{14}C CHOL/^{111}In-BLM liposomes will be presented according to lipoprotein type.

The means of ^{14}C radiolabel and ^{111}In radiolabel were compared (Students 't' test) for each fraction.

i) Chylomicrons.

Comparisons of the mean values presented for chylomicrons failed to show any significant differences between the amount of ^{14}C radioactivity and that of ^{111}In throughout the one hour period. The liposomes found in this density band ($d < 1.006$) were, therefore, probably intact liposomes.

The differences and the fall in total radioactivity over the time period of 1 hour is consistent with the hypothesis that this density contained the largest liposomes which would be cleared most rapidly from the circulation. The lack of statistical differences suggests that, if ^{111}In leakage is occuring during the circulation of these liposomes in the plasma, the ^{111}In radiolabel is prevented from localizing at this density (cf. Table 3:3 i and vi).

ii) VLDL.

The differences between ^{14}C and ^{111}In radioactivity in this fraction ($d = 1.006$) were not significantly different at any of the time points studied. However, the quantity of liposomes which intrinsically float at this density (21.3 $\pm$ 0.6 %. table 3:3, iv,v,vi) was markedly reduced to between 5 and 10 % of the control values. These liposomes may, therefore have been totally removed from the plasma or aquired a different density due to interactions with proteins.

iii) LDL.

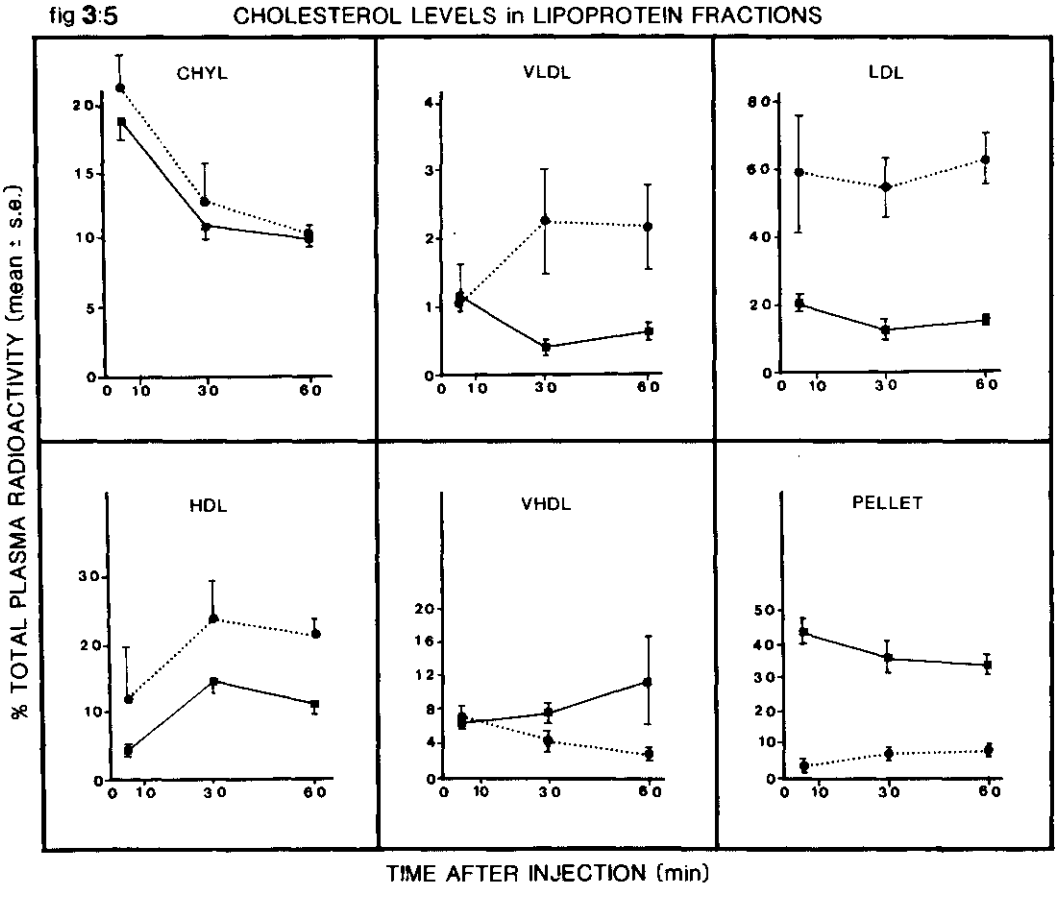
The comparison of the means in this fraction were statistically different at 30 min. ($p < 0.01$) and 60 min. ($p < 0.005$). In the absence of lipoproteins, the quantity of vesicles floating at this density was found to be 21 % (table 3:3.iv,v) and this was not statistically different from the amount of ^{111}In radiolabel found at any time in fig. 3:5. The quantities of ^{14}C cholesterol were found to be significantly higher than those of the controls at 30 min. ($p < 0.005$) and 60 min. ($p < 0.002$). In principle, therefore, this fraction does not represent whole liposomes - it has become enriched with ^{14}C cholesterol - suggesting that some ^{14}C radiolabel is being transfered to LDL. Since no measurement of the total cholesterol of this fraction was made it is impossible to say if the enrichment is the result of exchange of ^{14}C-CHOL between the liposome and the lipoprotein or if there is a net transfer of mass to the LDL which might signify the destruction of the liposome.

Legend Fig. 3:5.

CHOLESTEROL LEVELS IN LIPOPROTEIN FRACTIONS

Rats were injected I/V with 2 ml liposomes containing both 4-^{14}C-cholesterol (1.68 uCi; 4.4 umols) (circles - dotted lines) and ^{111}In-bleomycin(AP) (0.22 uCi; 0.135 ug BLM) (squares - solid lines).

At timed intervals after injection, rat plasma was subjected to ultracentrifugation to separate lipoprotein fractions. The amount of radioactivity due to the radiolabels was measured in each lipoprotein fraction and expressed as the mean (± S.E.) percent of the total plasma radioactivity (3 rats per time point).
Details of liposome preparation are given in table 3:2.



iv) HDL.

The radioactivity associated with the HDL density (1.063 - 1.21) showed a rise during the first 30 minutes post-injection but differences between the two radiolabels were not significant($p > 0.05$). Sixty minutes after injection the differences were significant ($p < 0.02$) and this fraction was also enriched in ^{14}C-CHOL. The proportion of liposomes (11.25 $\pm$ 1.41%) normally found at this density was not significantly different from the ^{111}In radioactivity at any time, ($p > 0.1$). This result implies that at 60 minutes post-injection there was a transfer of ^{14}C radioactivity to the HDL density range and this is more than the *in vitro* transfer (16.3 %, table 3:3 vi).

vi) VHDL.

These lipoproteins ($d > 1.21 < 1.25$) contained whole liposomes (^{14}C : ^{111}In radioactivity was not significantly different : $p > 0.1$) during the first 30 minutes after injection. One hour post-injection, the differences were significant ($p < 0.02$); the fraction had become enriched with ^{111}In and there was significantly more ($p < 0.01$) ^{111}In than was found in the control preparation (table 3:3 iv and v): the ^{14}C-cholesterol content was similar to the controls. However, in view of the difficulties of separating VLDL from the pellets and the very large standard error at 60 minutes, this result should be treated with some caution as it probably represents ^{111}In contamination from the fraction below.

vi) Pellet.

The control values (table 3:3 i, ii and iii) all showed that free ^{111}In localized with the pellet of plasma proteins at the tube bottom. The differences between ^{14}C and ^{111}In were highly significant throughout the time period ($p < 0.002$). The ^{111}In content was very different ($p < 0.001$) from the amount of liposomes found normally (table 3:3 iv) although there was no difference between these values and ^{14}C-CHOL. There was a similarity between the ^{111}In values in the pellet and those found for the *in vitro* controls (table 3:3 i and vi) or ^{111}In-BLM incubated *in vitro* with rat plasma (table 3:3 ii). These results all suggest that the location of ^{111}In radioactivity in this fraction is due to the presence of either free ^{111}In-BLM or free, plasma bound, radiolabel (probably the latter). The presence of this radiolabel in such quantities strongly suggests that the liposomes leak *in vivo*. The leakage rate is very fast (about 41 % in the first 5 minutes) although

it may level off later suggesting that either there is a population of liposomes which will not leak or that not all ^{111}In radiolabel is available for release. The presence of ^{14}C-CHOL in this fraction indicates that either whole liposomes are binding to, and sedimenting with, the plasma proteins (as in vi, table 3:3) or that cholesterol can be bound by some other (non-lipo) protein.

B) Interactions of ^{14}C-EPC with lipoproteins.

Similar experiments to those performed in the previous section were carried out using ^{14}C-EPC radiolabelled with ^{14}C-methyl iodide on the choline groups. In view of the low specific activity of the radiolabelled EPC (0.11 uCi/mg : 0.082 uCi/umol) and the small amount of material available control experiments similar to those shown in table 3:3 were not carried out. Although the liposomes used in these experiments were prepared in the exactly same way as those containing ^{14}C-cholesterol it cannot be guaranteed that, in the absence of lipoproteins, their distribution on the density gradients will be the same as shown in table 3:3 but the distribution of the ^{111}In radiolabel should be similar.

Table 3:4 shows the in vivo drug leakage from liposomes containing ^{14}C-EPC (LP) and ^{111}In-BLM (AP) following their I/V injection into fasted 350 g rats. In addition, the amount of radiolabel which remained associated with similar liposomes incubated with saline in vitro is shown for comparison (data taken from Section .. 2:2.iii)

The plasma clearance of ^{111}In (table 3:4) was different from that found in table 3:3 but these differences were not significant, nor were they different from fig. 3:3 ($p > 0.05$). The values for ^{14}C-EPC plasma clearance are not significantly different from ^{14}C-CHOL values (fig. 3:1 and table 3:2) at 5 and 30 minutes. The 60 min. ^{14}C-EPC value (single animal) may be lower than the ^{14}C-CHOL values if it is representative of the mean.

The ratio A values imply that the two labels are cleared approximately concurrently but the ratio A/ratio B values suggest that there is no leakage of the drug in plasma. However, as pointed out before, ^{111}In is known to bind to proteins and so the higher ratio A/ratio B values reflect the binding of this radiolabel to a slower moving plasma componant than EPC.

Fractionation of plasma lipoproteins from rats injected with ^{14}C-EPC/^{111}In-BLM (AP) liposomes is shown in fig. 3:6. A comparison of

Table 3:4.

Drug release from ^{14}C-EPC/^{111}In-BLM Liposomes in vivo and in vitro.

			Time (mins) post-injection or incubation.		
PREPARATION*			5'	30'	60'
	In vivo.				
			Mean % Radiolabel in Plasma.		
1)	^{111}In-BLM	(I)	86.01	70.94	40.21
	^{14}C-EPC	(E)	74.27	63.95	32.58
	RATIO A	(I/E)	1.16	1.11	1.23
	In vitro	(saline)			
			% drug remaining/100		
2a)	RATIO B	(I)	0.997	0.985	0.97
2b)	RATIO A / RATIO B		1.16	1.12	1.27

*

1) In vivo :

Liposomes were made from 80 umols (Methyl-^{14}C-choline) EPC (1.32 uCi): 22.8 umols CHOL: 11.4 umols PA dispersed with 3 ml of PBS containing 0.425 uCi ^{111}In (0.26 ug BLM). At injection, 1 ml of the liposome solution contained 6.4 umols ^{14}C-EPC (0.105 uCi) (93.21 % entrapped): 1.83 umols CHOL: 0.91 umols PA with 0.025 uCi ^{111}In and 0.015 ug BLM (73.6 % entrapped in AP); each animal received 1 ml of this solution. The animals were killed at 5, 30 and 60 minutes post-injection.

2) In vitro.

The identities and values of ratio B and ratio A/ratio B are the same as table 3:2 (Data taken from saline leakage Section 2:2 A iii page 76)

means (Students 't' test) of ^{14}C radioactivity (dotted lines) and ^{111}In radioactivity (solid lines) failed to find any significant differences ($p > 0.05$) in any fraction at 5 minutes and 30 minutes post-injection. Comparisons were not performed for 60 minutes values .

The values of ^{111}In distribution in vivo and in vitro were considered to be the same as found in table 3:3. In addition, table 3:3 has been used to give an approximate idea of the relative proportions of whole liposomes, from a MLV preparation, to be found in each fraction. Radioactive recoveries from the fractionations were between 87.5 and 110.2 %.

i) Chylomicrons.

The radioactivity associated with the CHYL density was similar to that found in the previous fractionation (fig. 3:5). This supports to view that these are whole liposomes and, moreover, that the size distribution of this liposome population was not very different from the previous experiment. The fall in total radioactivity in this fraction is again probably associated with the removal of these large liposomes from the plasma or with the relocalization of CHYL at a different density.

ii) VLDL.

As in the previous experiment the VLDL radioactivity was low compared to the quantity of liposomes which usually float at this density in the absence of lipoproteins (table 3:3iv). It appears, therefore, that these liposomes too have been removed from the circulation or relocated at a different density.

iii) LDL.

The total LDL associated radioactivity was different from the very high levels of ^{14}C-CHOL found before (fig. 3:5). In the case of ^{14}C-EPC and ^{111}In the levels are similar to those of control liposomes (table 3:3 iv, v, vi) and to the level of ^{111}In in fig. 3:5. It appears, therefore, that, unlike ^{14}C-CHOL, the LDL fraction contains whole liposomes (probably SUVs) which intrinsically float at this density.

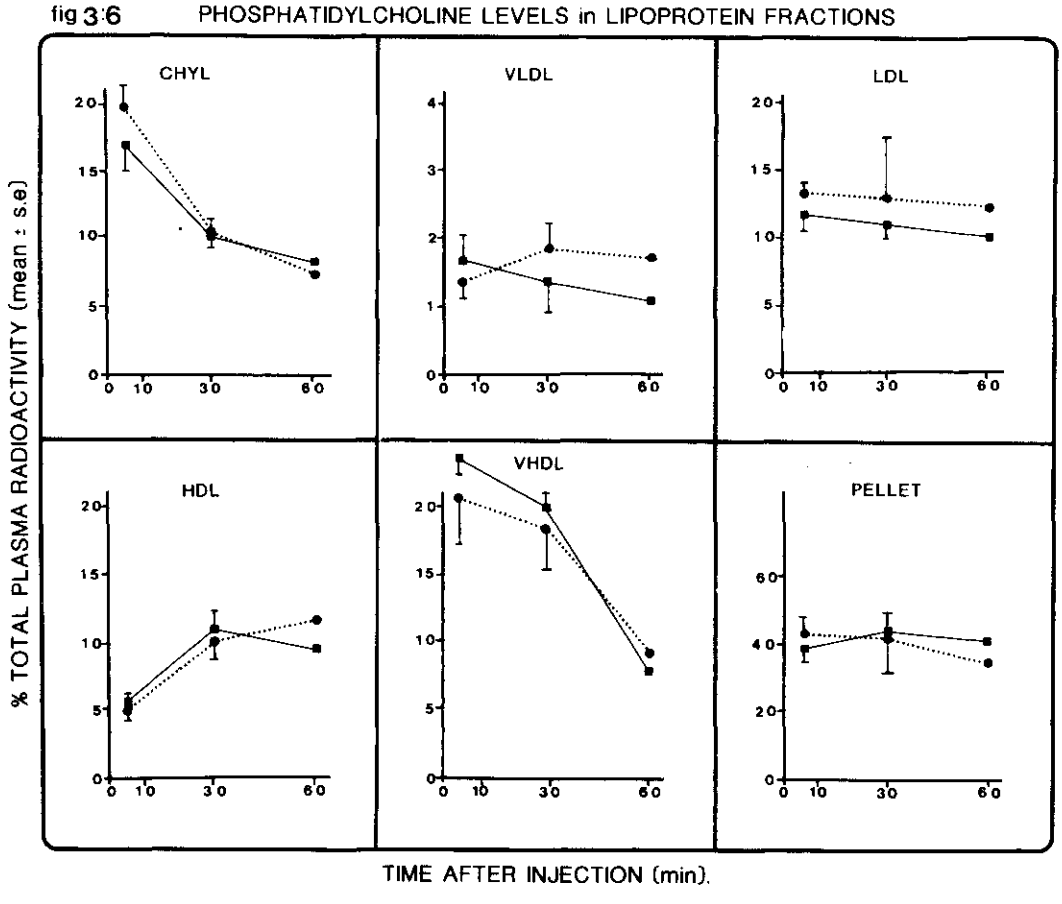
iv) HDL.

As in the previous three fractions, there was no difference between the ^{14}C and ^{111}In radioactivity in this fraction. Moreover, the total quantity of radioactivity due to ^{14}C-EPC was found to be about half of that found when ^{14}C-CHOL was used.

Legend Fig. 3:6

PHOSPHATIDYLCHOLINE LEVELS IN LIPOPROTEIN FRACTIONS.

Rats were injected I/V with 2 ml liposomes containing ^{14}C-EPC (0.21 uCi : 12.8 umol EPC)(LP) (circles - dotted lines) and ^{111}In-BLM (0.05 uCi : 0.03 ug BLM)(AP) (squares - solid lines). At timed intervals after injection, rat plasma was subjected to [redacted] ultracentrifugation to separate lipoprotein fractions. The amount of radioactivity due to the radiolabels was measured in each lipoprotein fraction and expressed as the mean (± S.E.) percent of the total plasma radioactivity (3 rats per time point except 60 minutes which is from 1 animal). Details of liposome preparation are given in table 3:4.



If this value represents whole liposomes then it appears to be in good agreement with the approximately 10 % of liposomes which can be floated at this density (table 3:3 v). The quantity of ^{111}In radiolabel found was similar to the previous experiments (fig. 3:5) and in vitro (table 3:3 vi). As with the ^{14}C-CHOL radiolabel the ^{14}C-EPC values increase with time.

v) VHDL.

Although the radioactivities due to each label were not significately different, the total radiolabel associated with the VHDL density at 5 minutes is two-fold greater than that found in the ^{14}C-CHOL experiments and between 4 and 10 fold higher than the quantity of whole liposomes which were normally found in this fraction (table 3:3.iv and vi). The radioactivity associated with the VHDL fell to these "normal" values during one hour in the plasma.

vi) Pellet.

The amount of ^{111}In radiolabel found in the pellet throughout this experiment was similar (40 %) to that found in the previous experiment (fig. 3:5, table 3:3 i, ii, and vi). However, there was an equal amount of ^{14}C-EPC radioactivity associated with the pellet and this has not been noted before either in vivo or in vitro. The reasons for this 8-10 fold increase are unclear but it seems unlikely that 40 % of the liposomes were bound to plasma proteins (this was one explanation for the small quantity of ^{14}C-CHOL found in this fraction) and so this may represent transformation of either whole liposomes or EPC into a more dense complex following interactions with proteins.

C) Discussion.

i) Serum induced drug leakage

A brief discussion has already been made in relation to the general leakage of drugs from liposomes (chapter 2, fig. 2:2) and in relation to the plasma clearance of drugs in the previous section (3:1). Here the concern is the role that liposome constituents and lipoproteins play in this effect.

In animal experiments Gregoriadis and Ryman (1972a) demonstrated that cholesterol and an entrapped protein were removed from the plasma concurrently, this appears to be the case for the CHOL / ^{111}In and EPC / ^{111}In presented here (tables 3:2, 3:4, ratio A).

However, examination of the lipoprotein fractions shows that leakage of ^{111}In occured *in vivo* (fig. 3:5) and this illustrates the problems of using AP markers which are not rapidly removed from the circulation when released from the liposome; a false impression of liposome integrity will be obtained. Gregoriadis (1973b) showed that for penicillin (but not ACT-D) entrapped in liposomes there was an accelerated diffusion of the drug out of the carrier in the plasma. The stability *in vivo* was apparently assumed in the early work, with good cause since entrapped proteins were not enzymatically or immunologically active in the plasma (Gregoriadis *et al.*, 1974b; Gregoriadis and Allison, 1974; Neerunjun and Gregoriadis, 1976).

This situation was changed when Scherphof *et al.*, (1975) very briefly reported that radiolabelled lecithin was cleared from the plasma at a slower rate than radiolabelled cholesterol co-entrapped in the same liposome. Immediately afterwards, Black and Gregoriadis (1976 and this work) and Krupp *et al.*, (1976) simultaneously showed that MLV-CHOL or SUV-E-CHOL and EPC could be found associated with plasma lipoproteins *in vivo*. Thereafter, the stability of the liposome, as an enclosed structure, could not be assumed when it was in contact with plasma.

Subsequently, Scherphof *et al.*, (1978) demonstrated that SUVs composed of lecithin alone were totally destroyed, and entrapped solute released, as a result of interactions with HDL. The occurence of such plasma-induced instability has been confirmed during *in vitro* incubations with rodent or human plasma (Chobanian *et al.*, 1979; Gregoriadis and Davis, 1979; Zirenberg *et al.*, 1979; Gregoriadis and Kirby, 1980; Kirby *et al.*, 1980a; 1980b; Scherphof *et al.*, 1980; Damen *et al*, 1981) although total vesicle destruction does not always occur (Kirby and Gregoriadis, 1981) nor are lipoproteins the only plasma proteins involved in the exchange/transfer of radiolabelled lipids to plasma components (Damen *et al.*, 1980).

There are differences between SUVs and MLVs in their interactions with plasma components. The MVLs leak entrapped solute (Gregoriadis and Davis, 1979; Hoekstra and Scherphof, 1979) but they are less susceptible than SUVs to protein and HDL attack, (possibly because only the outer bilayer is available for interaction) (Hoekstra and Scherphof, 1979; Scherphof *et al.*, 1979) especially when the quantity of entrapped cholesterol is high (Gregoriadis and Davis, 1979) or the liposomes are above their t_c (Scherphof *et al.*, 1979).

The role of cholesterol in the stability of liposomes has been amply demonstrated by the many reports which show that a high (> 33 mol %) CHOL content reduces or arrests the leakage of entrapped agents *in vivo* and *in vitro* (Finkelstein and Weissmann, 1979; Gregoriadis and Davis, 1979; Scherphof *et al.*, 1979; Kirby *et al.*, 1980b; Allen and Cleland, 1980) also, at the cholesterol content of the MLV liposomes used in this study (20 mol. %), Gregoriadis and Davis (1979) reported that the loss of laten cy *in vivo* in the first 2 min. post-injection into rats for a small entrapped molecule (6-carboxyfluorescein: 6-CF) was 55 % but only 30 % for a large protein (beta-fructofuranosidase); comparable values at 1 hour were 70 % loss (6-CF) but they remained at 30 % for the enzyme. These latter values are similar to the values (not shown) found here for ACT-D so that, although ACT-D is very much smaller than beta-fructofuranosidase, its entrapment in the lipid phase of liposomes probably retards its release (Juliano and Stamp, 1979). Assuming that most of the released ^{111}In radiolabel can be found in the CHYL + pellet fractions after lipoprotein fractionation (table 3:3 i and ii), the calculated release of free ^{111}In from the MLVs in this study totals between 60 % (5min.) and 40 % (60min.) i.e.: 40-60% loss of laten cy. The quantity of ^{111}In in the CHYL fraction falls over time so that the ^{111}In in the pellet may be a better measure of leakage; in this case leakage is very rapid (40-45 % in the first 5 minutes) but there is little leakage thereafter. Finkelstein and Weissmann (1979) have shown a leakage rate of 24.4 % hr. of entrapped radiolabelled inulin from MLVs (10 mol % CHOL) in plasma.

It is not surprising, therefore, to find that liposomes, of the composition used here (7:2:1), leak entrapped solute. However, this leakage will affect much of the subsequent data in this thesis because the study of liposomal drug uptake and distribution in tissues (chapter 4) will be difficult if, in the plasma, the drug is both inside and outside the carrier.

The identity of the plasma factor(s) responsible for solute leakage was not investigated here. The interactions of proteins with liposomes has already been mentioned (see chapter I) and will be further discussed in the following section. However, it is apparent that incubation of liposomes with BSA did not cause the release of entrapped ^{111}In *in vitro*. Whole rat plasma *in vivo* and *in vitro* can cause leakage but this has to be separated from the concurrent exchange/transfere of liposomal lipids to the plasma.

ii) ^{111}In-distribution.

Before discussing the results of the lipoprotein analyses further mention must be made of ^{111}In distribution. The interpretation of the interactions of radiolabelled lipids with lipoproteins depend upon the validity of ^{111}In as a marker for the aqueous space of the intact liposomes. Theoretically, removal of non-entrapped ^{111}In by predominantly, the plasma protein pellet means that the quantity of ^{111}In found in each fraction represents the quantity of whole liposomes in that fraction at the time when they were separated from the rest of the plasma proteins, irrespective of subsequent leakage of the label from the whole liposomes which may occur during the course of the analysis; but unlike the radiolabelled lipids which may bind to or exchange with lipoprotein fractions in vitro.

Problems arise when phospholipid experiments are performed using ^{111}In-BLM as a AP marker. As was shown in chapter 2 and by others (Huang and Mauk 1977) ^{111}In will bind to phospholipid polar head groups, especially PA, but it is uncertain whether the BLM is still attached to the ^{111}In label. Indium chloride will also bind to PA (chapter 2); the charge interaction is probably a general property of trivalent cations since it also occurs between lanthanum salts (La^{3+}) and PS in liposomes (Hammoudah et al., 1979). There is, however, no way of telling from these experiments :i) if ^{111}In-BLM is still present as the radiolabelled material, ii) what proportion of ^{111}In is in the aqueous spaces and what proportion is bound to the polar head group(s) iii) whether plasma proteins (mostly transferrin Thakur et al., 1973) can chelate ^{111}In away from the lipid bound material. There is a strong possibility, therefore, that in these experiments a proportion of the ^{111}In distribution is due to ^{111}In-PA as a complex either in whole liposomes or on lipoproteins. Since ^{111}In does not react with VLDL, LDL or HDL (table 3:3 i and ii) it seems possible that the radiolabel cannot interact with lipid already bound to the lipoprotein (but cf. chylomicrons), alternatively free ^{111}In is removed by other proteins before it can bind to the lipoproteins. In view of these facts it must be concluded that ^{111}In is not an ideal marker for aqueous phase in these experiments although it may be a valid marker for PA.

Evidence against this hypothesis comes from the control experiments where, apart from VLDL, very similar amounts of liposome-derived ^{111}In were found in the fractions in the absence of lipoproteins to those

in fig 3:5.

iii) Liposome-lipoprotein interactions.

The metabolism and structure of lipoproteins has been the subject of several recent reviews (Jackson et al., 1976; Smith et al., 1978; Nicoll et al., 1979; Tall and Small, 1979; Scanu and Landsberger; 1980). One of the major problems in the study of lipoproteins metabolism is that the lipoproteins are in a dynamic state in which both apoproteins and lipids continously exchange between the various lipoprotein classes. In the normal animal the lipoproteins can be assumed to be in a saturated state, but the addition of liposomal lipid to any individual lipoprotein may be expected to be reflected in compositional changes throughout the lipoproteins.

The majority of the published work involving lipoprotein-liposome interactions has been done using HDL and the major apolipoproteins derived therefrom. In addition, most of the in vitro work has been carried out using human HDL which differs from rat HDL in containing an extra apo-A lipoprotein (apo-AII) (Osborne and Brewer, 1977; Chapman, 1980) and this probably causes the differences in density flo.tation. Thus human HDL is more homogeneous, on density centrifugation, than rat HDL which has a density extending between 1.035 and 1.21g/L (Windmueller and Levy, 1967; Koga et al., 1969; Lasser et al., 1973; reviewed, Chapman, 1980) (c.f. human HDL: 1.063-1.21 g/L; Tall and Small, 1979). So the density fraction termed LDL in these studies ($d > 1.006 < 1.063$) will include some HDL (confirmed by immoelectrophoresis).
In addition, immunoelectrophoresis showed that CHYL and VLDL were not usually separated by this method and that VHDL was always contiminated by the pellet proteins.

Studies have indicated that in vivo and in vitro unilamella liposomes can be found in the LDL density range (Krupp et al., 1976; Kirby et al., 1980a; Tall, 1980). Whilst the work of others has shown that larger liposomes (MLVs) are found in the VLDL density range (Tall et al., 1978; Chobanian et al., 1979).

Many published studies have used liposomes (mostly SUVs) as model membranes in reassembly studies of lipids and apoproteins (Morrisett et al., 1977; Tall and Small, 1979). In view of the high avidity of apoproteins (and apolipoproteins) for lipids it seems unlikely that free apoproteins are ever found in vivo although they are produced artifactually by centrifugation (Eisenberg and Levy, 1975). Transfer of

apoproteins between lipoprotein classes and tissues is due to a very close approach of the individual lipoproteins to each other (so-called collision complexes). It does not seem, therefore, that apoprotein/liposome studies represent credible models for *in vivo* interactions; Eisenberg (1980) would agree. Moreover, delipidation of lipoprotein complexes e.g.: apo HDL (Scanu and Wisdom, 1972), which often results in conformational changes in the apoproteins (Tall and Small, 1979), cannot be said to be exact replicas of the situation *in vivo* either since in this state the complex will bind to many lipids.
Apolipoproteins, suitably relipidated with radiolabelled liposomal lipids, do, however, represent good models for studying exchange phenomena (Barter *et al.*, 1980; Gottlieb, 1980; Stein *et al.*, 1981). In parenthesis, it must be noted that to radiolabel purified normal HDL with cholesterol from liposomes requires cosonication (Scanu *et al.*, 1970) or prolonged incubation (24 hrs.) (Rothblat *et al.*, 1978) at very high liposome: protein ratios; situations which are unlikely to occur *in vivo*.

a) CHOLESTEROL

Reports of liposome-CHOL/ lipoprotein interactions have used SUVs rather than MLVs (Krupp, 1976; Kirby, *et al.*, 1980*a*, *b*; Kirby and Gregoriadis, 1980; Chobanian *et al.*, 1979; Tall, 1980), their results are not directly comparable with these. Several reports by Tall and co-workers (Tall and Small, 1977; Tall and Lange, 1978*a*; 1978*b*; Tall *et al.*, 1978) have used MLVs but only the first three reports studied the CHOL interaction. The accent in these published studies has been on HDL since this lipoprotein is known to interact with and dissolve either SUVs or MLVs in the absence of CHOL (see later).

It is clear from the plasma clearance curves of radiolabelled CHOL and EPC in SUVs (Scherphof *et al.*, 1975; Tall, 1980) and MLVs (Wharton and Green, 1978) that the two radiolabels are not cleared concurrently during the first hour after injection. It is unclear which radiolabel is cleared the fastest since Wharton and Green (1978) claim that EPC is removed more rapidly than CHOL whilst Tall (1980) and Scherphof *et al.*, (1975) reported that CHOL was faster than EPC. If the sizes of the liposomes used in these two present studies are roughly similar, the results presented here tend to confirm those of Wharton and Green i.e.: EPC is faster than CHOL. This may, therefore, represent differences between SUVs and MLVs. Krupp *et al.*, (1976) suggested that SUV esterified cholesterol (E-CHOL) was cleared at the same rate as EPC, which

may mean that E-CHOL is not handled by the plasma in the same way as CHOL. In the absence of additional CHOL in the SUVs this observation was confirmed by Kirby and Gregoriadis (1980) who found that the rates of plasma clearance of PC or E-CHOL were identical.

Other studies (Kirby et al., 1980a) have demonstrated that in vitro E-CHOL is transfered to HDL even when the SUV-CHOL concentration is high whilst transfer of PC is inhibited. Chobanian et al., (1979) have shown that in vitro CHOL-free-SUVs transfer E-CHOL to HDL more rapidly than EPC. The evidence, therefore, suggests that caution is required in the interpretation of E-CHOL transfer to lipoproteins not least because esterified cholesterol is not a good marker of vesicle fate in vivo in the absence of vesicular CHOL (Kirby and Gregoriadis, 1980).

If, as has been suggested (Krupp et al., 1976; Scherphof et al., 1978; Chobanian et al., 1979; Tall, 1980) dissolution of SUVs by HDL occurs (when vesicular CHOL is low or absent) and the lipids are transfered to the same lipoprotein particle it is difficult to explain why the plasma clearance rates of the two principle liposomal lipids (PC and CHOL) are so different even 2 minutes after injection (Tall, 1980) when exchange would be minimal. On the other hand, if CHOL was transfered to a different lipoprotein (with a different clearance rate of incorporated lipid) or if a system of differential exchange/transfer of PC to HDL operated (depending upon the CHOL content of the liposomes (Kirby et al., 1980a; Kirby and Gregoriadis, 1980; Tall, 1980; Damen et al., 1981) or their fluidity (Scherphof et al., 1979) with remaining vesicles becoming enriched with CHOL; (Tall and Lange, 1978a)) then differences in plasma clearance rates could be expected.

The study by Tall (1980) on the distribution of ^{3}H-CHOL-containing SUVs into rat lipoproteins showed that little or no cholesterol was exchanged with LDL in one hour. The HDL fraction received most of the ^{3}H-CHOL from vesicles of similar composition to those used here (but without PA) but the total HDL content of both CHOL and E-CHOL was not increased (i.e.:exchange rather than transfere). Chobanian et al. (1979) found the ^{14}C-E-CHOL in neutral SUVs did not exchange or transfer the label to LDL. Using whole human plasma Kirby et al. (1980a) also failed to find extra-vesicular E-CHOL associated with LDL although association with HDL was high; since release of entrapped solute did not occur in these experiments the authors claimed, and subsequently proved (Kirby et al. (1980b, 1981)), that complete liposomal lysis was not involved. Krupp et al. (1976) found approximately equal amounts of

both EPC and E-CHOL associated with HDL following I/V injection into rats; unlike their *in vitro* results, *in vivo* Kirby and Gregoriadis (1980) failed to find HDL-associated E-CHOL in mouse lipoprotein fractions.

Interactions of radiolabelled liposomal lipids with lipoprotein fractions other than LDL and HDL was studied by Kirby *et al*. (1980*a*) and Tall (1980). Cholesterol oleate was found to slightly enrich the VLDL (d = 1.006) fraction following incubation with human plasma (Kirby *et al*. (1980*a*) but Tall (1980) found only whole vesicles in this fraction. Interestingly, Tall also found PC radioactivity associated with the d > 1.21 fraction (3 - 13 % depending upon cholesterol content and time post-injection) but at no time was any cholesterol found there. Since this result agrees with the findings of Krupp *et al*. (1976), it seems possible the the CHOL radioactivity in VHDL and the pellet found in this study represents a further difference between MLVs and SUVs.

Relatively few studies have been performed on the interaction of VLDL with liposomes (Morrisett *et al*., 1977) but a study of VLDL lipids (not triglycerides) (Chajek and Eisenberg, 1978) in perfused rat hearts has shown a similar CHOL distribution to that found here.

In an analogous situation, the exchange of ^{14}C-cholesterol between radiolabelled erythrocytes (RBC) and lipoproteins is a well documented phenomenon. The mechanism of action of this exchange has yet to be established, probably because the quantity of cholesterol available for exchange is difficult to assess. In general, CHOL in RBC will exchange with both LDL and HDL (d'Hollander and Chevallier, 1972*a*, 1972*b*; Gottlieb, 1980) and perhaps VLDL as well (Bjornson *et al*. 1975). The HDL (but not LDL) exchange rate decreases over incubation time (6-24 hrs) (d'Hollander and Chevallier, 1972*a*) but the inital rates are the same to both HDL and LDL under approximately physiological conditions (Gottlieb, 1980). Exchange of cholesterol between RBC and SUVs is also rate limiting (Girand and Claret, 1979) and this is probably because only the outermost CHOL of the liposome membrane is available for exchange under equilibrium conditions (Poznansky and Lange, 1978).

Under non-equilibrium conditions *in vitro*, all the vesicle CHOL is available although its loss can be controlled by the fatty acid content of the SUV lecithin. In the experiments reported here no RBC-associated ^{14}C-radiolabel was found in any of the *in vivo* experiments. The reason for the difference between *in vivo* and *in vitro* experimental transfer of ^{14}C-CHOL from liposomes to RBC is unclear. Apart from the possibility that MLVs act differently from SUVs the difference may reflect

the presence of the lipoproteins *in vivo* which compete with the RBC for exchangeable material. Kirby *et al*. (1980b) reported that the presence of whole blood (as opposed to plasma or serum) tended to stabilize leaky SUVs; Gregoriadis and Davis (1979) showed a similar effect using MLVs. The reasons for this increased stability remain unexplained but it has been suggested that it could be due to donation of CHOL from erythrocytes to vesicles (Bruckdorfer *et al*., 1968).

Donation of free CHOL, adsorbed onto a celite surface, to lipoproteins has been observed (Ashworth and Green, 1964) in this way both human and bovine HDL have been shown (Jonas *et al*., 1978) to have a spare CHOL-binding capacity although the conditions used (37° C; 16 hours) and the HDL-CHOL levels produced are unlikely to be found *in vivo*. It was reported that LDL adsorbed very large amounts of the immobilized CHOL but this was not investigated further. Ashworth and Green (1964) claim that CHOL donated to HDL in this way does not behave in exactly the same manner as endogenous sterol.

Jonas and Maine (1979) studied the transfer of radiolabelled CHOL and EPC (2:1 molar ratio) from vesicles to HDL. The bovine HDL had been heat treated (to destroy LCAT) and then preincubated with SUVs to produce a larger "supersaturated" HDL particle containing 6 % by weight more lipid and 4 % by weight less protein than normal. When this particle was incubated with radiolabelled SUVs no release of protein from HDL occured nor was there any net transfer of lipid from the SUVs or from HDL. Exchange reactions took place in both directions and all the vesicle CHOL (but only 70 % of EPC) was available for exchange ; equilibrium was established in 5 hours at concentrations of SUV-CHOL : HDL-CHOL between 1:1 and 1:14 (approx.). Exchange was found to be dependent on the HDL-CHOL concentration.

Despite the *in vitro* work mentioned above, Sodhi and Gould (1967) have shown that apoHDL will not bind cholesterol. Presumably, the incorporation of CHOL from vesicles (Jonas and Maine, 1979) results from fusion of the vesicle membrane with the HDL membrane (Tall and Small, 1977, 1979). A study of the interaction of HDL apoproteins (A-I) (Tall and Lange, 1978a, 1978b) or native HDL (Tall and Small, 1979) with SUVs and MLVs demonstrated that the cholesterol/phospholipid ratio of the liposomes increased, these and other studies (Jonas and Kranjnovich, 1978) have led to the conclusion that, whatever the initial CHOL/PL ratio of the vesicles, HDL can only solubilize CHOL to certain extent. The CHOL/PL ratio in HDL reflects the initial vesicle concentration

but, as more PL is added to the HDL, cholesterol is actively excluded from the lipoprotein. The liposomes become more stable as their cholesterol content increases and less amenable to attack by HDL although this might make them more susceptable to attack by LCAT (lecithin : cholesterol acyl transferase) (Glomset and Norum, 1973).

Thus far, it has been assumed that the increase in ^{14}C cholesterol in the LDL (and HDL) fractions represents an exchange phenomenon. However, mass transfer of liposomal lipids cannot be ruled out since the total amounts of PL or CHOL were not measured before and after the injection into rats. Moreover, the total amounts of lipoproteins were not measured either; it is possible that ^{14}C cholesterol exchanged with lipoproteins which were subsequently removed from the plasma.

In summary, the primary sites for CHOL donation or exchange from RBC or other sources are LDL and HDL but uptake by HDL may be limited or saturatable. When the vesicle concentration of CHOL is low, SUVs transfere all their lipid (including CHOL) to HDL because their structure is unstable. However, MLVs, which are more stable even in the absence of CHOL, are not dissolved by HDL but are able to exchange CHOL with both LDL and HDL. The LDL-CHOL may additionally be due to exchange reactions between the lipoproteins. It seems possible that liposomal E-CHOL is not handled by lipoproteins in the same way as CHOL.

b) Phosphatidylcholine.

The last decade has seen a large number of reports concerning the interactions of liposomal PC with lipoproteins (mostly HDL). Only published results of *in vivo* experiments or incubations of liposomes with whole plasma (or serum) will be considered in relation to the results shown in here. Early reports on the use of phospholipid/triglyceride emulsions (intralipid) in animals (Thompson *et al*, 1965) and man (Thompson *et al.*, 1975) demonstrated that the phospholipid could be exchanged (without transfer) with LDL phospholipid. Since the intralipid emulsion contains EPC in liposome-like structures (Thompson *et al.*, 1975) the majority of which have a density < 1.006 (i.e. similar to MLVs) this would seem to indicate that HDL is not the only lipoprotein involved. This was confirmed later when Scherphof *et al*. (1978) and Damen *et al*. (1980) showed minor amounts of SUV-EPC in other lipoproteins fractions. Scherphof *et al*. (1978) showed that MLVs (composed of only EPC) would be transformed into a particle with a density similar to HDL when incubated with rat or human plasma. However, this same

group later (Scherphof et al, 1979; Hoekstra and Scherphof, 1979) demonstrated that non-CHOL-containing neutral or anionic MLVs, of exactly the same PC and PA composition as used in the present study (7:1), when incubated with serum showed almost no transformation into an "HDL-like" particle although SUVs of similar composition were transformed. To-date the distribution of MLV-EPC in lipoprotein fractions has not been published but in view of the interactions of SUVs, the data above indicate that there may be further differences between MLVs and SUVs apart from those discussed previously.

Here the most surprising result is the finding of 40 % of EPC associated with the plasma protein pellet. Such high amounts have not been previously reported by others (Krupp et al., 1976; Tall, 1980) although small amounts of SUV-EPC (2.8-13%) were so found. Scherphof et al. (1978) found 10 % of total EPC radioactivity, from CHOL-free MLVs, associated with this fraction but this was shown to be due to free fatty acids probably bound to albumin. This cannot be the case in this study, however, because the ^{14}C radiolabel is on the choline moiety of EPC. It is possible that the radioactivity in d > 1.21 represents lysolecithin (LPC) formed by the action of LCAT on the liposomes. Nichols and Gong (1971) have shown that MLVs and SUVs can act as substrates for this enzyme. No LPC (as judged by TLC) was present in the labelled EPC prior to liposome manufacture.

In a study of MLVs injected intramuscularly into rats, Zirenberg and Betzing (1979) have demonstrated that intact, unmetabolised, liposomes find their way from the injection site to the plasma and liver. Considerable amounts (40 % of total plasma radioactivity) of intact PC, LPC and fatty acids were found associated with the d > 1.21 fraction one hour after injection. Although the MLVs were admininstered by a different route, these data provide some confirmation of these present findings.

An explanation for the ^{14}C-EPC radioactivity in the pellet may come from studies on VLDL metabolism and transformation of this lipoprotein to LDL (Reviewed Eisenberg, 1980)*. Liposomes (MLVs) resemble CHYL and VLDL in their density at least : it is these fractions which decrease during the experiment although this may also be due to the plasma clearance of the liposomes. Chajek and Eisenberg (1978) found large quantities of PC (20 %) and LPC (30-35 %) from VLDL in the d>1.21 fraction (VHDL + pellet) during perfusion of rat hearts. The transformation of the VLDL particle was carried out by membrane-bound lipopro-

* See Corrections page 10 and 11.

tein lipase and the VLDL membrane lipids were released as discoidal particles. The role of lipoprotein lipase in lipoprotein metabolism has been reviewed by Nilsson-Ehle et al. (1980); the enzyme is found in endothelial cells lining blood vessels, to which liposomes may become adsorbed (chapter 4), and it has phospholipase as well as lipase activity. To-date the interaction of liposomes with lipoprotein lipase has not been studied.

The identity of the protein(s) in the pellet which are responsible for binding the ^{14}C radioactivity is not clear. If LCAT is involved in the process it is possible that the LPC so formed is bound by albumin (see following section). However, it must be remembered that it is the function of the lipoproteins to transport PC and CHOL. The presence of LCAT was not tested for in these present experiments but some enzyme inhibition during the in vitro separations can be expected from the presence of thimerosal in the plasma (Hatch and Lees, 1968). Tall (1980) reported that a small amount of LPC was produced following the injection of SUVs into mice but Chobanian et al. (1979), using human serum incubated with similar SUVs reported no LCAT activity; this was confirmed by Kirby et al. (1980a). It is possible that MLVs are better LCAT substrates than SUVs. An analysis of the lipid content of the lipoprotein fractions has not been made so it is not possible to state whether the pellet ^{14}C-radioactivity is due to the presence of EPC, or LPC.

Other proteins have been shown to bind to liposomes (see following section) and their presence could have altered the flotation density of MLVs. Of those which might concern these present experiments, Damen et al. (1980, 1981) have reported that the $d > 1.21$ fraction contains a protein(s) which assists in the transfer of PC to HDL and it also binds to liposomes; the identity and characteristics of this protein have not been defined. Scanu (1967) mixed liposomes, made from different HDL lipids, with apo HDL and found that they formed a complex which floated at a density of 1.24; the HDL/MLV complexes contained amounts of lipids proportional to the amount of PC added. However, the density of this complex has not been confirmed by the more recent work of Tall and Lange (1978b), using PC alone, who showed that it had $d = 1.11$ but this increased slightly when CHOL was incorporated into the MLV bilayers.

Apoproteins released from lipoproteins during centrifugation (Eisenberg and Levy, 1975) are also found in the $d > 1.21$ fraction but the well studied recombinants between these and liposomes do not remain

at the tube bottom (Tall and Small, 1977, 1979; Tall et al., 1978; Tall and Lange, 1978b).

The question must be asked if the ^{14}C-radioactivity in the pellet represents intact liposomes because its equivalence to the ^{111}In counts cannot be ignored; if ^{111}In did not leak from liposomes this would indeed be a logical assumption. On the other hand, the amount of ^{111}In found in the pellet is almost the same as that in the ^{14}C-CHOL experiments and in that case very little CHOL was associated with this fraction. As has already been pointed out ^{111}In could also be bound to phospholipid head groups (especially PA) and so, taken together, these two experiments might indicate that intact MLVs sediment with the proteins but that CHOL rapidly exchanges with LDL and HDL. Most of the other evidence is against this hypothesis because normally MLVs float at d < 1.006 (table 3:3), plasma clearance rates suggest that leakage of ^{111}In occurs in vivo and reports of leakage of entrapped material from MLVs containing low cholesterol in contact with plasma are very numerous.

There are, however, certain differences between the MLV preparations studied by others and those used here. An important, and to-date unstudied difference involves the use of a charge on the liposome. Anionic liposomes (MLVs or SUVs) are known to be cleared from the circulation faster than neutral or positive ones; if liposomal lipids undergo equivalent exchange/transfer reactions irrespective of charge, then their CHOL or PC plasma clearance rates could be expected to be similar. This, however, is not the case (Gregoriadis and Neerunjun, 1974; Juliano and Stamp, 1975). It seems possible that, in addition to the CHOL content, a further (but not exclusive) reason for these differences in lipid plasma clearance rates is the effect of charge on liposome/lipoprotein interactions.

Recently it has been demonstrated that for MLVs a further important parameter which determines lipoprotein interactions is the fluidity of the liposome membrane lipids. Thus, in the absence of CHOL, Scherphof et al. (1979) showed that lipids close to their t_c, irrespective of size, will be destroyed by the effects of plasma. Tall's group (Tall and Small, 1977; Tall and Lange, 1978b) using pure dimyristolyphosphatidylcholine (DMPC : t_c 23°C) incubated with HDL at 27°C found an enhanced dissolution of the liposomes; EPC-MLVs are not destroyed (Hoekstra and Scherphof, 1979).

It must be strongly stressed, that there is no evidence which suggests that, above the gel to liquid t_c of EPC (-15°C), MLVs are broken down by contact with plasma. Indeed, the evidence suggests that EPC-MLVs remain intact (Scherphof et al., 1979; Hoekstra and Scherphof, 1979) although they will leak. Whilst increasing CHOL in the bilayer will reduce the leakage of solutes neither its presence, nor the presence of charged lipids is necessary for liposome stability. Therefore, reactions between MLVs and lipoproteins probably only involve exchange phenomena. The presence of a population of SUVs mixed in with the MLVs will be treated differently from MLVs and, in this case, the vesicles may be broken down and EPC transfer (rather than exchange) is expected.

In the EPC experiments, the amount of ^{111}In, representing whole liposomes (either entrapped in the aqueous space or bound to the bilayer), found in each fraction was similar to that found in the fractions of the CHOL experiment and in the controls. The equivalence of ^{14}C-EPC and ^{111}In in these fractions, therefore, suggests that these levels represent whole (unexchanged) liposomes. If, however, exchange has taken place, it would appear that PA (^{111}In marked) exchanges (or transfers) at the same rate as EPC but that the amount of CHOL exchange is about 5 fold higher into the LDL fraction and 2 fold higher into HDL.

In view of the fact that the MLV results presented here contradict much of the published work on the interactions of SUVs with lipoproteins, these latter will be outlined here (reviewed by Morrisett et al, 1977; Tall and Small, 1979).

According to Tall and Small (1977, 1979) HDL can solubilize (DPPC) MLVs and SUVs with the formation of discoidal phospholipid/apoprotein complexes. The high density HDL_3 species can be transformed into lower density "HDL_2-like" particles, enriched in apo A-I; a similar enrichment occured when liposomes were incubated HDL_2. The mechanisms of interaction are complex and depend upon the lipid : protein ratio. Thus, spherical HDL gives up part of its apo-AI to the liposomes to form a discoidal PC/apo-A-I particle. In addition it appears that phospholipid might insert directly into the surface of HDL. (Tall et al., 1978).

Under conditions of complete incorporation of SUV phospholipid into HDL (low vesicle PL:HDL ratios), the HDL fraction was found to be homogeneous by election microscopy and equilibrium density gradient ultracentrifugation (Chobanian, et al., 1979). At higher ratios, trans-

formation was slower and an apoprotein vesicle complex was isolated after short incubations. These findings suggest that incorporation of SUVs into HDL may depend upon dissociation of the apo-A-I and perhaps instability of the resulting vesicle, but also that the HDL particle may have a limited capacity for direct uptake of phospholipid into its surface.

More recently Jonas et al., (1980) have studied the interaction under rigorous conditions and have found that human apo-A-I interaction with pure PC SUVs gives only two types of stable complexes at equilibrium: SUV/apo-AI complexes of about 1000 mols PC and containing 3-4 apoprotein monomers/complex, and apo-A-I micellar complexes with PC containing about 100 PC/apo-A-I and 3 protein monomers/particle. The former complex is formed at high PL/HDL ratios, the latter at low ratios.

Morrisett et al., (1977) have reviewed the interaction of the C-apoproteins with MLVs and SUVs. Unlike the interactions with HDL or apoprotein-A mentioned above, the C-apoproteins do not cause dissolution of EPC-containing SUVs or MLVs although they will dissolved DMPC vesicles. The differences are thought to be primarily due to the degree of saturation of the acyl chains of the PC (Scherphof et al., 1979). The interactions of the apoproteins of the C group suggest that transfer of these proteins from VLDL (where they are the major components) to EPC-containing MLVs and SUVs, in an analogous manner to that already described for apo-A-I from HDL, will probably not occur although fusion of the VLDL and CHYL particles with liposomes may be possible.

Interactions of liposomes with LDL or apo-B have not been studied to any great extent. Chobanian et al., (1979) did not find PC associated with this fraction although a slight association with VLDL did occur (As also suggested from the work of Kirby et al., 1980a and Tall, 1980). However, Thompson et al., (1975) and Scherphof et al., (1978) have suggested that some MLV-EPC can be found associated with LDL fractions. Studies on the association and/or dissolution properties of apo-B with liposomes have been hindered by the total insolubility of this protein, whose molecular weight remains unknown, in aqueous solutions (Bradley et al., 1980) and no clear idea of the structure of the LDL particle although some suggestions have been made (Luzzati et al, 1979).

In summary, the SUVs in the injection are known to interact with lipoproteins and transfere their EPC to HDL. The MLVs which remain in

the plasma are probably stable perhaps because their multilamella structure or surface charge prevents interaction with lipoproteins. Exchange of MLV-EPC with lipoproteins may only be slight. On the other hand, MLV-EPC appears to interact with other plasma proteins perhaps through the mediation of lipoprotein lipase or LCAT. An alternative, but less likely, explanation may be that proteins adsorb to liposomes and the consequent increase in density causes liposomes to sediment with the plasma proteins.

SECTION 3 : 3

Interactions of liposomes with plasma proteins.

In addition to the lipoprotein interactions, discussed in the previous section, it was evident that liposomes interacted with other plasma proteins. This section describes attempts to identify these other proteins.

Introduction.

The serum-induced leakage of entrapped solutes has already been discussed previously. Such leakage presupposes that there is a close interaction between the protein and the liposomes but binding of protein to liposomes may not be an absolute prerequirement for solute release, indeed, the two may not be directly connected (Scherphof et al., 1981). As a starting point for the discussion of liposome-protein interactions the divisions suggested by Papahadjopoulos et al., (1975), with some modifications (table 3:5), have been used.

Types 0, I and II interactions are the most likely to occur in plasma. Type 0 reactions, by definition, tend to be reported less often than the others. In the absence of binding data and isolation of the vesicle/protein complexes it is difficult to descriminate between type 0 and type I interactions since no apparent leakage of solute will occur. Indeed, in studies on the effects of various proteins on the uptake of liposomes by cells (Tyrrell et al., 1977; Hoekstra and Scherphof, 1979; Mayhew et al., 1980) there is always the possibility that the protein is acting by changing the cell membrane rather than binding to the liposome and promoting or retarding the uptake of the liposome/protein complex.

The type III interactions have already been considered previously in relation to the findings of apoproteins associated with MLVs and SUVs (Section 3:2).

Reports of type IV reactions are not numerous. Klausner et al., (1980) noted that a hepatic receptor protein for asialoglycoproteins interacts hydrophobically with lipid but does not induce release of entrapped solutes. However, since this is essentially a cell protein rather than a soluble plasma protein, type IV interactions may represent a special case in that they are typical of proteins which are normally membrane bound and these will not be further considered here.

The reactions of plasma proteins with liposomes containing :

Table 3:5.

CLASSIFICATION OF LIPOSOME/PROTEIN INTERACTIONS*

Type of interaction	Effect on liposomes.		
	Permeability	ΔH. Enthalpy of acyl chain melting	ΔT_c. Temperature of acyl chain melting
0 (no action)	nil	nil	nil
I (electrostatic)	nil or small increase	increase	none or increase.
II electrostatic/ hydrophobic	large increase	decrease	decrease
III hydrophobic	large increase	decrease	none
IV orientated hydrophobic	nil or small increase	decrease	decrease

* modified from Papahadjopoulos et al. (1975).

i) hapten sensitized PL s (Kinsky, 1972, 1980; Kinskey and Nicolotti, 1977);

ii) proteins co-valently coupled to liposome surfaces (Heath et al., 1980; Huang et al., 1980; Leseman et al., 1980a, b; Torchillin et al., 1980).

iii) adsorbed and entrapped liposomal proteins (Gregoriadis and Neerunjun, 1975b; Gregoriadis, 1979a, 1980a, 1980b; Gregoriadis and Meehan, 1981) will not be discussed here since these liposomes must also be considered to be special cases of reactions not normally taking place. It must be stressed, however, that drug containing liposomes may react with proteins in a different manner to empty liposomes because drug entrapment has the theoretical capacity to alter such factors as membrane fluidity and surface characteristics.

SECTION 3 : 3

A) Micro-electrophoresis of liposomes.

Results.

A suggestion that proteins might become associated with liposomes was found from electrophoresis mobility data (table 3:6) of liposomes electrophoresed in the presence of 10 % plasma.

Empty liposomes were prepared from twice the standard quantities (see methods) of lipid and dispersed with 0.145 M NaCl (adjusted to pH 7.4 with NaOH if necessary: this solution was used throughout these electrophoresis mobility studies and for washing of liposome pellets). The liposomes were hand-shaken for 5 minutes but not sonicated since small liposomes cannot be seen in the electrophoresis cell. Three preparations of liposomes were made containing either i) 10 mol % PA (anionic), ii) 10 mol % SA (cationic) or iii) neutral. Each liposome solution (2.5 ml) was diluted 1:1 with fresh pooled (3 rats) plasma (heparinized) and incubated in a waterbath at 37° C for 1 hour. Meanwhile, a sample of each liposome preparation was diluted 1:10 with 0.145 M NaCl and subjected to microelectrophoresis according to the method of Bangham et al. (1958a). The results of this microelectrophoresis are shown in A (table 3:6). These values (and the others of table 3:6) are the means (± S.E.) of at least 5 individual large MLVs.

After incubation the plasma samples were diluted 1:5 with 0.145 M NaCl to give the same lipid concentrations as in A but in 10 % rat plasma. The electrophoretic mobilities of these liposomes are shown in B of the table.

A sample (5 ml) of each diluted incubation mixture was washed and finally resuspended in NaCl. The mobilities of these washed pellets are shown in C (table 3:6). In preliminary experiments (not shown), a sample of each pellet was subject to microelectrophoresis after each wash. At least six washes were required to give constant mobilities for anionic MLVs. The mobilities of cationic and neutral liposomes became constant after 3 and 4 washes respectively.

A comparison of means of the values in table 3:6 showed that anionic liposomes did not give significantly different values from the saline data in the presence of plasma or in the washed pellets. However, it was apparent from the mean values that the negative charge was being quenched (liposomes became more cationic) in plasma and that the

Table 3:6.

Electrophoretic mobility of liposomes.

Preparation**	Charge of liposomes	Direction of movement	Electrophoretic mobility*
A.			
liposomes in NaCl	-	Anode	1.41 ± 0.11
	+	Cathode	1.54 ± 0.17
	NIL	-	0.00
B.			
liposomes in 10%			
Plasma	-	Anode	1.23 ± 0.14
	+	Anode	1.18 ± 0.19
	NIL	Anode	1.46 ± 0.26
C.			
liposomes in NaCl	-	Anode	0.83 ± 0.23
post plasma	+	Anode	0.35 ± 0.06
incubation	NIL	-	0.00

* units = (um. $s.^{-1}$ V^{-1}. cm^{-1}) mean (± st.dev).

** Microelectrophoresis of liposomes in saline (A) or 10 % rat plasma (B) was carried out according to the method of Bangham et al. (1958a; 1958b). Following B, liposomes were centrifuged, washed 6 times and then re-electrophoresed in saline (C). Lipid concentrations (per ml) electrophoresed were 0.8 umol EPC : 0.23 umol CHOL: 0.11 umol charged lipid (PA; anionic : SA; cationic). Final volume was 5 ml in every case.

Comparisons of means (Students 't' test : N/S = not significant).

i)	Anionic	A vs. B: N/S.	B vs. C: N/S.	A vs. C: N/S.
ii)	Cationic	A vs. B: $p<0.001$	B vs. C: $p<0.002$	A vs. C: <0.001.
iii)	Neutral	A vs. B: $p<0.002$	B vs. C: $p<0.002$	A vs. C: N/S.

washed pellet moved more slowly than the liposomes in NaCl. Cationic liposomes and neutral liposomes were significantly different from their saline conterparts in plasma and in the washed pellet (cationic only). It was also apparent that the neutral liposomes lost their acquired charge when washed. The cationic liposomes had their charged quenched and reversed in the presence of plasma and this reversal remained even after washing.

In 100 % plasma (i.e. in vivo) it can be anticipated that, whatever their bilayer charge, all liposomes have the appearance of being anionic due to the interactions with plasma components but, because of the charge quenching effects the order of electronegativity is neutral > anionic > cationic.

b) Discussion.

The results demonstrate that in the presence of plasma, cationic and neutral liposomes appear to attain a negative charge whilst anionic liposomes have their charge quenched (not significant).
However, this change in electrophoretic mobility is not permanent in the case of neutral liposomes since it is removed on washing. This suggests that for neutral liposomes, at least, the interaction is transient (probably type I).

Anionic liposomes, in the absence of protein, were found to have a mobility in NaCl very similar to that found by Bangham (1968) and the zeta potentials, calculated as suggested by Papahadjopoulos and Watkins (1967), for the cationic preparation in saline were the same as was found by these authors using 10 mol % SA.

Microelectrophoresis of liposomes in the presence of plasma has not been previously reported. However, Bangham et al., (1958b) have investigated the plasma coating of clean (acid washed) glass particles. (10 um. diameter). At pH 7.4 in NaCl, the clean glass had an electrophoretic mobility (-1.55 units) very similar to the anionic liposomes used in this study. After the glass particles were incubated with plasma and washed well their electrophoretic mobility proved to be less than the untreated particles and strikingly similar (-0.8 units) to the electrophoretic mobilities of the washed anionic liposomes : other charges were not investigated. These results confirmed that plasma adsorbtion onto inert particles occurs, as first proposed by Abramson (1929 : quoted in Bangham et al., 1958b).

Wilkins and Myers (1966) coated polystyrene latex beds with char-

ged polylysine (cationic) or gum arabic (anionic) to form coacervates; these charged particles were electrophoresed in rat serum (100 %). These authors reported that, in serum, the coacervates all exhibited a net anionic charge. Even highly charged cationic particles attained an anionic charge which was very similar to that attained by anionic coacervates. In addition, anionic coacervates had their electrophoretic mobility quenched from 1:1 units to 0.7 units.

These results are similar to those reported here for liposomes and confirm that protein coating and anionic charge acquisition are a common phenomenon for particles in the circulation.

In other studies, Kimelberg and Papahadjopoulos (1971a) reported on the electrophoretic mobilities of liposomes composed of 100 % PS (anionic) and found mobilities of -9.55 units i.e.: about 7 times faster than the anionic liposomes used here (10 mol % anionic charge). Moreover, these authors showed that the addition of basic proteins (polylysine, lysosyme, cytochrome C) caused a charge reversal (polylysine > lysosyme > cytochrome C). This was used as evidence of an electrostatic interaction. However, ribonuclease A, also a basic protein, only reduced the anionic mobility of PS without reversing the polarity of movement (i.e.: similar to the effect of plasma proteins on anionic MLVs here).

Bangham et al., (1958b) have stated that in 0.145 M NaCl the mean thickness of the ionic double layer will be about 1nm. so that only those charge groups within this distance from the particle surface will contribute to the electrophoretic mobility; this thickness will be reduced if impurities are present in the suspending electrolyte, especially if there are ionic species. Therefore, the presence of 10 % plasma during electrophoresis can be expected to reflect the mobilities of the plasma components nearest the surface (which may not necessarily be those bound to the underlying charged groups) whilst thorough washing will remove those proteins not firmly bound, increase the thickness available for mobility studies and so represent the true motion of the liposomes.

The acquisition of an anionic charge by the cationic liposomes can be anticipated from electrostatic interactions (most proteins are electronegative at physiological pH's). Does this mean, therefore, that anionic liposomes are absorbing basic (cationic) proteins since their mobility is reduced after washing ? i.e. : will the absorbed proteins be different in each case ? This is considered in the following expe-

Table 3:7. Amount of protein associated with liposomes.

Liposome type	Incubation mixture totals		Washed pellets		% Recoveries		ug Protein/ ug P.	ug Protein/ mg PL.*
	Lipid P ug	Protein mg	Lipid P ug	Protein ug	Lipid P	Protein		
anionic	301.7	53.06	229.5	397.3	76.06	0.748	1.731	41.872
cationic	248.3	51.08	171.22	500.0	68.95	0.978	2.92	70.63
neutral	261.9	56.65	163.42	462.8	62.4	0.816	2.827	68.38
NIL	-	55.82	-	70.61	-	0.126	-	-

* Calculated assuming molecular weight of EPC = 750.

Abbrev. Lipid P Lipid phosphorus
P. phosphorus
PL. phospholipid.

Liposomes were prepared as stated in Chapter 2 (methods). The large-(unsonicated)-empty MLVs were incubated at concentrations of 8 umols EPC: 2.28 umols CHOL: 1.4 umols charged lipid (omitted from neutral) in 50 % rat plasma and then washed as before (microelectrophoresis study). In addition, 50 % plasma without added liposomes was treated in the same way. After final wash, the amount of lipid phosphrus and protein associated with the pellets was measured.

riments but here it must be stated that, according to the results of others (Bangham et al., 1958b) only if the protein is more than 1nm thick on the surface the liposome will it reflect the mobility of that protein. Furthermore, if the reaction is of type II an electrostatic charge reversal is not a necessary phenomenon. In the case of liposomes it seems possible that a polyvalent protein(s) might bind to phospholipid per se perhaps after an initial electrostatic attraction. Since it is possible that phospholipid charged head groups are not randomly arranged in liposome membranes (at least at high levels of anionic phospholipid) (Papahadjopoulos et al., 1978; Szoka and Papahadjopoulos, 1980) it also seems possible that the influence of polycations and proteins from the plasma might induce a lateral phase separation of charged lipid groups into patches of membrane covered with protein and other patches of anionic lipid. Depending upon the thickness of the protein (and its relative abundance) some of these anionic patches will be within the plane of sheer whilst others may be too far away (therefore their charges will be apparently quenched). Such liposomes, containing mixed domains of protein and lipid, could be expected to have a varying electrophoretic mobility. Indeed, this appears to be the case in this study (not shown) where washing anionic liposomes more than four times still decreases their anodal electrophoretic mobility although this has little effect upon the neutral liposomes.

As to the identity of the adsorbed proteins, little can be deduced from these experiments. However, the electrophoretic mobility of washed erythocytes is uneffected by the presence of proteins such as:-albumin, casein, gelatin, fibrinogen or haemoglobin (Ambrose, 1966). Yet, other proteins e.g.: antibodies (but not those of the Rh system) will adsorb onto erythocyte surfaces and alter their mobility; similar alterations can be seen in bacteria incubated with serum (Ambrose, 1966).

c) Identification of adsorbed proteins.

Following the discovery that plasma proteins could become adsorbed onto liposomes, the identity of these protein was sought.

The results (table 3:7) show that, even in the absence of liposomes, some protein can be found in the pellet. The identity of this protein was not studied further but a preliminary immunoelectrophoresis (IE) suggested that more than one type of protein (including alpha-$_2$-macroglobulin) was present (not shown). This protein pellet probably represents aggregates of proteins, protein bound to the walls of the

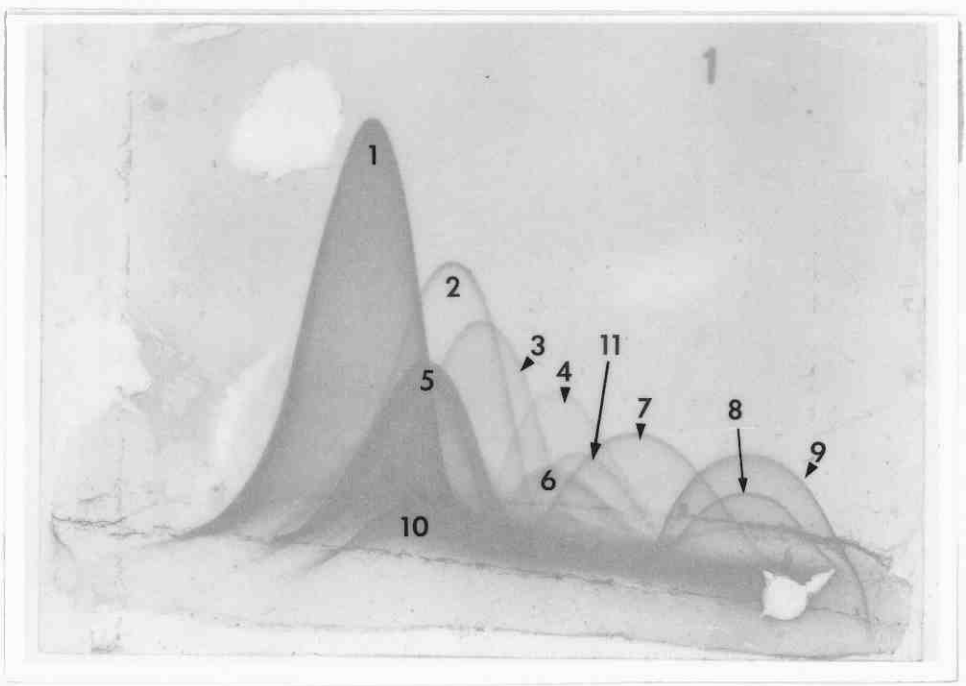
Legend : Plate 1.

Double Immunoelectrophoresis (Clarke and Freeman, 1968) of whole rat plasma (pre-centrifuged for 6 x 10^6 g.min) mixed with anionic sonicated liposomes (4 mols. EPC : 1.14 mols CHOL : 0.57 mols PA) per ml plasma. Sample (5 ul) run against 1 ul/cm^2 whole rat plasma antiserum stained with 0.1 % amidoblack.

The overlay identifies the plasma proteins (where known) in comparison with similar patterns of human proteins.

Proteins :

1. Albumin. 2. Alpha-1-antitrypsin. 3. G_c-Globulins. 4. Alpha-2-HS-glycoprotein. 5. Alpha-I-acid glycoprotein (orosmucoid). 6. Alpha-1-macroglobulin. 7. Transferrin. 8. Immunoglobulin (Ig) G. 9. Ig A. 10. Alpha-lipoprotein. 11. Ceruloplasmin.



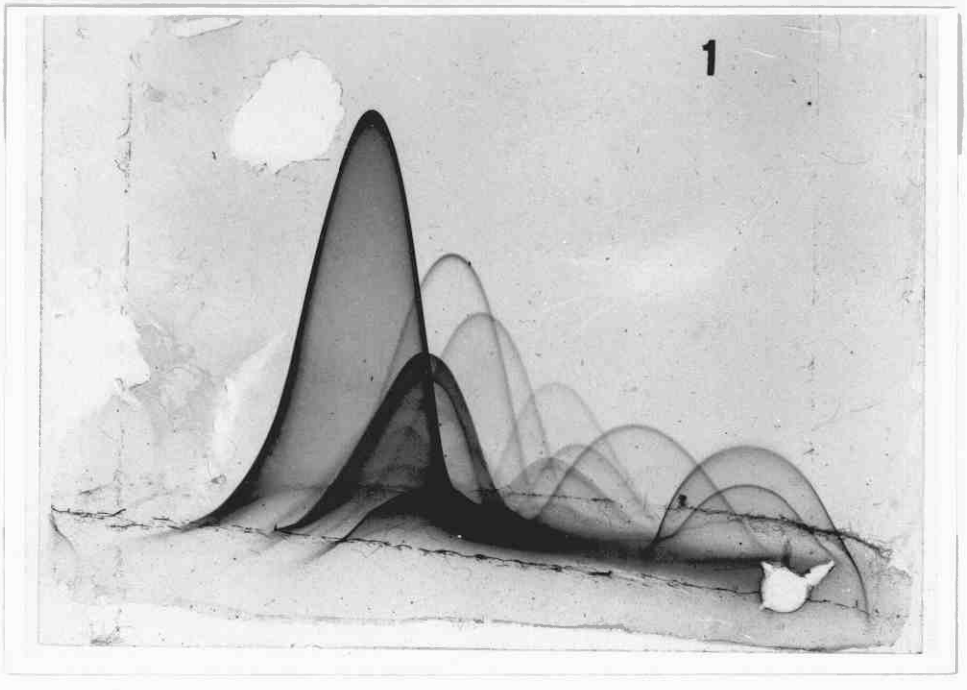
Legend : Plate 1.

Double Immunoelectrophoresis (Clarke and Freeman, 1968) of whole rat plasma (pre-centrifuged for 6 x 10^6 g.min) mixed with anionic sonicated liposomes (4 mols. EPC : 1.14 mols CHOL : 0.57 mols PA) per ml plasma. Sample (5 ul) run against 1 ul/cm^2 whole rat plasma antiserum stained with 0.1 % amidoblack.

The overlay identifies the plasma proteins (where known) in comparison with similar patterns of human proteins.

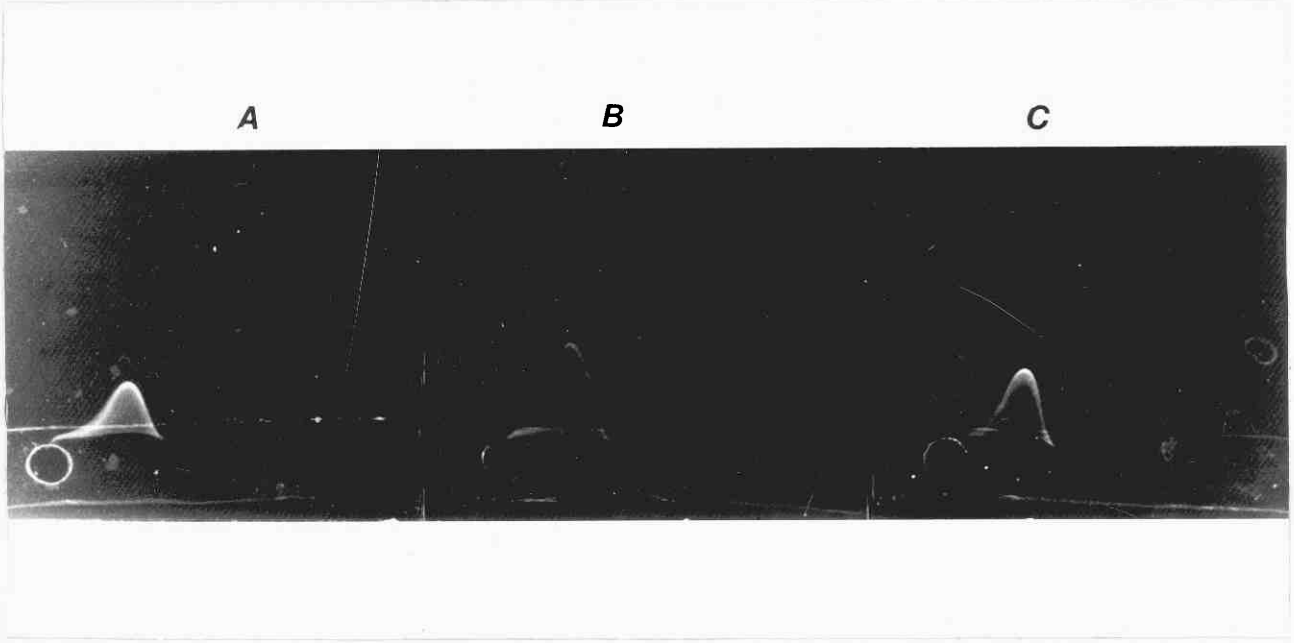
Proteins :

1. Albumin. 2. Alpha-1-antitrypsin. 3. [illegible] globulins. 4. Alpha-2-HS-glycoprotein. 5. Alpha-I-acid glycoprotein (orosmucoid). 6. Alpha-1-macroglobulin. 7. Transferrin. 8. Immunoglobulin (Ig) G. 9. Ig A. 10. Alpha-1-lipoprotein. 11. Ceruloplasmin.



Legend : Plate 2.

Immunoelectrophoresis of washed pellets of unsonicated liposomes previously incubated in rat plasma A - anionic; B - cationic; C - neutral. Sample (5 ul) run against 2 ul/cm^2 whole rat plasma antiserum (Negative print).



centrifuge tube and/or very high molecular weight proteins. This result determined that for future experiments plasma was precentrifuged at 100 000 g for 1 hour before incubation with liposomes. This proce dure did not alter the amount or species of protein found liposome associated (not shown).

The results in table 3:7 demonstrate that protein associates with all liposomes irrespective of charge. Association of protein with neutral liposomes, which was not inferred from the microelectrophoresis studies, suggests that this interaction may be of type II. The amount of protein associated with liposomes was quite constant irrespective of liposome type. The lowest amount of protein was found with the anionic liposomes and the highest amount with the cationic liposomes. Lipid recoveries of unsonicated (U/S) liposomes were not as large as expected and the differences probably reflect liposomes which do not sediment following 100.000 g x 1 hour centrifugation.

Laurell double immunoelectrophoresis (IE) of the washed pellets from plasma incubations was performed on unsonicated MLVs as well as sonicated MLVs. Each liposome type was incubated for 1 hour in either rat or human plasma. The washed pellets were resuspended in 1 ml NaCl.

Identification of rat alpha-1-macroglobulin (alpha-1-M) was made by the addition of pure rat alpha-1-macroglobulin (6 mg/ml), dissolved in 0.145 M NaCl, to the washed pellet samples before IE ("spiked" samples). A sample of this rat-alpha-1-M gave a single protein peak when run against rat plasma antiserum (not shown).

In one experiment, human plasma was heated at 56° C for 1 hour, to destroy complement, before being incubated with the liposomes.

Results.

Plate 1 shows the IE pattern of whole rat plasma mixed with anionic sonicated liposomes and run against rat plasma protein antiserum. The identity of all the peaks has not yet been firmly established for rat plasma proteins. The overlay and corresponding foot note to plate I gives the identity of the known proteins by comparison with published human IE patterns (Clarke and Freeman, 1966; Crowle, 1973; Kroll, 1968, 1969, 1970; Gregoriadis et al., 1974b) and data supplied by Dako-immunoglobulins (antiserum manufacturers).

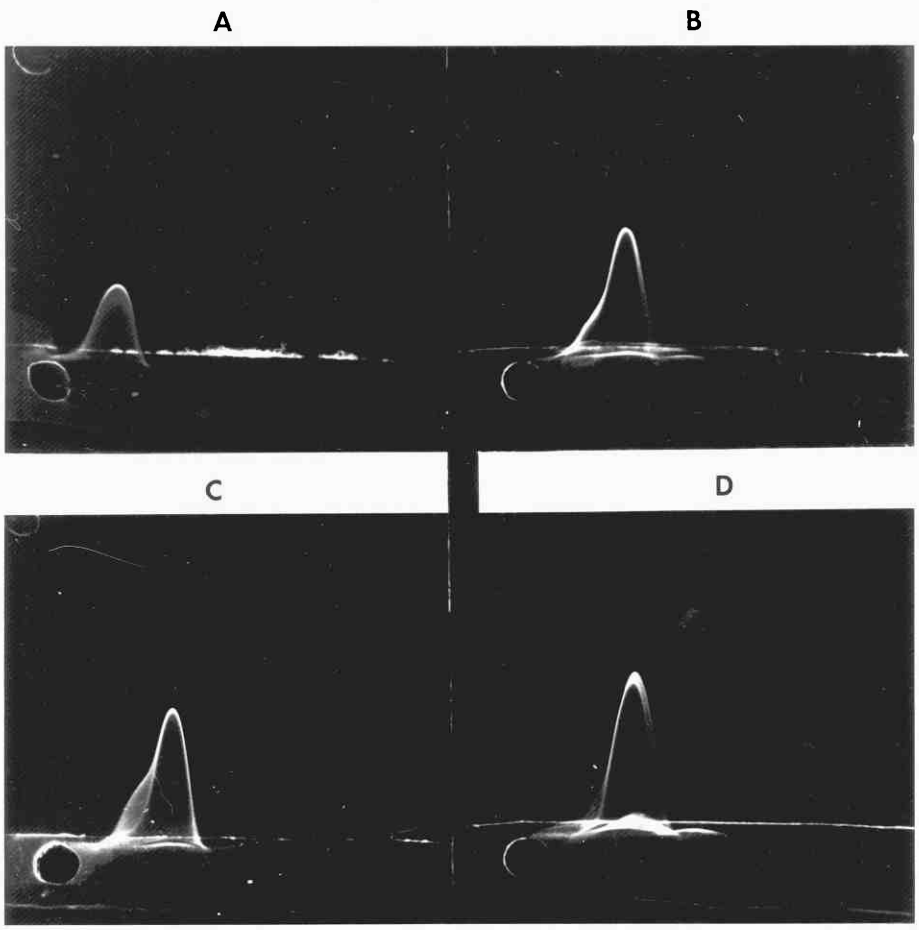
Plate 2, A, B, C, are prints of negatives showing the IE pattern of washed pellets of anionic (A), cationic (B) and neutral (C) U/S MLVs previously incubated in rat plasma. There is only one major protein

Legend : Plate 3.

Immunoelectrophoresis of washed pellets of unsonicated liposomes previously incubated in plasma A - anionic; rat plasma. B - cationic; rat plasma. C - anionic; human plasma. D - cationic; human plasma.

Samples A and B (5 ul) run against 2 ul/cm^2 whole rat plasma antiserum. Samples C and D (5 ul) run against 2 ul/cm^2 whole human plasma antiserum.

(Negative print).



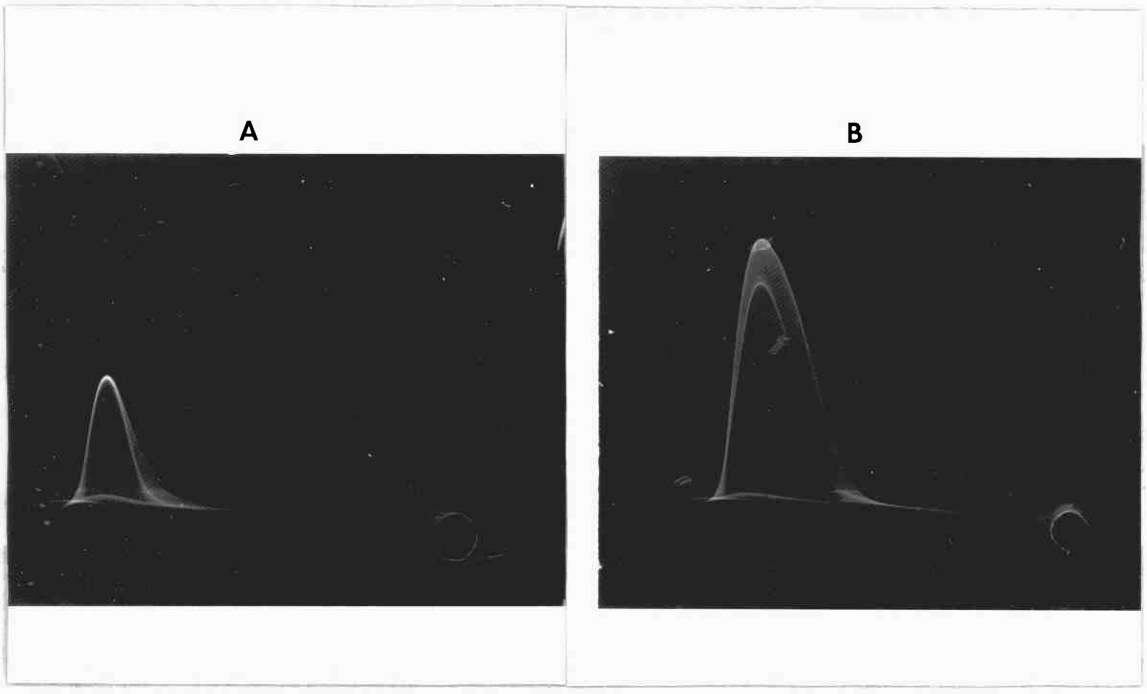
Legend : Plate 4.

Immunoelectrophoresis of washed pellets of unsonicated anionic liposomes :

A) incubated in rat plasma (sample 10 ul run against 2 ul/cm^2 whole rat plasma antiserum).

B) as for A except 2 ul of pure rat alpha-1-macroglobulin (6 mg/ml in NaCl) has been added (sample 12 ul).

(Negative print).



peak in each case. Plate 3 shows negative prints of the IE patterns from washed pellets of anionic U/S MLVs incubated in rat (A) and human (C) plasma. For cationic U/S MLVs incubated in rat (B) and human (D) plasma, the IE-patterns are shown alongside. Plate 3D indicates that in the case of cationic MLVs/human plasma there is some contamination by other proteins but this is minimal compared to the amount of the major peak.

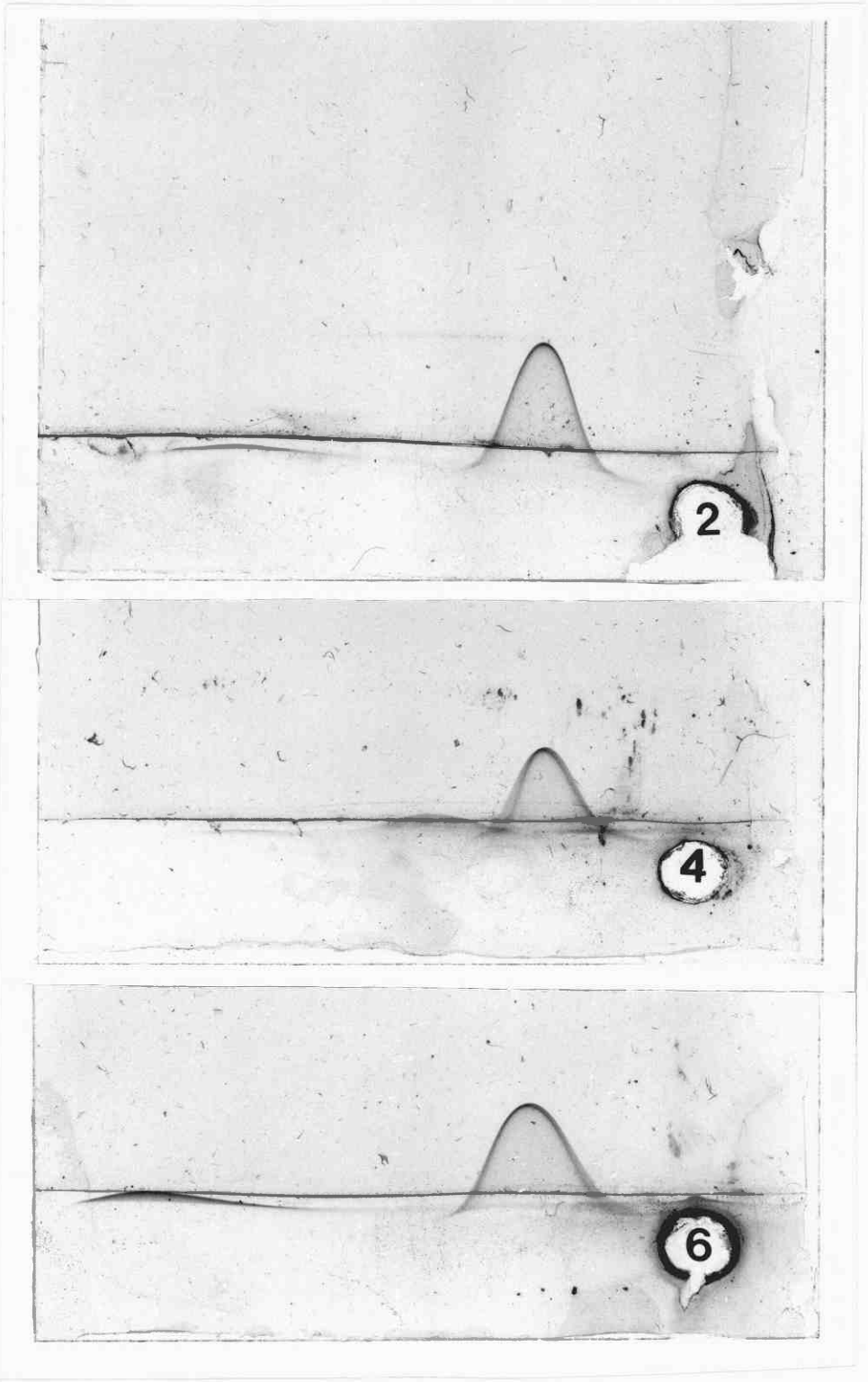
Plate 4 (A) is the washed pellet of anionic U/S MLVs in rat plasma and 4B, is exactly the same preparation, run at the same time, except that pure rat alpha-1-M (6 mg/ml) has been added to the liposomes. These plates demonstrate that the effect of the alpha-1-M is additive and does not change the position of the liposome peak nor produce additional peaks. This, therefore, is direct confirmation that the protein associated with the liposome pellet behaves in a similar manner to purified rat alpha-1-M. Exactly the same results were obtained with the pellets from cationic and neutral U/S MLVs when "spiked" with the pure protein (not shown). Sonicated MLVs of all types incubated with rat plasma and run at the time as Plate 4 gave similar results although the peak hights of the pelleted material were lower (but still obviously due to the same single protein : not shown). At the same time, some samples of U/S MLVs pellets were "spiked" with pure rat albumin 5 ul (3 mg/ml) (not shown). These IE patterns showed a clear separation of the albumin peak from the pellet-protein peak. In a single experiment (not shown) anionic U/S MLVs were found to associate with the pure rat alpha-1-M but not the pure albumin solution. Calculation of the peak areas before and after the addition of rat alpha-1-M (6 mg/ml) allowed an estimation of the total amount of this protein associated with the liposome pellets. In each case the addition of the pure protein resulted in an increase of approximately 0.84 square units. Using this data the amounts of protein calculated for U/S MLV washed pellets were : -anionic 416.2 ug; cationic 483.9 ug and neutral 439.1 ug. Comparison of this data with table 3:7 suggests that at least 94 % of the protein in every case is alpha-1-macroglobulin. Comparative values for protein associated with the sonicated preparations were 187.5 ug (anionic), 203 ug (cationic) and 155.4 ug (neutral). The quantities of lipid used in the incubation with plasma were the same in each case (8 umols EPC : 2.28 umols CHOL : 1.14 umols charged lipid) but there is no guarantee that the same quantities of sonicated MLVs were pelleted in each case. Lipid phosphorus was not measured.

Legend : Plate 5.

Immunoelectrophoresis of washed pellets of unsonicated liposomes previously incubated in human plasma

2 : anionic; 4 : neutral; 6 : cationic;

Samples : 10 ul run against 2 ul/cm^2 whole human plasma protein antiserum.



Legend : Plate 6.

Immunoelectrophoresis of washed pellets of sonicated liposomes previously incubated in human plasma

9 : anionic; 10 : cationic; 15 : neutral;

Samples : 5 ul run against 3 ul/cm^2 whole human plasma protein antiserum.

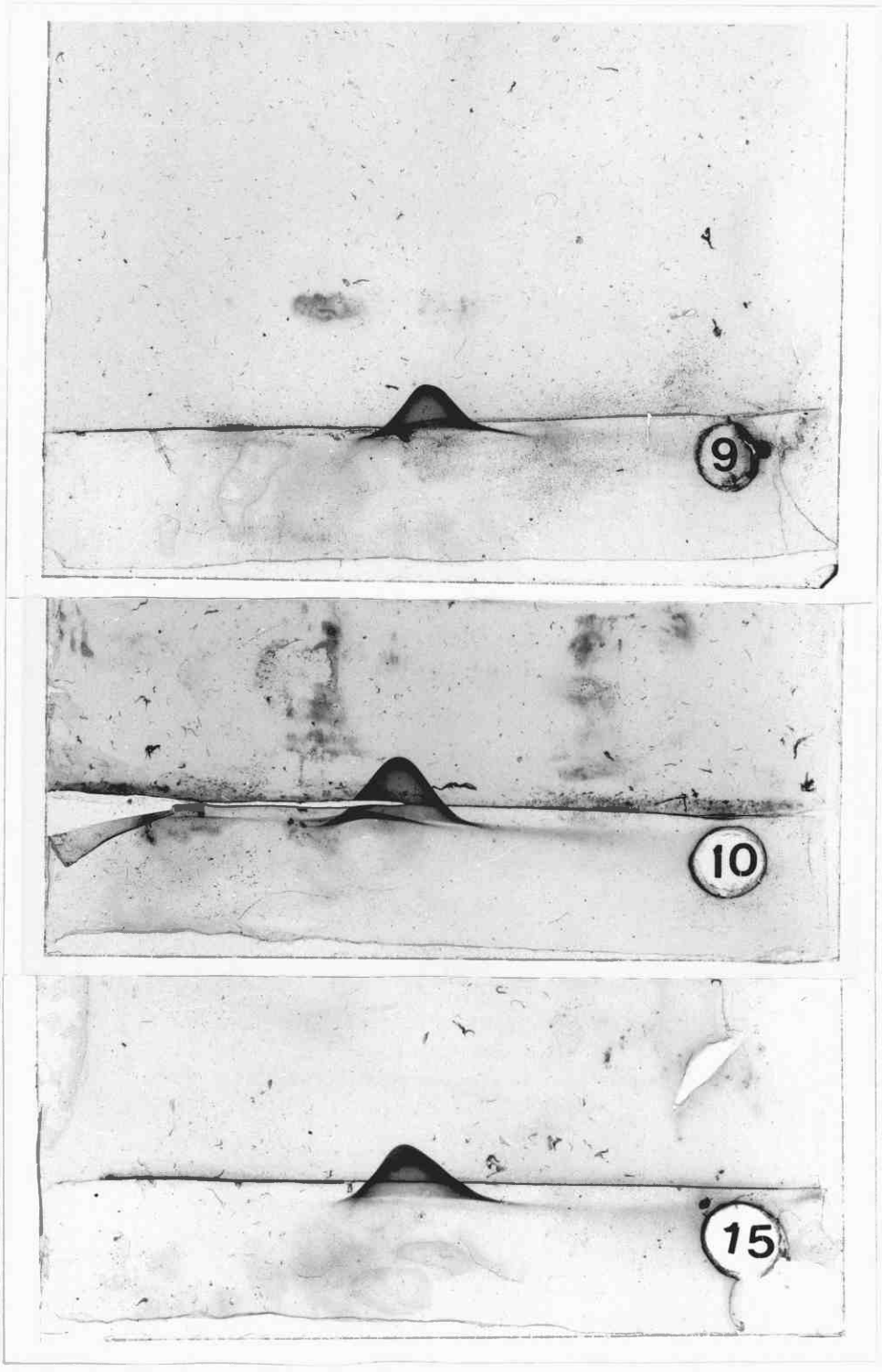


Plate 5 shows the U/S MLV pellets of anionic (2), neutral (4) and cationic (6) liposomes incubated with human plasma and run against whole human-plasma-protein antiserum. The results demonstrate that although there were some differences in peak areas (cationic > anionic > neutral) only one major protein peak was found (some very mild albumin contamination in 2 and 6 can be seen and a very small amount of an unidentified protein to the left of the alpha-2-M peak in 4). In a similar experiment using sonicated liposomes (Plate 6) incubated with human plasma, the peak areas were reduced in comparison to the unsonicated MLVs. In this case, the amount of protein associated is in the order cationic (10) > neutral (15) > anionic (9). Once again there was only one major protein peak with no albumin contamination although the cationic preparation (10) appeared to be very slightly contaminated with another unidentified protein (below left of peak). The reasons for the lower amounts of protein seen associated with these sonicated preparations are probably due to the presence of SUVs in the incubation mixture. (These smaller vesicles will not be sedimented at 100,000 g). There ought, therefore, to be less lipid (not tested) in these pellets. A further factor may be the influence of liposome size on the amount of protein bound (individual U/S MLVs have a larger surface area than sonicated MLVs).

Following the identification of the rat liposome-associated protein, samples of liposome pellets were electrophoresed against an antiserum specific for human alpha-2-macroglobulin. Plate 7 shows anionic sonicated MLVs run against whole plasma antiserum (8) and the same sample run against alpha-2-macroglobulin antiserum (7).

Plate 8 shows the same experiment as in plate 7 using cationic sonicated MLVs. In this case the unidentified protein contamination (c.f. plate 6 (10)) was clearer when the sample was run against whole antiserum (11) although it disappears when specific human alpha-2-M antiserum was used (12). Similar results were obtained with the neutral sonicated preparation.

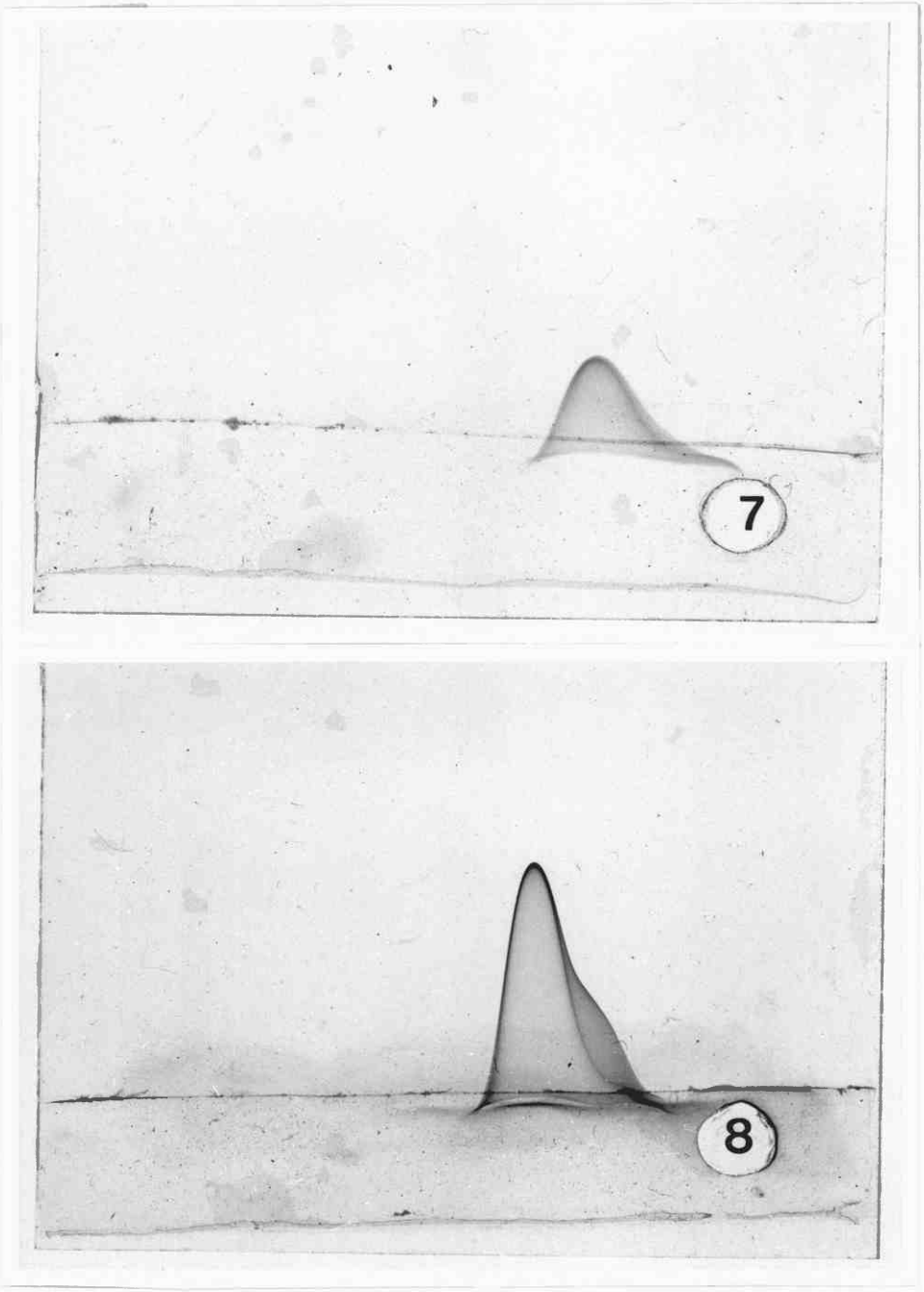
Finally Plate 9 shows neutral U/S - MLVs, which had been incubated with normal human plasma (14) or complement destroyed human plasma (16). Samples were run against specific human alpha-2-M antiserum. Similar results were obtained using anionic and cationic preparations. The plates show that destruction of complement does not affect the binding of alpha-2-M to liposomes although the quantity bound may be affected (peak area 16 is less than 14). However, this lower binding

Legend : Plate 7.

Immunoelectrophoresis of washed pellets of sonicated liposomes previously incubated in human plasma

8 : 15 ul sample run against 2 ul/cm^2 whole human plasma protein antiserum.

7: 5 ul sample run against 4 ul/cm^2 specific human alpha-2-macroglobulin antiserum.

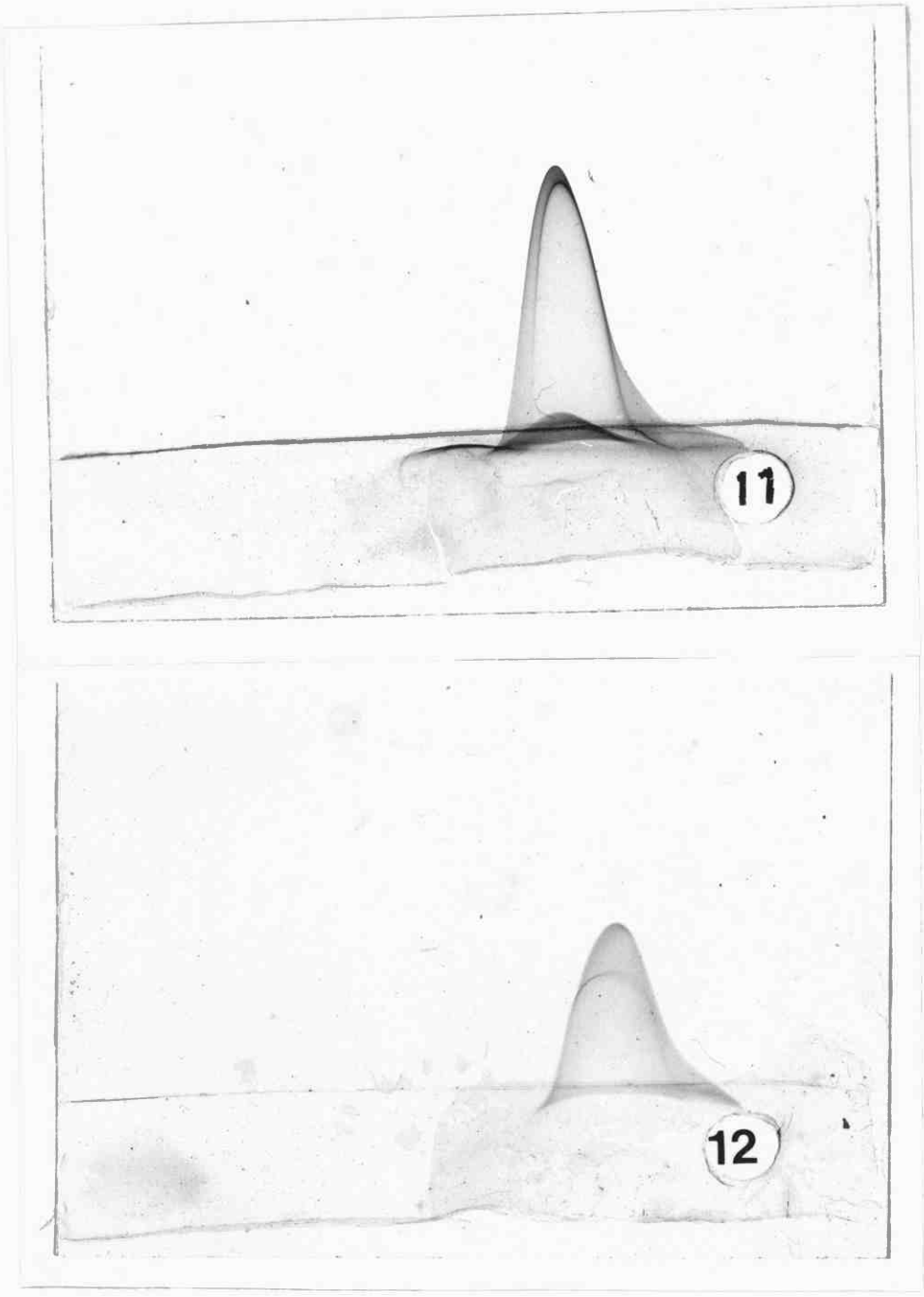


Legend : Plate 8.

As for Plate 7, except the samples are of sonicated cationic liposomes.

11 : 15 ul sample run against 2 ul/cm^2 whole human plasma protein antiserum.

12 : 5 ul sample run against 4 ul/cm^2 specific human alpha-2-macroglobulin antiserum.



cannot be proved since the lipid content of these pellets was not measured but a similar reduction was seen with the other liposome types. The distance moved by the peaks is not the same because the experiments were not carried out on the same day although the same liposome preparations were used in each case. This experiment was not carried out with sonicated preparations.

In other experiments (not shown), which were designed to test the type of interaction occuring between the liposomes and the protein, the electrophoresis of U/S MLVs, incubated in human plasma, was carried out in the presence of 4 M urea (final concentration). Using equivalent sized samples and antiserum concentration, the presence of this agent did not increase the peak hights. This result suggests that either : i) electrostatic binding of the proteins does not occur; * or ii) electrophoresis of liposomes removes all the bound protein. In similar experiments, 0.05 % Triton-X-100 (final concentration) was also used. In this case only cationic liposomes showed an increase in the alpha-2-M peak area (not found in the contaminating protein) but this was very minimal. These experiments do not, therefore, give any conclusive evidence as to the type of interaction occuring.

In summary, sonicated and unsonicated liposomes will bind to proteins of the plasma of both rat and humans irrespective of charge. In all cases there is only one dominant protein found associated with the liposomes and this has been identified as an alpha-macroglobulin. In the case of human proteins, destruction of complement does not effect this protein association.

d) Discussion.

As can be seen centrifugation does not fully separate liposomes from contaminating proteins which can also be sedimented. The device of precentrifuging the plasma, whilst effectively preventing co-sedimentation of proteins, also runs the risk of removing certain high molecular weight proteins or complexes which might interact with liposomes (Juliano and Lin, 1980).

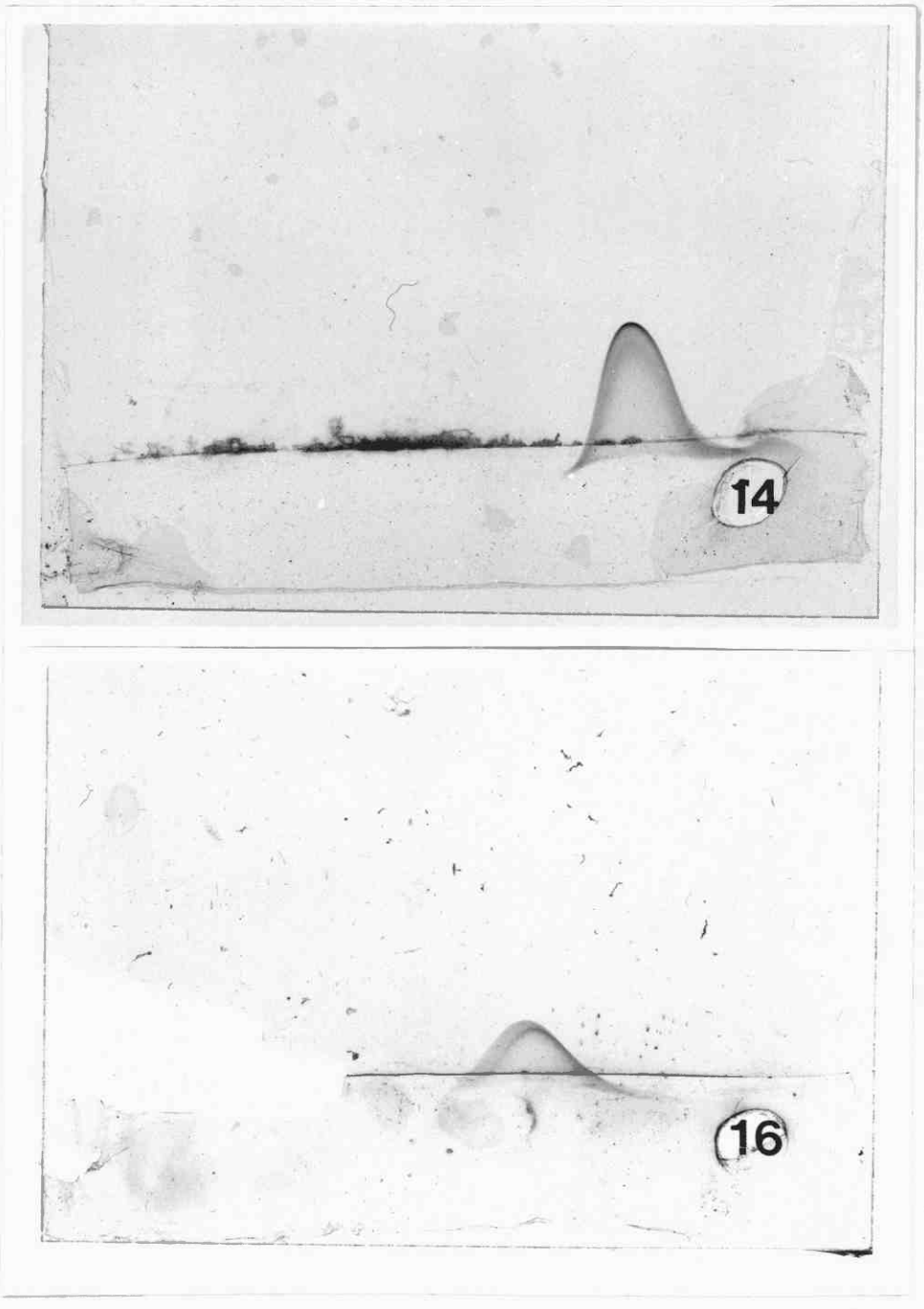
Plate 1 demonstrates 11 major peaks from rat plasma proteins. Unfortunately, published data on rat proteins using this IE method is not common (Gregoriadis et al., 1974b). Identification of these proteins has therefore been made by comparison with human plasma proteins. Apart

* See Corrections page 10 and 11.

Legend : Plate 9.

Immunoelectrophoresis of washed pellets of unsonicated neutral liposomes previously incubated in human normal (14) or complement destroyed (16) human plasma.

Samples : 15 ul run against 4 ul/cm^2 specific human alpha-2-macroglobulin antiserum.



from albumin, alpha-2-M and lipoproteins (whose IE patterns were investigated in the course of this work) other identities cannot be assurred in the absence of specific antiserum. Further, since this plate is for illustrative purposes only, no attempt was made to examine the less numerous proteins, which undoubtedly exist, but can only be visualized by sample dilution or other techniques (Crowle, 1973). The failure of the electrophoretic mobility studies to reflect protein association with neutral liposomes remains a puzzle since the binding of both rat and human plasma is obviously not of the type which allows its removal by simple washing. It must be supposed, therefore, that the alpha-1-M in contact with neutral liposomes becomes differently charged but the mechanism remains unclear.

Apart from the general finding that only one major protein is associated with liposomes, the most surprising fact is the absence of lipoproteins associated with the vesicles. (see previous section). Column chromatography of EPC or DMPC vesicles in the presence of plasma HDL has shown that apo-A-I can be found associated with the liposomes (see previous references). The contaminating human protein associated with cationic MLVs (plate 6:10 and plate 8:11) might in fact be HDL from its electrophoretic position. However, in general the lack of lipoproteins associated with liposomes serves to confirm the report of the previous section that EPC-MLVs do not interact with these proteins. Since SUVs could not be studied in this method, these results are not incompatible with the results of others (e.g.: Tall and Small, 1977; Kirby et al., 1980b; Gregoriadis and Kirby, 1980) who showed HDL-SUV complexes. Hoekstra and Scherphof (1979) demonstrated that the vast majority of anionic EPC-MLVs did not contain protein but a small amount of lipid radioactivity could be found bound to other proteins. It is most likely that this radioactivity is due to lipid exchange rather than transfer, however, if this small fraction contains whole liposomes they may not have the same density as other MLVs and so will not sedimented in these experiments.

The amount of protein associated with various MLV pellets represent very small quantities of the total available material. In rat plasma the alpha-1-M concentration is about 9.1 mg/ml (Ganrot, 1973 cf. about 3.0 mg/ml alpha-2-M in humans : Schultze and Heremans, 1966) so that between 4 and 5 % of the macroglobulin available is liposome bound. Hoekstra and Scherphof (1979) found approximately 2.5 fold higher values (per umol PL) of a whole alpha-globulin bovine plasma

fraction bound to liposomes (very similar to those used here) but this binding was dependent upon the amount of protein available. Thus, using 1.5 umol EPC these authors found about 8 % of the alpha-globulin fraction bound. But in contrast to the results presented here, Hoekstra and Scherphof also found that albumin, beta-globulins and gamma-globulins bound to anionic MLVs when incubated with plasma or pure fractions of these proteins. In the case of albumin, these authors found that, at the same concentration (3 mg/ml) use in the present experiments, only very small amounts (13 ug/umol lipid) were bound to the liposomes and they pointed out that sensitive protein assay methods are needed to detect this level. Using the above figure, the amount of albumin associated with the sample on the IE plate would be approximately 500 ng and this should be theoricically detectable.

The findings of beta and gamma-globulins bound to liposomes (Hoekstra and Scherphof, 1979) is not confirmed in the present findings. Tyrrell et al., (1977) showed that beta-globulins could cause leakage of entrapped solutes from liposomes but they failed to find similar effects when whole serum, albumin or gamma-globulins were used. This result suggests that beta-globulins do not have the same effects when they are incubated separately as when they are a constituent of whole plasma serum.

This point raises a major criticism of work which purports to show protein associated with liposomes. It seems likely that many proteins will bind liposomes if they are: - i) at a high enough concentration, ii) incubated for long enough and iii) not subjected to competition from other plasma proteins of different avidity. A further point must be considered as well, the type of interaction between liposomes and the protein is very important. A protein which binds electrostatically (type I) may not remain bound even in the absence of a dissociating agent. It may be in equilibrium with unbound material especially when the number of "binding sites" are limited. Further, these electrostatic interactions may well be disrupted by mechanical means (such as 7 centrifugations at 100.000 g for 1 hour each!). Whereas an interaction of the type which involves hydrophobic insertion into the bilayer (type II) should remain more stable until such time as the bilayer is disrupted (or, as implied earlier, the electrophoretic current is high enough to "pull" the protein out of the bilayer).

The work of Tyrrell et al., (1977) suggested that gamma-globulins and albumin were only type I interactions because no leakage occurs

from the liposomes, in this work these results with albumin were confirmed (section 3:1). Hoekstra and Scherphof (1979) state that bovine serum albumin is only loosely bound since almost half of it is lost from the vesicle surface when these liposomes are added to serum. Interactions of liposomes with albumin have been much studied and there have been conflicting reports of the type of interaction involved. Most of the work has considered albumin in isolation from other plasma proteins. At low pH, bovine albumin (BSA) (Sweet and Zull, 1969; Sogor and Zull, 1975; Bartholow and Greyer, 1981) and ovalbumin (OA) (Oshima and Nagasawa, 1973) will bind to liposomes although this is dependent upon the presence of anionic charge (Sweet and Zull, 1969) or the purity of the albumin (Bartholow and Geyer, 1981). At certain concentrations of OA liposomes are aggregated at low pH (Oshima and Nagasawa, 1973) but this is inhibited by increasing the ionic strength or by cations (i.e.: probably type I). Bound BSA has been shown to perturb the membrane structure (Sogar and Zull, 1975) as well as causing solute leakage (Sweet and Zull, 1969) (Type II). At physiological pHs, BSA (Sweet and Zull, 1969; Weissmann et al., 1974; Kimelberg, 1976; Kitagawa et al., 1976; Tyrrell et al., 1977; Kaye and Ryman, 1980; Weinstein et al., 1981), O A (Weistein et al., 1981), alpha-lactalbumin (Hassens et al., 1979) and human serum albumin (HSA) (Finkelstein and Weissmann, 1979; Juliano and Lin, 1980) bind strongly to liposomes and do not cause leakage irrespective of the charge on fluidity of the vesicles (Tyrrell et al., 1977; Finkelstein and Weissmann, 1979). (Type I interactions). However, if the liposomes contain LPC (> 25mol%) then leakage does occur (although CHOL will protect against this), furthermore, methylated BSA will also cause solute leakage even without LPC in the membrane but BSA can prevent the leakage induced by the addition of exogenous LPC (Kitagawa et al., 1976) (Type II effects). Zborowski et al., (1977) reported that albumin was capable of binding to, and causing the destruction of, liposomes composed of rat PC. Later, this group suggested that this might be due to impurities (lipoproteins) in their BSA (Scherphof et al., 1981). Nevertheless, in the single experiment reported here no rat albumin bound to liposomes of any composition. However, an albumin may be one of the minor proteins seen in a few of the IE patterns (plate 5 (2 and 6)).

Weissmann et al., (1974) have shown that liposomes bind IgG molecules when co-incubated (anionic almost 2 fold higher than cationic) and that this can be increased if the protein is heat aggregated but this does

not happen with albumin. This gamma-globulin effect seems to be a type II interaction since it causes solute leakage, however, Tyrrell et al., (1977) using a similar system were not able to confirm that leakage occurred. From the results shown here it does not appear that gamma-globulins, in the presence of other proteins, bind to liposomes.

In a study of MLV binding to human plasma proteins Juliano and Lin (1980) investigated the effect of charge and fluidity of the liposome membrane on protein interactions. Since in many respects their investigations are comparable to those carried out here, this report will be discussed in some detail.

Firstly, the amount of protein bound per mg lipid was very similar to that found in this work at least in the case of cationic liposomes (50ug/mg) but the anionic and neutral liposomes were found to bind considerably less (12ug/mg and 8ug/mg respectively) than was found here. The reasons for these differences are unclear although there are two important differences between this work and that of Juliano and Lin's (1980); i) the results were obtained with human serum and ii) the lipid composition (EPC:CHOL = 1:1; anionic charged imparted by PS) is not the same as used here. It is possible that the increased cholesterol content changes the binding (probably the type II interactions) of some proteins (cf. the results of others discussed in the lipoprotein section).

Secondly, these authors have found differences in the species of proteins bound by individual types of liposomes. Anionic (PS) liposomes (EPC or DPPC) bound a random assortment of proteins but few high molecular weight (> 200.000 daltons) components. In contrast, cationic and neutral selectively bound these larger proteins and neutral liposomes apparently failed to bind proteins of the molecular weight range 60-150 x 10^3 daltons. There was only one slight difference between DPPC and EPC-containing cationic liposomes; cationic DPPC vesicles bound more of an unidentified protein in the 60-150 x 10^3 dalton range.

Interestingly, the binding of proteins was found to be temperature and time dependent. Less protein was bound at 4°C than at 37°C. Further, the high molecular weight proteins bound instantaneously whereas other proteins (e.g.: albumin) took up to 15 minutes to bind.

As to the identity of the bound proteins, Juliano and Lin (1980) have shown a wide variety of protein bands on polyacrylamide gels of liposomes incubated with plasma. A major difference in the methodology

between these two reports is that the MLVs were only washed 3 times (not considered sufficient in this work) and centrifugation was carried out at 10000 rpm for 20 minutes which would probably result in the loss of some smaller MLVs. These authors have identified proteins solely upon their molecular weight using standard markers.

By this method, it is claimed that only very small amounts of alpha-2-M are liposome-associated whilst large amounts of albumin (especially) and 2 (or 3) high molecular weight proteins also bind. Unfortunately, Juliano and Lin (1980) incorrectly claim that the molecular weight of alpha-2-M is 185000. In fact, the molecular weight of human alpha-2-M is 7.25×10^5 (Steinbuch, 1971; Harpel, 1973, 1976; James, 1980) whilst it is generally recognized that the protein consists of 4 subunits each of 185,000 mol.wt (Harpel, 1973, 1976) and at least 2, but more usually 4, of these subunits are required for total activity (Starkey and Barrett, 1977). Since, as Juliano and Lin (1980) point out, there are very few plasma proteins of > 200,000 mol.wt, it seems possible that one of their high molecular weight liposome-associated proteins could be alpha-2-M. These authors also found two other proteins of high molecular weight and it is conceivable that these could be dimers or timers of alpha-2-M.

In the lower molecular weight range, in addition to albumin and immunoglobulin heavy and light chains, Apo-AI and sever other unidentified proteins were also shown to bind. Fibronectin was also shown to bind to all the types of liposomes tested. Since this assortment was not found in the present study a further difference between these two reports must be considered. Juliano and Lin (1980) used human serum for their studies. In view of the findings on liposome stability (Gregoriadis and Davis, 1979; Kirby et al., 1980b) in the presence of blood, plasma and serum it is obvious that there are differences between the interactions of liposomes with plasma and with serum (stability, plasma > serum). Moreover, it has been known for some time that alpha-2-M must be isolated from plasma and not serum because, in addition to its well known ability to bind to proteinases, it will also bind to thrombin, Hageman factor (clotting factor XII), plasmin and Kallikrein (Steinbuch et al., 1975).

Finally, Juliano and Lin (1980) having washed the MLVs, then treat the pellet with ethanol which precipitates the proteins and removes some of the lipids. This

method ensures that even lightly bound proteins (which may be bound to other proteins - see electrophoretic mobility results) will be precipitated whereas the centrifugal method used here probably removes these.

The addition of dissociating agents (chaotropic ions, urea), however, failed to change the gel pattern whilst typsin treatment removed the high molecular weight proteins (suggesting type II interactions). The binding of the fibronectin, at least, could not be displaced by incubation of these "coated" liposomes with whole serum.(Juliano and Lin 1980).

Juliano and Lin (1980) also studied the effects of liposomes upon the clotting characteristics of human plasma. Charged liposomes altered the clotting times by, it was suggested, binding to clotting factors. Anionic liposomes apparently caused depletion of serum factors VIII (antihaemophillic factor), XII (Hageman factor) and possibly others as well. Several factors (V and VIII) in the clotting cascade are known to be activated by phospholipids and Ca^{2+} (Mahler and Cordes, 1971); Bangham (1961) showed that increased clotting activity was associated with increased liposome surface charge.This was further investigated by Papahadjolpoulos et al. (1962) who reported that the in vitro clotting activity of anionic liposomes at pH 7.4 was low when 10 mol. % PA, PS or PE were incorporated into the membrane; they concluded that the activity was due to surface charge density rather than any phospholipid species. It is surprising therefore that Juliano and Lin (1980) found that anionic liposomes (10 mol % charge) could deplete the plasma of factors VIII, XII and possibly VII; the effect on factor VII was also found with 10 mol % cationic liposomes. It seems possible that the reversal of the surface charge of cationic liposomes coupled with the presence of alpha-2-M to remove clotting factors may account for these results, especially since neutral liposomes had no effect on the clotting times but did lower factor VII levels in the plasma.

Removal of clotting components does not necessarily mean that the clotting components are bound to the liposomes. Two plasma proteins, alpha-2-M (Steinbuch et al., 1975) and fibronectin (Yamada and Olden, 1978) are known to bind clotting factors. It seems possible that clotting factor depletion might be due to removal of specific factors rather than activation of the whole clotting mechanism. In addition, this removal of factors may not necessarily result in the increased tendancy to bleed since alpha-2-M also removes plasmin, the enzyme

responsible for the breakdown of clots.

The effects of liposomes on clotting factors (Juliano and Lin 1980) are not in agreement with the findings of Gregoriadis (1978) who reported no effects of liposome administration on the haematology of rats and dogs treated with liposomes I/V and, later (Gregoriadis, 1980a), failed to find any effects of liposome administration on either the clotting mechanism or plasma alpha-2-M levels in a patient treated over a 3 year period. The differences may be due to the method of taking the blood or other factors which have yet to be recognised.

Although complement did not appear to play a role in the binding of alpha-2-M to liposomes in this study and Juliano and Lin (1980) did not find complement depletion following the addition of liposomes to human serum, other workers have reported that liposomes can activate the complement system. Kaplan and Volanakis (1974) and Volanakis and Kaplan (1974) found that C-reactive protein (CRP), an acute phase reactant, interacted with PC/CHOL emulsions (probably liposomes) to activate the human classical complement pathway with a concomittant depletion of haemolytic complement ability. More recently, Richards et al. (1977) reported that CRP will bind to certain liposome types (i.e. those which are cationic and contain galactosylcerebrosides) and, in the presence of human serum, this results in the consumption of each of the components of the classical complement pathway (C_1 -C_9) with the resulting release of liposome entrapped solutes. This reaction appears to be specific for liposomes containing PC as their major bilayer constituent.

Liposomes composed of lipids which are not haptenated or do not contain carbohydrate moieties are not intrinsically immunogenic nor do they cause depletion of complement (Kinsky, 1972; Kinski and Nicolotti, 1977; Kinsky, 1980). However, it seems highly likely that the incorporation of drugs, proteins or other molecules into immunologically inert liposomes may cause hapten groups to become available on liposome surfaces; in this case binding of proteins (e.g. immunoglobulins and complement) may be different from non-hapten-containing liposomes.

In conclusion, whilst alpha-1-macroglobulin (rat) or alpha-2-macroglobulin (human) may not be the only protein bound by liposomes, it may be one of the major proteins which associates with the vesicles in vitro.

The presence of an alpha-globulin associating with liposomes has been confirmed by Tyrrell *et al* (1977), Hoekstra and Scherphof (1979) and Juliano and Lin (1980). The extent to which alpha-2-M (and other plasma non-lipoproteins) associate with liposomes *in vivo* awaits determintation. The role that alpha-2-M may play in *in vivo* interactions will be discussed in the next chapter.

CHAPTER 4.

Tissue uptake and action of free and entrapped drugs following intravenous injection.

INTRODUCTION

The previous chapter has shown that liposomes will interact with plasma components following a single intravenous injection. In this chapter the tissue distribution of the liposome entrapped drugs will be followed and this will be compared to the distribution found for the same drug given in its free form. The liver uptake of the entrapped drugs will be studied in greater detail, since this is the organ which receives most of the injected dose. An examination of the sub-cellular distribution of both the free and entrapped agents in this organ will be made. Finally, the activity of the entrapped agents in the liver will be examined by studying their affects on nucleic acid synthesis in the regenerating liver.

Section 4:1.

Tissue distribution of free and liposome entrapped drugs.

The distribution of ACT-D, BLMs and ASPase into tissues other than the liver (for which see following sections) was followed over timed periods after the intravenous injection of either non-entrapped or entrapped drugs into rats (100-125 g body weights). The preparation of drugs and liposomes has already been mentioned (Chapter 3, section 1) in relation to the plasma clearance of these compounds. Since the results in this section and section 4:2 and 4:4 were obtained from the same animals as in Chapter 3:1 only a summary of the doses and composition of the liposomes will be given here (Table 4:1). This will serve as a reference for this section and sections 4:2 and 4:4. Liposomes and free drugs used in section 4:6 were different and will be outlined in that section.

The mean weights of tissues removed for the study of drug distribution were given in table 2:1. Generally, the radioactivities in each tissue will be expressed as % of the injected dose found per gm of tissue (not corrected for blood contamination).

i) Actinomycin-D.

The tissue distribution of free and entrapped ^{3}H-ACT-D is shown in fig. 4:1. A comparison of the means was calculated using Student's 't' test. The results of these comparisons are given in table 4:2 which

Table 4:1.

Liposome Compsosition* and drug doses used for tissue distribution studies.

Drug Preparation E = Entrapped F = Free	Quantities injected**				Dose/Kg***	
	EPC umol.	CHOL umol.	PA umol.	DRUG ug.	EPC mg.	DRUG ug.
^{3}H-ACT-D (F)	-	-	-	10	-	90.91
(LP) (E)	1.5	0.43	0.21	10	10.25	105
^{111}In-BLM (F)	-	-	-	64.0	-	512.0
(AP) (E)	4	1.14	0.57	55.7	24	445.6
ICL-^{125}I-BLM (F)	-	-	-	0.531	-	4.25
(AP) (E)	3.68	1.05	0.52	0.517	22.06	4.13
B/H-^{125}I-BLM (F)	-	-	-	0.875	-	7.0
(AP) (E)	3.58	1.02	0.51	1.0	21.5	8.0
^{125}I-ASPase (F)	-	-	-	1.44	-	12.02
(AP) (E)	1.6	0.45	0.23	1.35	17.77	11.24

* All liposomes were anionic MLVs (7:2:1, EPC:CHOL:PA, molar ratio) (2.5 min. sonicated). Details of preparation can be found in chapter 3:1. Each animal received a volume of 1ml in every case.

** Assuming 100 % recovery of EPC in liposomes.
Specific activities of drugs : -
^{3}H-ACT-D; (F) 10 uCi/mg, (E) 25 uCi/mg. ^{111}In-BLM; 1.66 mCi/mg. ^{125}I-BLM(ICL); 429 uCi/mg. ^{125}I-BLM(B/H); 236 uCi/mg. ^{125}I-ASPase; 72.4 uCi/mg.

*** Normally the rats were of equal weight $\pm$ 5 %.

Legend Fig. 4:1.

Uptake of ^{3}H-actinomycin-D into tissues.

Free (closed bars) or entrapped (open bars) ACT-D was injected I/V into rats (see table 4:1 for details of doses). The histogram shows the percentage of the injected dose associated with each gram of tissue at timed intervals after injection.

Each bar represents the mean (± st.dev.) from 2 (free) or 3 (entrapped) treated animals.

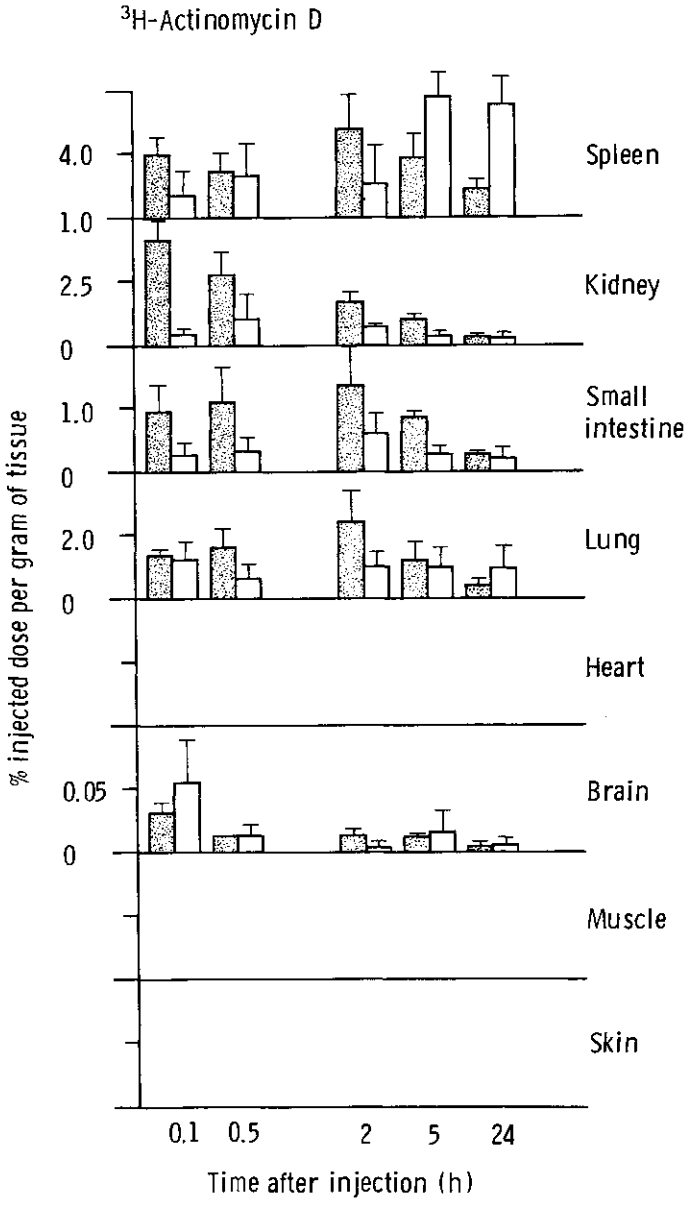


Table 4:2.

Significance values (Students 't'-test)** for the comparison of means between free and entrapped drug concentrations in tissues.

^{3}H-Actinomycin D.

TISSUE	Time after Injection					
	5'	30'	120'	5h.	24h.	72h.
Spleen	N/S*	N/S	N/S	< 0.02	< 0.005	N/S
Kidney	< 0.001*	N/S*	< 0.05*	< 0.01*	N/S*	N/S*
Small Gut	< 0.05*	N/S*	N/S*	< 0.02*	N/S*	N/S*
Lung	N/S*	N/S*	N/S*	N/S*	N/S	N/S
Brain	N/S	N/S*	N/S*	N/S	N/S*	N/S*

* Free drug concentation (% injected dose/gm wet weight tissue) higher than entrapped drug concentration. Otherwise entrapped drug concentration is higher than free.

** 2 degrees freedom. N/S Not significant ($p > 0.01$).

also includes addition information at 72 hours post-injection (not shown in fig. 4:1).

These results demonstrate that there was an increased localisation of entrapped-ACT-D in the spleen at 5 and 24 hours after injection. In the kidney the free drug values were always higher than the entrapped values and significantly higher during the first 24 hours (except 30 min post-injection). Although there was no significant differences between the free and entrapped values in the lungs and brain at any time, the levels of the free drug in the small intestine were significantly higher 5 minutes and 5 hours after injection.

Free drug levels in the kidney and brain appear to reflect the decrease in the plasma concentration of ACT-D (fig 3:1) with time whereas the liposome ACT-D-levels in spleen and lung suggest that localization of the drug is occurring in these tissues although only the levels in the spleen were significantly higher.

ii) Bleomycins.

a) The tissue distribution of ^{111}In labelled BLM (^{111}In-BLM) is shown in fig. 4:2. The results of the 't'-test comparisons are shown in table 4:3. This table also shows additional distributions into the femur (bone), testes and thymus; and into all tissues at 48 hours.

b) The tissue distribution of ^{125}I-BLM (iodine monochloride : ICL-BLM) is shown in fig. 4:3. The results of the 't' test comparisons are shown in table 4:4 (data at 48 hours post-injection also shown).

c) The tissue distribution of ^{125}I-BLM (Bolton and Hunter : B/H-BLM) is shown in fig. 4:4. The results of the 't' test comparisons are shown in table 4:5 (48 hours post-injection also shown). A comparison between the tissue levels of the different entrapped BLMs is shown in table 4:6.

iia) ^{111}In-BLM. (fig. 4:2, table 4:3)

The tissue distribution values of the ^{111}In entrapped drug were consistently and significantly higher in the spleen throughout the time period, whereas the values of the free drug found in the kidneys were always higher than the entrapped drug and significantly higher at most time intervals (except 2 and 24 hours). Preferential location of the liposomal-drug into the small intestine, bone and thymus did not start

Legend Fig. 4:2.

Uptake of ^{111}In-bleomycin into tissues.

Free (closed bars) or entrapped (open bars) ^{111}In-BLM was injected I/V into rats (see table 4:1 for details of doses). The histogram shows the percentage of the injected dose associated with each gram of tissue at timed intervals after injection.

Each bar represents the mean (± st.dev.) from 3 animals.

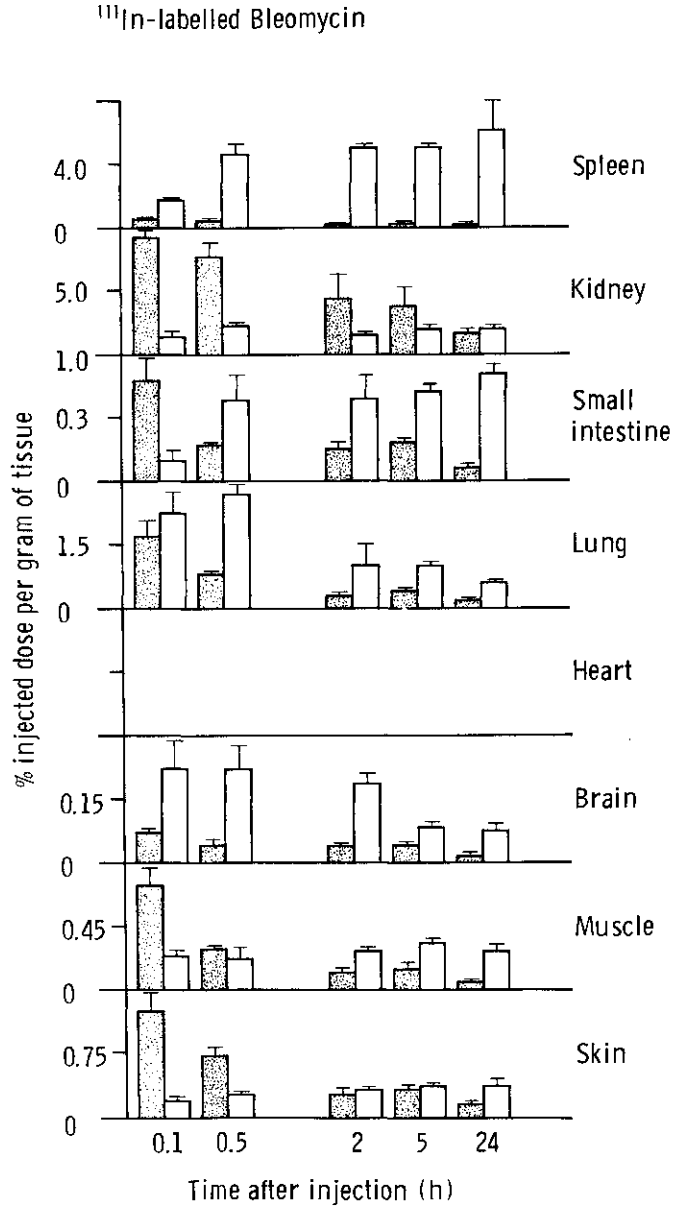


Table 4:3.

Significance values (Students 't'-test)** for the comparison of means between free and entrapped drug concentrations in tissues.

^{111}In-Bleomycin.

TISSUE	Time after Injection					
	5'	30'	120'	5h.	24h.	48h.
Spleen	< 0.001	< 0.001	< 0.001	< 0.001	< 0.01	< 0.001
Kidney	< 0.001*	<0.001*	N/S*	< 0.01*	N/S	< 0.05
Small Gut	N/S	N/S	< 0.02	< 0.001	< 0.01	< 0.001
Lung	N/S	< 0.002	N/S	< 0.001	< 0.001	< 0.001
Brain	< 0.05	< 0.02	< 0.001	< 0.01	< 0.001	< 0.001
Muscle	< 0.01*	N/S*	< 0.001	< 0.01	< 0.001	N/S
Skin	< 0.01	N/S*	N/S	N/S	< 0.01	< 0.001
Bone	N/S*	N/S	< 0.01	< 0.001	< 0.005	< 0.01
Testes	< 0.05*	N/S	< 0.001	< 0.001	< 0.001	< 0.001
Thymus	N/S	N/S	< 0.05	< 0.001	< 0.001	< 0.01

* Free drug concentation (% injected dose/gm wet weight tissue) higher than entrapped drug concentration. Otherwise entrapped drug concentration is higher than free.

** 4 degrees freedom. N/S Not significant ($p > 0.01$).

until after 30 minutes post-injection but thereafter it remained significantly higher than the free drug; a similar picture was seen in the muscle and testes although immediately after injection there were significantly higher free drug concentrations in these tissues. In the lungs there was a higher liposome localization of drug throughout the period, this was significantly greater than the free drug levels at 30 minutes and again after 5 hours. Levels of entrapped drug in the brain were significantly higher than the free drug thoughout the 48 hours period.

A comparison of these results with the plasma clearance curve of ^{111}In-BLM, free and entrapped, suggests that the decline in free drug values in the tissues (e.g.: kidney, small intestine, lung, brain, muscle and skin), especially during the first 2 hours post-injection, was due to removal of free ^{111}In-BLM (or ^{111}In-protein complexes) from the circulation i.e. these levels can be due to blood contamination of these organs. Localization of the drug in the tissues would be expected to give plateau levels in the organs at least for some time after injection and before metabolism and excretion of the radiolabel commenced. The free drug levels in the skin, muscle, brain and perhaps lung tissue during the period 2-24 hours may reflect these genuine uptake levels, the plasma concentration of free ^{111}In-BLM is relatively constant over this period and so these tissue levels may be due to radiolabel in the plasma or tissue fluids.

The liposome radiolabelled uptake by many of the tissues also mirrors the plasma concentration (e.g.: brain, testes, thymus) and is probably due to blood contamination of the tissues. However, since the ^{111}In label leaks from the vesicles, it is difficult to descriminate between genuine tissue localization of liposome-entrapped ^{111}In-BLM and the combination of free (protein bound) ^{111}In and liposomal drug.

In four cases (spleen, small intestine, lung and bone (not shown)) there appears to be increasing tissue uptake of radiolabel associated with the liposome carrier; this is most marked in the spleen and small intestine. In the lungs the initial localization may be due to larger liposomes, which are cleared from the plasma first, and the later concentration due to smaller liposomes alone or in combination with protein-bound leaked radiolabelled.

iib) ^{125}I-BLM (ICL). (fig. 4:3, table 4:4)

The levels of liposome associated ^{125}I-BLM (ICL) in the spleen,

Legend Fig. 4:3.

Uptake of ^{125}I-bleomycin (iodine monochloride) into tissues.

Free (closed bars) or entrapped (open bars) ^{125}I-BLM(ICL) was injected I/V into rats (see table 4:1 for details of doses). The histogram shows the percentage of the injected dose associated with each gram of tissue at timed intervals after injection.

Each bar represents the mean (± st. dev.) from 3 animals.

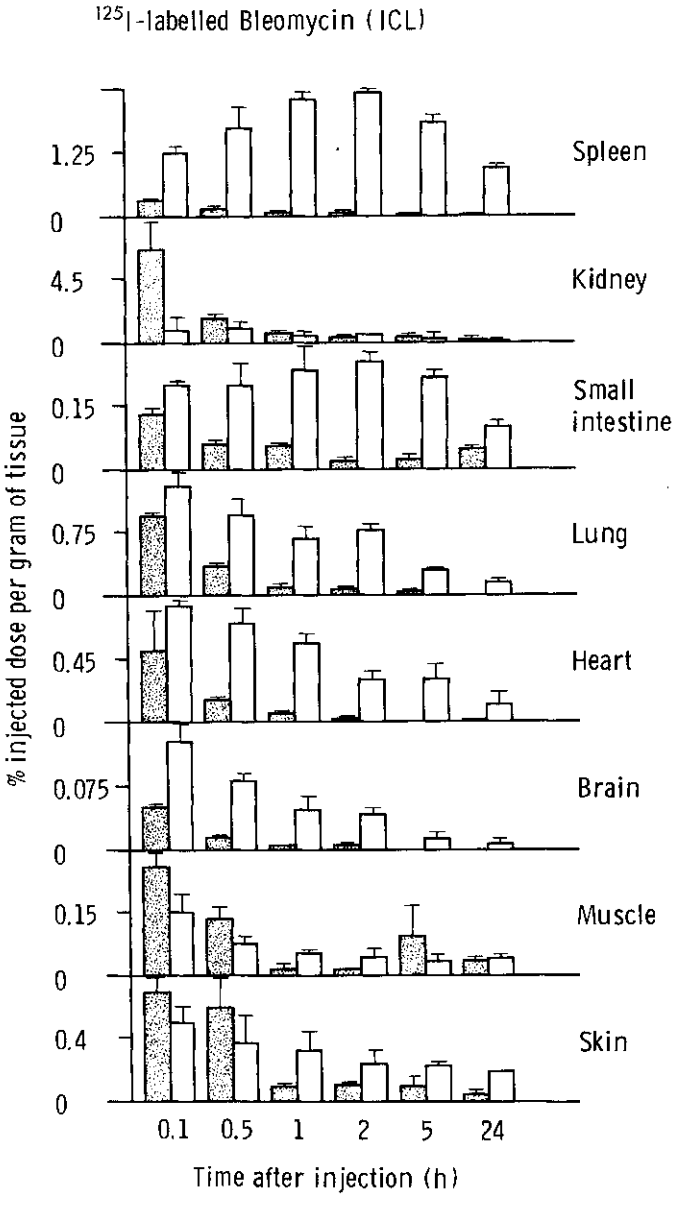


Table 4:4.

Significance values (Students 't'-test)** for the comparison of means between free and entrapped drug concentrations in tissues.

^{125}I-Bleomycin (ICL).

TISSUE	Time after Injection						
	5'	30'	60'	120'	5h.	24h.	48h.
Spleen	<0.001	<0.005	<0.001	<0.001	<0.001	<0.001	<0.01
Kidney	<0.02*	<0.05*	N/S*	<0.02*	N/S*	N/S*	<0.02*
Small Gut	<0.005	<0.05	<0.02	<0.001	<0.001	<0.005	<0.001
Lung	<0.05	<0.01	<0.005	<0.005	<0.001	<0.001	<0.002
Heart	N/S	<0.002	<0.001	<0.001	<0.02	N/S	<0.01
Brain	<0.05	<0.001	<0.01	<0.001	N/S	N/S	<0.01
Muscle	<0.05*	N/S*	<0.005	N/S	N/S	N/S	<0.01
Skin	N/S*	N/S*	<0.05	N/S	N/S	<0.01	N/S

* Free drug concentation (% injected dose/gm wet weight tissue) higher than entrapped drug concentration. Otherwise entrapped drug concentration is higher than free.

** 4 degrees freedom. N/S Not significant ($p > 0.01$).

small intestine and lung were significantly higher than the free drug levels throughout the 48 hour period. As in the previous experiment (^{111}In-BLM) the levels of the free drug were consistently higher than the entrapped drug in the kidneys although these differences were not always significant. In the muscle and skin tissue the drug levels were very similar at all time intervals although some differences were found to be significant, notably at 1 hour post-injection where liposome drug was higher than the free.

Tissue concentration of the liposome entrapped drug in the heart and brain were usually higher than the corresponding free drug levels but, at least in the brain, these levels were not maintained over time.

The plasma clearance of ICL-labelled BLM showed a rapid removal of the free drug compared to the entrapped species. This removal is paralleled in the falling free drug concentrations in all the tissues studied. However, although a similar fall was seen in the tissue levels of the entrapped drug (e.g.: brain, muscle, skin and perhaps heart), the other tissues exhibited levels of radiolabel which suggest that tissue location of liposomes is taking place (i.e.: especially the spleen but also small intestine and lungs).

iic) ^{125}I-BLM (B/H). (fig. 4:4, table 4:5)

Distribution of this radiolabelled BLM was similar to that of ICL-BLM. Some notable exceptions were seen however. In the muscle and skin differences between free and entrapped drug were much more significant so that, unlike the levels in the heart, brain and kidney, the liposomes appear to be preferentially locating in the former tissues. Distribution into the spleen, small intestine and lung (at early time points) also represent localization.

The leakage of ^{125}I and ^{111}In radiolabels from liposomes has already been commented upon (fig. 2:2, section 3:2). In the plasma clearance of free ^{125}I-BLM (ICL) and ^{125}I-BLM (B/H) it was shown that there was no significant difference between these levels during 24 hours. A similar comparison of means was made between the levels of both free drugs in the tissues. No significant difference was found in the free drug levels in the spleen, small intestine, heart, brain, muscle or skin at any time. However, in the kidney there were significantly higher amounts of free ^{125}I-BLM (B/H) ($p < 0.01$-0.001) than free ^{125}I-BLM (ICL) at 30, 60 and 120 minutes after injection. Similarly, in the lung the values of the B/H - BLM were significantly higher at 5 and

Legend Fig. 4:4.

Uptake of ^{125}I-bleomycin (Bolton and Hunter) into tissue.

Free (closed bars) or entrapped (open bars) ^{125}I-BLM(B/H) was injected I/V into rats (see table 4:1 for details of doses). The histogram shows the percentage of the injected dose associated with each gram of tissue at timed intervals after injection.

Each bar represents the mean (± st.dev.) from 3 animals.

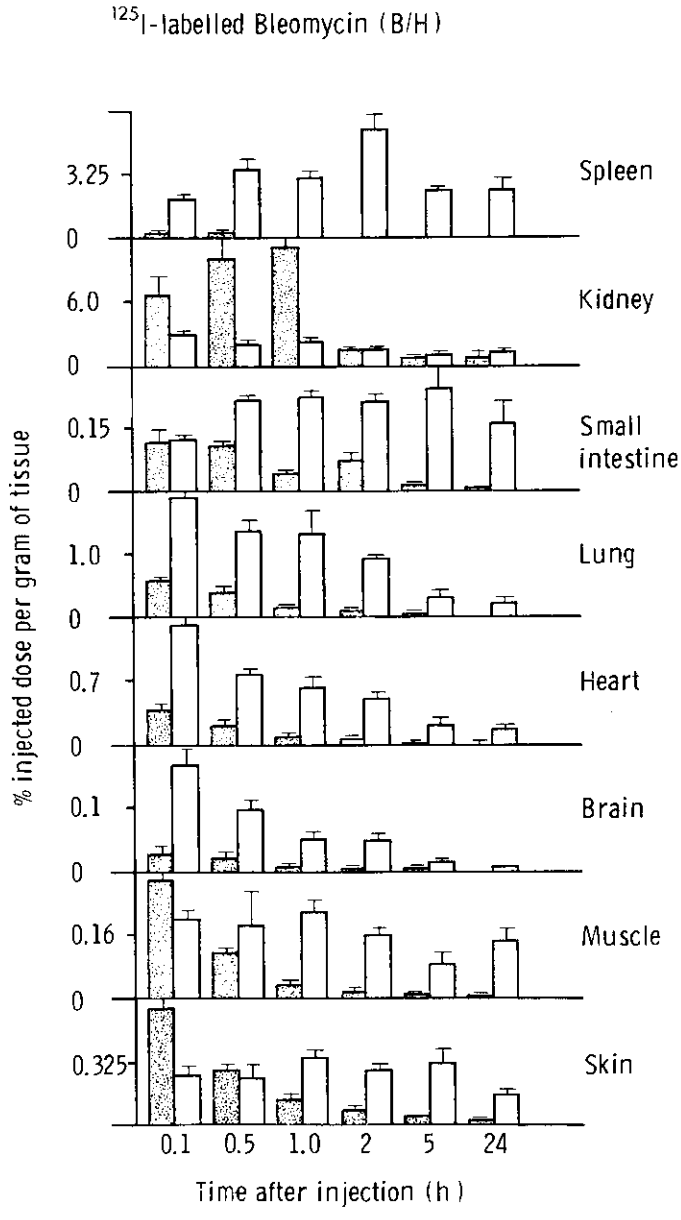


Table 4:5.

Significance values (Students 't'-test)** for the comparison of means between free and entrapped drug concentrations in tissues.

^{125}I-Bleomycin (B/H).

TISSUE	Time after Injection						
	5'	30'	60'	120'	5h.	24h.	48h.
Spleen	<0.002	<0.001	<0.001	<0.001	<0.001	<0.005	<0.001
Kidney	<0.05*	<0.01*	<0.001*	N/S	N/S	N/S	<0.002*
Small Gut	N/S	<0.001	<0.001	<0.001	<0.005	<0.005	<0.001
Lung	<0.001	<0.001	<0.01	<0.001	<0.005	<0.01	<0.005
Heart	<0.001	<0.001	<0.001	<0.001	<0.002	<0.002	<0.001
Brain	<0.002	<0.002	<0.001	<0.001	<0.001	<0.002	<0.05
Muscle	<0.01*	N/S	<0.001	<0.001	<0.002	<0.005	<0.01
Skin	<0.001*	N/S*	<0.005	<0.001	<0.002	<0.001	<0.001

* Free drug concentation (% injected dose/gm wet weight tissue) higher than entrapped drug concentration. Otherwise entrapped drug concentration is higher than free.

** 4 degrees freedom. N/S Not significant ($p > 0.01$).

30 minutes post-injection ($p < 0.002$ and 0.05, respectively). In a comparison of means between the tissue distribution of free ^{111}In-BLM and ^{125}I-BLM (B/H), it was found that the ^{111}In label tissue levels were significantly higher that the ^{125}I label in all comparable tissues at most times after injection (overall $p < 0.01$). Exceptions to this were found during the first 30 minutes post-injection, where the kidney and brain the differences were not significant.

In comparison of the liposome entrapped BLMs, the B/H labelled BLM was compared with the ICL labelled and the ^{111}In-BLM material. These results are shown in table 4:6. The B/H material was chosen as the standard since in general its tissue values fall between the ICL material (lowest) and the ^{111}In material (highest). The table demonstrates that the distribution of the three radiolabels were not the same in the tissues studied. In the small intestine, heart, brain, muscle and skin the two ^{125}I-labelled species have a similar distribution over time although some statistical differences were found. The distribution of the indium labelled material in these tissues was similar to the ^{125}I-labels but it gave consistently higher amounts of radiolabel. In the kidney the levels of B/H-^{125}I and ^{111}In are similar during the first 5 hours post-injection and both of these are very much higher that the entrapped ICL-^{125}I levels. This fact, taken in conjunction with the levels of the free drug in this tissue, tends to suggest that the B/H material might also leak or loose its radiolabel as has been shown for the ^{111}In label. The levels of the entrapped compounds in the spleen, which is known to concentrate liposomes, were lower in the case of ^{125}I-BLM (ICL) than the other two labels and, initially, these latter two labels were not very different from each other. Of course, these differences need not necessarily reflect leaked material but may also reflect differences in the size of the liposomes in the population injected. In this respect the lungs, which, like the spleen, take up more of the larger liposomes (see discussion) showed some similarities between the two ^{125}I-radiolabelled whilst the ^{111}In labelled liposomes were somewhat different (^{111}In higher than ^{125}I).

In summary, the injection of liposomes containing radiolabelled bleomycin into rats shows that higher amounts of radiolabel can be found in the spleen, small intestine and lung than for the free drug; these levels probably reflect drug uptake rather than blood contamination. Conversely, the free drug levels in the kidneys and perhaps

Table 4:6. Significance values (Students 't'-test)** for the comparison of means between liposome entrapped ^{125}I-BLM (B/H) and either ^{125}I-BLM (ICL) or ^{111}In-BLM drug concentrations in tissues.

TISSUE	B/H-BLM versus :	Time after Injection						
		5'	30'	60'	120'	5h.	24h.	48h.
Spleen	ICL	< 0.05*	< 0.02*	N/S*	< 0.001*	< 0.005*	< 0.01*	N/S
	In	N/S*	N/S	-	N/S*	< 0.001	< 0.05	< 0.001
Kidney	ICL	< 0.05*	<0.02*	< 0.001*	< 0.001*	N/S*	< 0.001*	< 0.001*
	In	< 0.005*	N/S	-	N/S	N/S	< 0.02	< 0.001
Small Intestine	ICL	< 0.005	N/S	N/S	N/S	N/S*	N/S*	N/S*
	In	N/S	N/S	-	< 0.05	< 0.02	< 0.001	< 0.001
Lung	ICL	< 0.005*	N/S*	N/S*	< 0.001*	N/S*	N/S*	< 0.05*
	In	N/S	< 0.001	-	N/S	< 0.001	< 0.002	< 0.001

continued on following page.

Table 4:6.
(continued)

TISSUE	B/H-BLM versus :	Time after Injection						
		5'	30'	60'	120'	5h.	24h.	48h.
Heart	ICL	< 0.002*	N/S*	N/S*	< 0.05*	N/S	N/S	N/S
Brain	ICL	N/S*	N/S*	N/S*	N/S*	N/S*	N/S*	N/S*
	In	N/S	< 0.02	-	< 0.001	< 0.001	< 0.001	< 0.001
Muscle	ICL	N/S*	N/S*	< 0.02*	< 0.001*	< 0.05*	N/S*	N/S*
	In	N/S	N/S	-	< 0.001	< 0.002	< 0.05	N/S
Skin	ICL	< 0.05	N/S	N/S	N/S*	N/S*	N/S*	N/S*
	In	N/S*	N/S	-	< 0.05	N/S	N/S	N/S

* ^{125}I-BLM (B/H) tissue concentration higher than comparative drug concentration. Otherwise it is lower

** 4 degrees freedom.

N/S Not significant ($p > 0.01$).

muscle and skin are higher than the liposome entrapped drug levels. Other tissues, e.g.: heart and brain, have drug levels which reflect the plasma concentration either the free or the entrapped drug.

iii) Asparaginase. (fig. 4:5, table 4:7).

The values in fig. 4:5 are for the total radioactivity of ASPase (TCA precitatable material not tested). The results of the 't' test comparisons are shown in table 4:7 (48 hours time points also included) The tissue distribution of free and entrapped ASPase showed marked differences from the ACT-D and BLM results. The major difference was the fact that the free drug levels were always higher than the entrapped levels in all tissued throughout a 48 hour period. At periods more than five hours after injection, the majority of tissues (except spleen and kidney) did not show any significant differences between the two preparations.

For the free drug the lung, heart, brain, skin and kidney levels of the radiolabel probably reflect the plasma concentrations of the drug in that they fall with time. However, the spleen, small intestine and muscle tissue levels may represent localization of the drug in these tissues. It might be expected that the very rapid initial plasma clearance of aggregated ^{125}I-ASPase (which might be reflected in high levels in the reticuloendothetial system) would also give high initial levels in the spleen. However, the spleen levels do not reflect this but continue to rise up to 2 hours post-injection : this uptake is reflected, to a certain extent, in the lungs at 5 minutes. In general, the higher free drug levels of ASPase are probably due to the longer plasma half-life of this protein in comparison to the liposomes.

In the case of the carrier, the liposomal ASPase was found to be markedly different from the BLM results discussed previously. In the spleen, kidney, lung, heart and brain, the liposome levels appear to fall over time and probably reflect the plasma concentration of the entrapped drug. Some localization of the entrapped drug (or radiolabel) in the small intestine, muscle and perhaps skin may have occured.

The reasons for the differences between drugs are not at all clear, the proportion of the injected liposomal ASPase associated with the spleen, kidney, lung and brain are much lower than those found for ACT-D, ^{125}I-BLM (B/H) and ^{111}In-BLM but not significantly different (overall $p < 0.05$) from those of ^{125}I-BLM (ICL). It is possible that

Legend Fig. 4:5.

Uptake of ^{125}I-Asparaginase into tissues.

Free (closed bars) or entrapped (open bars) ^{125}I-ASPase was injected I/V into rats (see table 4:1 for details of doses). The histogram shows the percentage of the injected dose associated with each gram of tissue at timed intervals after injection.

Each bar represents the mean (± st.dev.) from 3 animals.

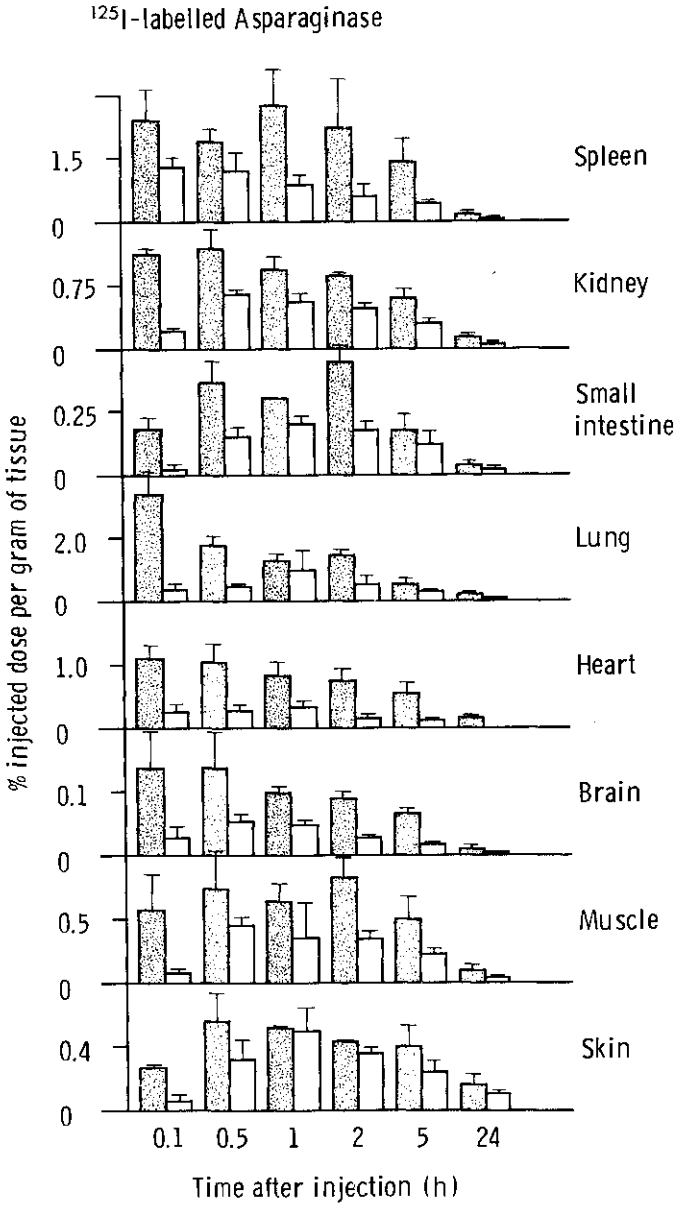


Table 4:7.

Significance values (Student's 't'-test)** for comparisons of means between free and entrapped drug concentrations in tissues.

^{125}I-Asparaginase.

TISSUE*	Time after Injection						
	5'	30'	60'	2h.	5h.	24h.	48h.
Spleen	N/S	N/S	N/S	<0.01	<0.05	<0.02	N/S
Kidney	<0.001	<0.05	<0.05	<0.001	<0.02	<0.02	<0.001
Small Gut	N/S	<0.02	<0.01	<0.02	N/S	N/S	N/S
Lung	<0.005	<0.001	N/S	<0.01	N/S	N/S	N/S
Heart	<0.005	<0.02	<0.05	<0.02	<0.01	N/S	N/S
Brain	<0.05	<0.002	N/S	N/S	N/S	N/S	N/S
Muscle	<0.05	<0.02	N/S	<0.01	N/S	N/S	N/S
Skin	<0.001	N/S	N/S	N/S	N/S	N/S	N/S

* Free drug levels higher than entrapped drug levels at all times and in all tissues.

** 4 degrees freedom. N/S Not significant ($p > 0.1$).

the liposome-ASPase preparation contained higher proportions of SUVs than the other preparations, this would account for the lower spleen and lung levels, but is similarity to the BLM-ICL preparation (which was cleared fastest from the plasma) suggest that this may not be the entire explanation. The possibility of ASPase (or radiolabel) leakage from the liposomes cannot be ruled out although substantial leakage (eg: destruction of the liposomes in the plasma) could be expected to produce very different tissue distributions into most tissues (especially kidney and lung) at early times post-injection. A possible explanation for the lack of uptake of entrapped ASPase (by the spleen especially) is that the presence of entrapped protein in (or on) the liposome surface may be preventing the interactions of the vesicles with other plasma components or causing the carrier to be "directed" to other sites (e.g.: other body fluids).

SECTION 4:2

Liver uptake of free and liposome entrapped drugs.

It has been known for sometime (Gregoriadis and Ryman, 1972a, 1972b) that the liver takes up the greatest quantities of intravenously injected liposomes. In addition to the tissue distribution studies shown in the previous section, the uptake of liposome-entrapped drugs into the liver was also studied. These results are shown in figures 4:6 (ACT-D), 4:7 (BLMs) and 4:8 (ASPase). In addition, the uptake/gm of liver tissue for these drugs is shown in table 4:8.

i) ACTINOMYCIN-D.

The percentage of the injected dose found localized in the liver at timed intervals following I/V injection of either free or entrapped ACT-D is shown in fig. 4:6. A comparison of means revealed that the means were significantly different (table 4:8) at all times up to 5 hours ($p < 0.02$) with the entrapped drug always being the higher. However, at 24 and 72 hours (not shown fig. 4:6) there was no significant difference between the amount of free and entrapped ACT-D found in the liver. The plasma clearance curves of ACT-D suggests that the rapid initial plasma disappearance of both preparations was reflected in the liver values as early as 5 minutes post-injection. This was much more obvious in the case of the liposomal drug. The percentage uptake of ACT-D per gram of liver tissue (table 4:8) demonstrates that this organ takes up the greatest quantity of entrapped drug. The fall in the entrapped ACT-D liver concentration, which continues for up to 72 hours ie: long after the plasma concentration of both free and the entrapped drug has diminished to almost zero, is probably due to excretion of the drug.

ii) Bleomycins.

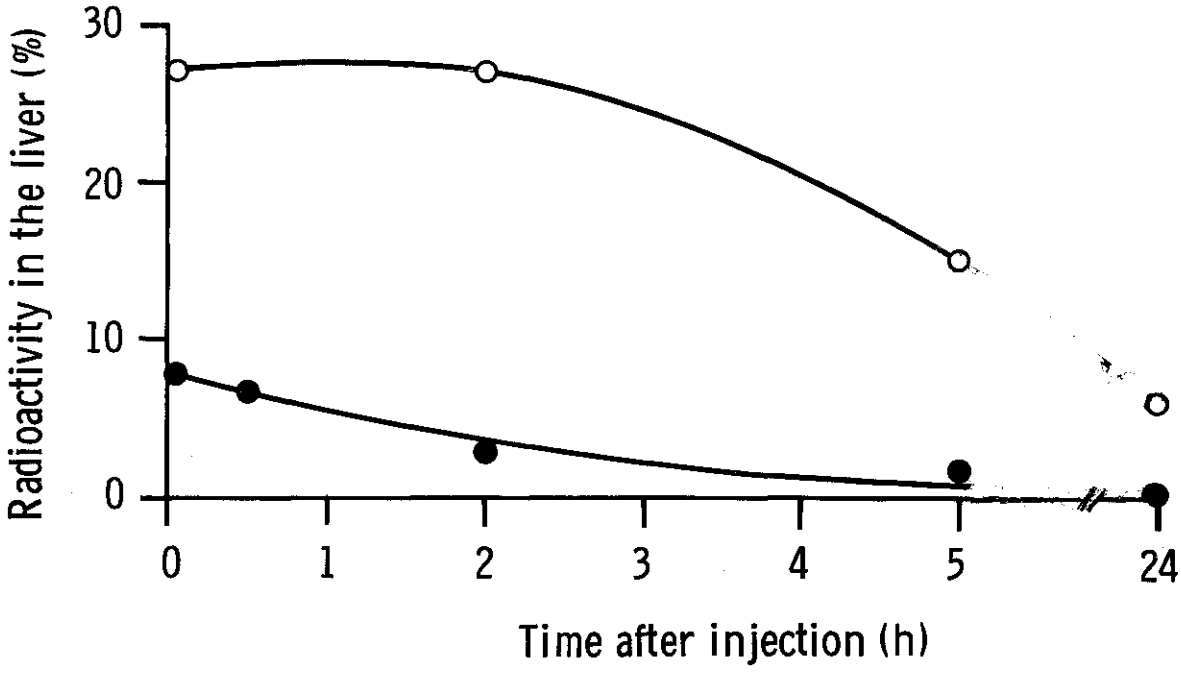
The hepatic capture of radiolabelled BLM's is shown in fig. 4:7. The free drug levels remain at low levels throughout the 24 hours period (i.e.: between 0.2 and 3.3 % of the injected dose). A comparison of means between the free drugs showed that for all time points up to 24 hours the ^{125}I-BLM(B/H) was significantly higher ($p<0.001$) than the ICL-BLM. Differences between the higher B/H-BLM values and ^{111}In-BLM were significant ($p < 0.001$) except 2 and 5 hours post-injection.

Legend Fig. 4:6.

Liver uptake of ^{3}H-actinomycin-D.

Free (closed circles) or entrapped (open circles) ACT-D was injected I/V into rats (see table 4:1 for details of doses). The figure shows the percentage of the injected dose associated with the whole organ at timed intervals after injection.

Each point represents the mean (± st.dev.) from 2 animals.



The hepatic uptake of the liposome entrapped drugs was significantly different ($p < 0.001$) from their appropriate free drug levels at all times. The uptake of ^{111}In-BLM was significantly higher ($p < 0.001$) than that of the ^{125}I-labelled BLMs at all times after 5 minutes. A comparison of the means between the two radioiodinated species was made and this showed that these entrapped drugs did not have significantly different liver uptakes except at 1 and 2 hours post-injection when the ICL-BLM was significantly higher ($p < 0.01$, $< p$ 0.001 respectively). The data on the comparisons between the free and entrapped species (mean % injected dose/gm liver) is shown in table 4:8.

In general, all the entrapped BLMs demonstrated a rapid initial uptake to give high levels in the liver. These levels remained elevated for up to 48 hours post-injection. Peak values did not occur at the same time for all the preparations and were probably determined by the proportion of SUVs in the injected preparation and the amount of drug leakage from the liposomes. The peak levels were obtained at 2 hours for ICL-BLM and 5 hours for ^{111}In-BLM and B/H-BLM. The fall from the peak levels was attributable to drug metabolism (i.e.: loss of radiolabel from the drug but not necessarily total breakdown of the BLM nucleus) and excretion.

The higher level of liposomal ^{111}In-BLM in the liver may be due to the fact that leaked radiolabel escaping from liposomes in the circulation will bind to plasma proteins; these newly-labelled proteins may also be removed into the liver (see section 4:4), whereas leakage of ^{125}I-radiolabelled drugs from liposomes probably leads to urinary excretion.

iii) Asparaginase.

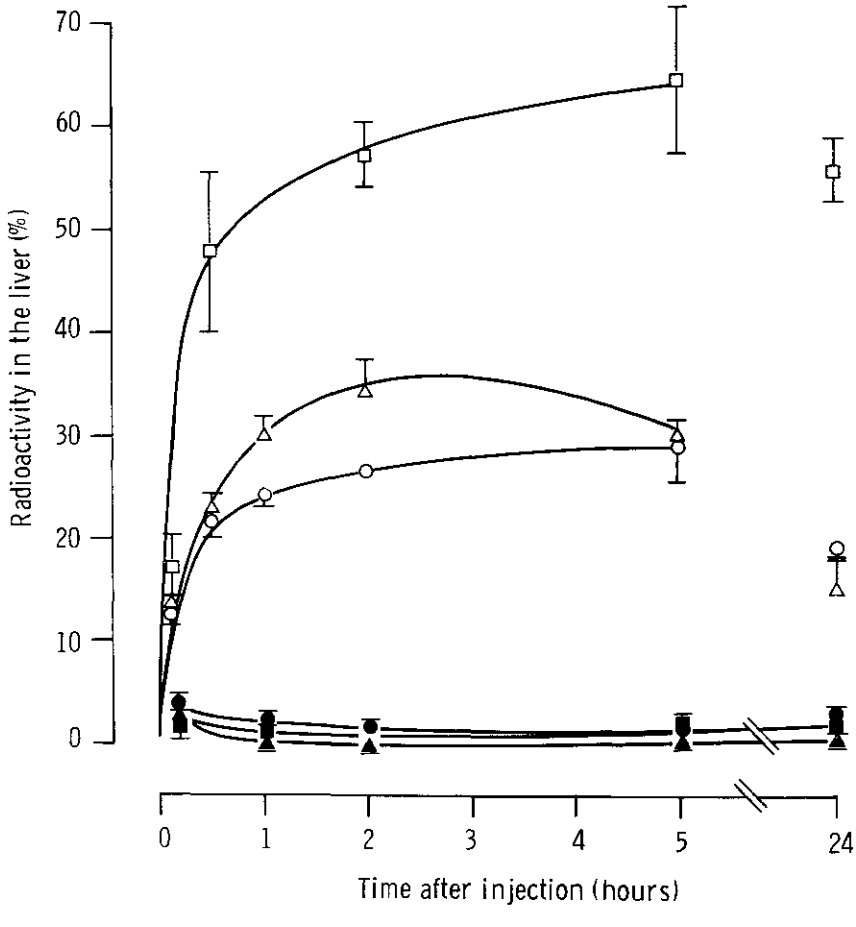
As may be seen in fig. 4:8, the capture of ^{125}I-ASPase entrapped in liposomes was very similar to the uptake of the free drug and different from the liver uptake seen using other liposome-entrapped agents. In the figure the circles represent the material precipitatable by trichloroacetic acid (TCA) whilst the squares represent the total ^{125}I counts. The liver concentration of both the free drug and the entrapped drug paralleled the plasma clearance curves with the highest amounts of drug being found at 5 minutes post-injection (c.f. BLMs : fig. 4:7). This uptake suggests that either the liver levels were due to blood contamination or that if uptake had occured the protein and/or its radiolabel were rapidly excreted. A comparison of means between

Legend Fig. 4:7.

Uptake of Bleomycins by the liver.

Details of doses are given in table 4:1. Animals were injected I/V with i) free (closed squares) or entrapped (open squares) ^{111}In-BLM. ii) free (closed triangles) or entrapped (open triangles) ^{125}I-BLM-ICL. iii) free (closed circles) or entrapped (open circles) ^{125}I-BLM-B/H.

The figure shows the percentage of the injected dose associated with the liver at timed intervals after injection. Each point represents the mean (± S.E.) from 3 animals.



free and entrapped drug levels in the normal material (not shown) and in the TCA precipitatable (TCA pptable) material (table 4:8) demonstrated that the liposomal hepatic levels were significantly higher than the free drug levels during the first hour after injection but not thereafter. In addition, a comparison of means between the ^{125}I counts per gm liver and the TCA ^{125}I counts showed that these were not significantly different at any time for the livers from the liposome treated animals. However, in the free drug treated animals, the total ^{125}I counts were significantly higher ($p < 0.05 - 0.002$) than the TCA ppt counts at all time intervals except 24 and 48 hours post-injection. The free ^{125}I-ASPase, which is rapidly cleared from the circulation, appears to loose its radiolabel in the liver but this may also be due to uptake of free radiolabel from the plasma at 1 to 5 hours post-injection although free ^{125}I would normally be expected to be excreted in the urine or concentrated in the thyroid gland (Welt and Blythe, 1965). The liposome preparation, although also rapidly cleared, protects the entrapped ASPase from loss of radiolabel but its localization in the liver is not as great as other liposome drugs. Indeed because they contain a protein these liposomes may not localize in the liver to any great extent. Taken with the data presented on the tissue distribution of ASPase (fig. 4:5), these results suggest that liposomal ASPase is localizing at other sites (eg: small intestine, muscle and perhaps skin) or in other fluids. Since its distribution to other organs is not different from the free drug, it seems possible that the presence of the entrapped protein could be determining the distribution of the liposomes. An alternative explanation may be that these vesicles are less stable in the plasma than other drug-containing liposomes. In the plasma, liposomes did not loose radiolabelled ASPase until about 2 hours post-injection and after 24 hours this loss no longer occured. In the liver, the uptake of liposomes was only significantly different from the free drug up to 1 hour after injection although the liposomes which are removed from the plasma protected the entrapped protein from loss of label. In view of the clearance kinetics of different sized liposomes, these data imply that it is the SUV population which is unstable whilst the MLV population stays relatively more intact.

Legend Fig. 4:8

Uptake of Asparaginase by the liver.

Details of dose are given in table 4:1. Animals were injected I/V with free (closed symbols) or entrapped (open symbols) ^{125}I-ASPase. Each point represents the mean (± S.E.) from 3 animals.

Circles : TCA precipitated radioactivity.
Squares : Total radioactivity.

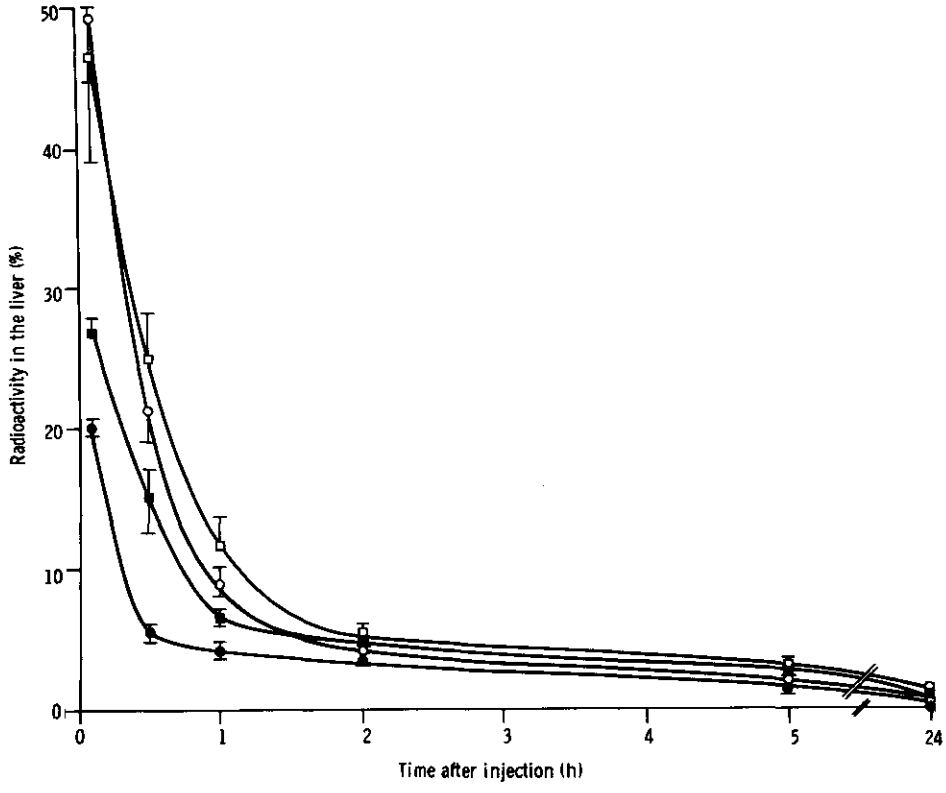


Table 4:8. Hepatic uptake of free and entrapped agents following I/V injection.

Preparation		Mean % injected dose/gm liver (significance between entrapped and free drug)						
F = Free drug E = Entrapped drug		Time after Injection						
		5'	30'	60'	120'	5h.	24h.	48h.
ACT-D	F	2.091	1.85		1.402	0.489	0.128	0.041
	E	6.158	4.32		4.606	3.987	1.491	0.395
	p =	(<0.02)	(<0.005)		(<0.002)	(<0.005	(N/S)	(N/S)
^{111}In-BLM	F	0.295	0.145		0.156	0.191	0.244	0.260
	E	2.43	7.32		8.178	9.238	8.192	9.548
	p =	(<0.001)	(<0.001)		(<0.001)	(<0.001)	(<0.001)	(<0.001)
^{125}I-BLM (ICL)	F	0.265	0.121	0.052	0.0057	0.050	0.047	0.042
	E	2.458	4.037	5.388	6.199	5.56	3.391	1.944
	p =	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
^{125}I-BLM (B/H)	F	0.554	0.388	0.326	0.236	0.187	0.087	0.052
	E	1.912	3.171	3.536	3.725	4.519	2.745	1.601
	p =	(<0.002)	(<0.002)	(<0.001)	(<0.001)	(<0.001)	(<0.001)	(<0.001)
^{125}I-ASPase	F	3.288	0.946	0.724	0.645	0.297	0.073	0.063
	E	9.456	3.372	1.510	0.685	0.371	0.130	0.091
(TCA.ppts)	p =	(<0.002)	(<0.005)	(<0.02)	(N/S)	(N/S)	(N/S)	(N/S)

Section 4:3.

Discussion of tissue distribution.

A) Free drugs.

i) Actinomycin-D.

The tissue distribution of radiolabelled ACT-D has been extensively studied by Schwartz and co-workers (Schwartz et al, 1968a; 1968b) and others (Ro and Busch, 1965; Galbraith and Mellet, 1975; Kaye et al, 1981). From these studies and those of others (Weissbach et al., 1966) there is no evidence that the drug can be metabolised or altered in vivo.

Following the rapid plasma clearance of an I/V injected dose into rats, a significant proportion of the drug (15-20 % of the injected dose in 24 hours) is excreted in the urine, further large quantities (up to 30 % of the injected dose in 4 hours) are secreted into the bile (Schwartz et al., 1968a). This biliary ACT-D appears to originate in the liver because the transport of the drug from the small intestine via the entro-hepatic circulation is limited. This means that the liver has an efficient excretory mechanism and indeed may compartment ACT-D intracellularly to reduce damage to vital functions and enhance biliary excretion (Schwartz et al, 1968a). Moreover, this also means that the liver is not a good site as a depot for re-release of ACT-D after uptake: pace Kaye et al, (1981).

The rapid plasma clearance of ACT-D also ensures that one hour after injection all the tissues have drug concentrations exceeding the blood levels (Schwartz et al, 1968a, 1968b) and this is confirmed in the tissues examined in this work and by Kaye et al. (1981). The principal organs involved in the uptake of free ACT-D in this present work were found to be the spleen (highest uptake per gm tissue) > kidney (up to 2 hours post-injection) > lung > liver and small intestine (these latter two tissues were equal initially but the intestine retained the drug longer). A similar order of distribution was found by Kaye et al. (1981) and Schwartz et al. (1968a, 1968b). Using ^{14}C-ACT-D Ro and Busch (1965) found higher levels in the kidney and liver than in the spleen at 15 minutes after injection but this was not confirmed in these studies since the splenic uptake was consistently the highest even 5 minutes after injection. The amount of ACT-D found by Kaye et al. (1981) to be sequestered in the spleen, liver, lungs kidney and small

intestine was not reproduced in this study, these authors found more than twice as much drug per gram of tissue in nearly all the tissues they studied at both 3 hours and 24 hours after injection. These differences are probably due to the fact that Kaye et al. (1981) and Schwartz et al. (1968b) used mice for their studies. Mice are much less susceptible than rats to the toxic effects of ACT-D (LD_{50} : mice 1.2 mg/kg - rats 0.6 mg/kg) (Schwartz et al., 1968a) and the same authors have found differences in ACT-D localization between certain rat and mouse tissues. Moreover, using rats, Ro and Busch (1965) found quite similar levels to those of this study 15 minutes after injection.

The toxicity of ACT-D has been equated with the drug concentration and the time during which susceptible tissues remain in contact with toxic levels of drug (Schwartz et al., 1968b). Tissues which normally undergo cell proliferation as part of their function can be targets for both chemotherapy and toxicity. In the case of spleen, thymus and bone marrow, a reduction in organ size has been reported following a single I/V injection of 0.4 mg/kg ACT-D into rats; this is further reflected in a progressive depletion of blood cells during the following 6 days (Schwartz et al., 1968a; Rahman et al., 1978). The small intestine concentrates free drug to reach a plateau level after 30 minutes (Schwartz et al., 1968; Rahman et al., 1975 and this work) and this level is maintained for some time (3-4 hours), such a period is long enough to effect the proliferative cells of mucosa.

In a study of other tissues Ro and Busch (1965) showed that both testis and brain took up lower amounts of ACT-D at 15 minutes than those found in the brain in this study, the reasons for this difference are not clear but in mice Rahman et al. (1975) found similar amounts of ACT-D in the brain as was found in this study.

In general, therefore, the distribution of free ACT-D in rat tissue presented here is not substantially different from the data of others. Most authors agree that the highest uptake is to be found in the spleen, liver, small intestine and lung tissue and that is confirmed in these studies.

ii) Bleomycins.

The use of BLM as a tumour imaging agent (Rasker et al., 1975) has meant that tissue distribution studies have been performed in both rats and mice as well as humans. Only those studies which have administered the drug by intravenous injection will be considered here.

In mice (Konikowski et al., 1977) more than 75 % of a ^{111}In-BLM injection was excreted in the urine in 2 hours whilst in man (Rasker et al., 1975) urinary output of cobalt-labelled BLM accounted for almost 90 % of the injected dose in a 72 hour period with 54 ± 20 % of unmetabolised drug excreted in the first 6 hours. The excretion of ^{111}In-BLM in mice is likely to reflect labelled drug rather than released ^{111}In radiolabel because not only is the plasma clearance of $^{111}In^{3+}$ slower than that of the labelled drug (Robbins et al., 1974; Konikowski et al., 1977) but similar excretion rates have been measured for the unlabelled drug by bioassay procedures (Ohnuma et al., 1974).

Several publications have reported that BLM can be inactivated by enzymes in tissues (Müller et al., 1972; Umezawa, 1974; Ohnuma et al., 1974; Onishi et al., 1975). The enzyme responsible can be found in the cytoplasm of almost all rat tissues; high activity occurs in the small intestine, kidneys, liver, spleen (highest) and brain whilst skin, muscle and uterine tissue have low or absent levels, some inactivation occurs when the drug is in contact with plasma, erythocytes, lung and pancreatic tissues. The activity of the enzyme appears to be restricted to the terminal amide group of BLM and so it is similar to an aminopeptidase; (Umezawa, 1974).

Following I/V injection, the tissues which took up the maximum amount of ^{3}H-radiolabelled BLM in rats were kidneys > liver > lung > intestine > spleen and heart >> muscle and skin (Ryo et al., 1975). Over a 72 hour period the order of uptake remained the same but the levels fall in all tissues except the kidneys. Much higher overall radioactivity was found by Hayakawa et al., (1974) in mouse tissues following the administration of ^{14}C-BLM but bioassays for tissue located drug revealed much reduced levels of actual drug in most tissues especially in the kidneys and liver (^{14}C label on amino groups). In general, the distribution of ^{125}I-labelled BLMs found in this study was similar to that of ^{3}H-BLM in rats (Ryo et al, 1975). Some important exceptions were seen, B/H-BLM had similar kidney concentrations to the ^{3}H-BLM but ICL-BLM was lower. Much higher levels of both iodinated drugs were found in the skin and muscle tissue at all times post-injection than the ^{3}H-BLM of Ryo et al., (1975). Since these tissues do not contain high levels of inactivating enzyme it is possible that iodination has changed the tissue distribution of BLM, or that the ^{3}H-label is unstable, although some dissociation of the radiolabel from BLM in the plasma is also possible (see section 3:1).

Krohn et al., (1977), using ICL-labelled BLM, have demonstrated that its tissue distribution in mice is similar to that found for both the iodinated BLMs used in this study especially in the skin and muscle tissue but the kidney distribution of their ICL-BLM was higher than the ICL-BLM in this study and similar to the B/H-BLM. The reasons for this difference are unclear.

The distribution of free ^{111}In-BLM has been studied by several authors in mice but not in rats. There is general agreement that high levels of radiolabel can be found in the kidneys at all times post-injection (Robbins et al., 1974; Konikowski et al., 1975; Krohn et al., 1977) but these are much lower than the levels of $^{111}InCl_3$ (Robbins et al., 1974). Uptake of ^{111}In-BLM by the skin was significantly higher in the work of Robbins et al.(1974) than that of the other authors, and than that of the present study. However, since Ryo et al., (1975) demonstrated that in the rat skin concentrations of both ^{3}H-BLM and ^{64}CU-BLM were the same, it seems possible that the higher ^{111}In-BLM levels found in mice are due to species differences . The results of the skin location of iodinated and ^{111}In-labelled BLMs used in this study appear to fall between the low levels of Ryo et al., (1975) and the almost 50 fold higher levels in mice.

Similarly, higher levels of ^{111}In-BLM were found in mouse liver, spleen and small intestine than were found here where values for these organs are not very different from the rat values of Ryo et al., (1975) who used two differently radiolabelled BLMs. Data presented here on the lung disposition agrees with that of Ryo et al., (1975) in rats but it is not similar to the studies of Krohn et al., (1977) and Robbins et al., (1974) who found up to five fold higher levels in their mice; these last authors also reported very much higher lung levels in mice injected with $^{111}InCl_3$. Levels of ^{111}In-BLM found in the rat brain agree with the data of Krohn et al., (1977) in mice.

iii) Asparaginase.

Despite its clinical use as an antineoplastic agent, the tissue distribution of I/V injected APSase has not been fully studied. Broome (1968) reported that, following I/P injection of ASPase, mice retained very small amounts of the drug in the blood and spleen whereas the liver had five times more enzyme activity than untreated controls. Putter (1970) concluded that ASPase could be found in the extravascular spaces and in tissues following I/V injection into dogs. Furthermore,

following I/P injection into mice this author reported maximum tissue uptakes 90 minutes post-injection. At this time the blood contained about 17.5 % of the injected dose whilst the uptake by other tissues was : - spleen (6.6 % dose/gm) > kidney (4.5 %) > liver (4.2 %) > heart muscle (2.2 %), skeletal muscle and brain tissue contained 0.8 and 0.2 % respectively. These levels are similar to those found in this study for the liver, skeletal muscle and brain but not for the other tissues examined. These results cannot, however, be directly compared since the species and the route of administration were different from those of this study.

A further consequence of the different plasma half-lives of ASPase from different bacterial species (see chapter 3) was pointed out by Hall (1970) who reported that, following I/V injections of ASPase into sheep, the E.coli enzyme was found in high amounts in the lymph 30 minutes post-injection whereas only trace amounts of the Erwinia carotovora enzyme were found in this fluid. Since the lymphatic drug concentration may determine the levels of the drug transported to other tissues, it is clear that the origin of the ASPase will also effect the tissue distribution.

In clinical studies, Schwartz (1970) failed to find a correlation between plasma ASPase levels and therapeutic efficacy implying that the lowering of plasma asparagine levels, which is known to occur following I/V administration of the enzyme (Rudman et al., 1971), may not be the only mechanism of its action. Further, this author reported very low levels of enzyme activity in cerebrospinal fluid and urine of patients who had high plasma levels. Moreover, an examination of enzyme levels in blood cells and in tissues at post-mortem failed to find significant increases in ASPase content despite high plasma levels.

These data raise a further problem of interpretation of ASPase results. Numerous studies (reviewed; Patterson, 1975) have reported that the enzyme will inhibit protein and nucleic acid synthesis and delay mitosis both in isolated cells and in vivo, however, since ASPase will hydrolyse asparagine wherever it is found it is extremely difficult to separate the effects of low asparagine levels in tissue fluids from the presence of intracellular ASPase. Thus, studies which show, for example, supression of the functions of both B (bone marrow derived) and T (thymus derived) lymphocytes and transient necrosis of the thymus, lymph nodes and spleen germinal centers following ASPase treatment (Patterson, 1975), do not necessarily mean that the administered

enzyme concentrates in these tissues.

Indirect evidence that the reticuloendothelial system (RES) is not totally responsible for the removal of free ASPase from the circulation comes from the work of Ho et al., (1971) who could not find any change in the plasma clearance of the enzyme when dogs and guinea pigs were pretreated with the RES partial blocking agent Zymosan; but others (Wriston and Yellin, 1973) reported a blockade of ASPase capture by lymphoid tissue in mice pretreated with colloidal carbon or steriod drugs. In the present study, mention has already been made (chapter 3) of the tendency of ASPase to aggregate (Hall, 1970), the extent to which this aggregation will alter the kinetics and distribution of the free drug has not been assessed but it is likely that higher levels than normal might be found in RES.

In summary, the data presented here on the tissue distribution of free ASPase suggests that concentration in the liver, spleen and kidneys occurs; lower levels in the heart, skeletal muscle and brain were found. The data is in general agreement with the published data although additional localization of the drug in the small intestine, lungs and skin of rats was also found here.

Factors which control the general uptake of proteins by tissues are still the subject of investigation. Morell et al., (1971) have presented evidence of a general mechanism for the hepatic uptake of glycoproteins and this has recently been further expanded by many workers (Sly, 1980; Stahl and Schlesinger, 1980). The tissue uptake of many proteins now appears to be mediated by carbohydrate moieties on the proteins which interact with receptors on the surfaces of many cells (particularly hepatocytes). However, the E.coli and the E.carotovora enzymes probably do not contain carbohydrate (Patterson, 1975) unlike the F.tricinctum enzyme, which its cleared very rapidly from the plasma and is inactive against tumours.

B) Tissue distribution of liposome entrapped drugs.

The data of this study will be compared with published material related to the same entrapped species (where this exists) and this will be followed by a general discussion on the distribution of liposome entrapped agents.

i) Actinomycin D.

While this work was being carried out Rahman et al., (1975) published the results of a study on the tissue distribution of neutral unsonicated liposomes containing ACT-D in the AP or LP. These studies showed that ACT-D (AP) liposomes leaked drug faster than if the drug was entrapped in the lipid phase. Consequently, the distribution of ACT-D (AP) was not very different from the free drug in many tissues and this preparation will not be further considered here. In agreement with the present studies, using ACT-D (LP) in unsonicated MLVs Rahman et al., (1975) also demonstrated high entrapped drug levels in the liver and spleen throughout a 48 hours period whilst the uptake by the small intestine and kidneys was lower than the free drug. Unlike these data, however, these authors reported high liposomal ACT-D levels in the lungs but this may be due to the larger size of their liposome preparation (Hunt et al., 1979). This size effect may also influence the uptake by the brain since Rahman et al. (1975) found more than twice the drug concentration in this tissue than was found in this present study but drug leakage from liposomes is a more likely explanation.

In the liver Rahman et al., (1975) found very similar levels of liposomal ACT-D as is shown here except that the initial drug concentration reached a value of close to 45% of the injected dose, moreover after 24 hours the liver concentration had fallen to only 12 % (cf. fig. 4:6 ; 5 %) this difference may be due to the larger size of the population or to differences between mice and rats. These authors also reported that the bone marrow concentrated liposomes a up to a maximum of about 10 % of the injected dose at 24 hours post-injection.

Previously, Gregoriadis (1973b) had shown that ACT-D (LP) entrapped in cationic liposomes was not concentrated in the rat small intestine or kidney which results in this thesis confirm, however, the hepatic uptake of these cationic liposomes did not exceed the free drug until at least 20 minutes after injection, and this is not confirmed by these results nor by the work of others (Rahman et al., 1975). It is unclear if this difference is due to the fact that cationic liposomes are cleared more slowly than anionic ones (Gregoriadis and Neerunjun, 1974; Juliano and Stamp, 1975) or perhaps due to the presence of a substantial population of SUVs in Gregoriadis's (1973b) preparation, as is suggested from its plasma clearance curve.

Juliano and Stamp (1978) and Juliano et al. (1978) studied the pharmacokinetics of ACT-D (LP) entrapped in cationic SUVs and found a

similar distribution after 3 hours with these vesicles as was found here; the main exception being that they found negligible levels of entrapped drug in brain tissue. These authors also found that uptake by cardiac and skeletal muscle was low in comparison to the free drug.

Finally, Kaye et al., (1981) have recently investigated the tissue distribution and antitumour activity of ACT-D (LP) in small cationic MLVs injected into mice. These authors found higher quantities of entrapped drug (per gm tissue) at all times (3-72 hours) than were found in these studies with the exception of splenic tissue. Moreover, at all times, all the tissues (apart from liver) had higher amounts of free drug than entrapped drug and this is generally true of the results presented here. Interestingly, these authors found that the level of liposomal ACT-D in murine liver and spleen remains high for long periods and only falls to 50 % of its initial level after 7 days. A similar effect was not seen in this study where the liver looses 50 % of its initial level in about 6 hours. These authors were not able to confirm the preferential uptake of liposomal ACT-D by the bone marrow which had been reported by Rahman et al., (1975).

Several workers have studied the effects of liposome-entrapped ACT-D against tumour cells. Papahadjopoulos et al., (1976) demonstrated that anionic SUVs (ACT-D.LP) enhanced the uptake of drug into a cell line which was resistant to ACT-D, thus overcoming its resistance. Gregoriadis and Neerunjun (1975a) demonstrated that liposomal ACT-D would prolong the survival time of tumour bearing mice beyond that found with equivalent doses of free drug, and Rahman et al., (1974) found a similar effect in mice bearing a different tumour. However, Kaye et al., (1981) failed to find preferential (i.e.: > free drug) location in vivo of liposomal ACT-D in a tumour sensitive to the drug or in a resistant subline of the same tumour. Moreover, although these authors confirmed the prolonged survival time of tumorous mice following treatment with entrapped ACT-D, they stated that this was due to a general reduction in tissue toxicity of the drug when in the entrapped form since there was no apparent effect upon the tumour weight in long term survivors. The entrapped drug also failed to effect an ACT-D resistant tumour subline in vivo.

The toxicity of entrapped ACT-D has been investigated by Rahman et al., (1978) and Kaye et al., (1981). The first report showed that ACT D(LP) in neutral-handshaken-MVLs was no less toxic than the free drug to cell proliferation in the bone marrow and intestine. However,

the liposomal ACT-D was less immunosupressive than the free drug. These authors (Rahman et al., 1974) had previously shown that I/V administered ACT-D was less toxic when given in the entrapped form (LD_{50} Free ACT-D: 0.43mg/kg, ACT-D Liposomes: > 1 mg/kg). Kaye et al., (1981) reported that no deaths occurred in mice treated with 8 mg/kg ACT-D entrapped in cationic small MLVs.

ii) Bleomycins.

Gregoriadis and Neerunjun (1975b) and Dapergolas et al., (1976) reported that ^{111}In-BLM MLVs were taken up by both the liver and an implanted tumour following I/V injection. The smaller SUVs were taken up by the liver to a lesser extent than the MLVs but the opposite was true for the tumour tissue. However, these latter authors noted significant differences between the liver and tumour localization in different strains of mice.

Gregoriadis et al., (1977a) failed to find differences in the plasma clearance of ^{111}In-BLM liposomes injected into rats or mice but the uptake by tissues was different. Thus, although most of the dose was recovered in the livers and spleens of mice 20 and 50 hours after injection, the levels in the same organs in the rats was 4 (liver) and 6 (spleen) times lower. In both species, a reduction in the size of the liposomes led to a less radioactivity being taken up by the liver and spleen but to an increase in localization in the lungs, kidney, skeletal muscle and brain. At all times and for all tissues studied the uptake (per gm tissue) was higher in the mouse than the rat. The levels of radioactivity found in the rat tissues studied by Gregoriadis et al. (1977a), using the same liposomes, were almost identical to the levels found in this study. These authors found significantly higher amounts of liposome derived ^{111}In-BLM in mouse livers and spleens than other authors have found using similar liposomes containing ACT-D (Rahman et al., 1975; Kaye et al., 1981). This fact suggests that ^{111}In-BLM leaks less rapidly from liposomes than ACT-D and confirms previous results of this study (section 2:2 and 3:2). However, both Dapergolas et al., (1976) and Gregoriadis and Neerunjun (1975b) have claimed that ^{111}In-BLM does not leak from liposomes and, whilst the present data does not agree with this, a contribution by protein-bound ^{111}In to the distribution of "entrapped" ^{111}In in tissues cannot be ruled out.

Following intravenous injection of ^{111}In-BLM-containing liposomes into humans Segal et al., (1976) failed to find increased radiolabel

localization in hepatic tumour tissue and this is in contradiction to the tumour location found by others in animals (Dapergolas et al., 1976; Gregoriadis et al., 1977a). Moreover, it did not agree with previous studies in humans using liposomal ^{131}I-albumin (Gregoriadis et al., 1974a). In addition, much of the radioactivity was found to be present in the bone marrow. At autopsy, 8 days after liposome administration, significant quantities of radiolabel were found in the liver and spleen but also in the lungs, kidneys, bone marrow and, in one patient, the brain and tumour mass (Segal et al., 1976)

The distribution of ^{111}In in SUVs has also been studied by Hwang and Mauk (1977), Mauk and Gamble (1979) and Hwang et al., (1980). These results are not strictly comparable to those of this study since the radiolabel is chelated to another molecule (nitrilotriacetic acid) and not BLM. Three hours after I/V injection into mice, Mauk and Gamble (1979) found that anionic and neutral SUVs concentrated to the greatest extent in the liver (although the blood levels of these liposomes was always very high) but cationic SUVs exceeded these liver concentrations by 2.5 to 3 fold. The uptake by other tissues was low (e.g.: kidney and spleen < 3 % of the injected dose) but about 5 % of the dose was found in the small intestine although lower levels of cationic vesicles were found in this tissue. After 24 hours, the liver concentration of the cationic SUVs remained significantly higher than that found with neutral or anionic liposomes, the spleen levels were lower than at 3 hours whilst the levels in the small intestine had increased. Uptake of all vesicle types by the tissues of the chest and into the skin was very similar (7-10% of injected dose) but the cationic preparation remained lower than the others in all tissues apart from the liver. Comparison of these data with figs 4:2 and 4:7 confirms the reports of others (Tyrrell et al., 1976a; Gregoriadis et al., 1977a) that the spleen captures the larger liposomes. The uptake by the small intestine, skin, lungs and brain was less (% dose/gm tissue) in the current studies in rats than was found at both 3 hours and 24 hours in mice by Mauk and Gamble (1979) but this may be due to higher blood levels of radioactivity contaminating these tissues. In a further series of experiments using SUVs containing the ^{111}In complex but made of sphingomyelin and CHOL, Hwang et al.,(1980) reported that the plasma half life of these vesicles was 16.5 hours and that the liver concentration of the radiolabel continued to increase over a period of 23 hours (unlike fig. 4:7). Similar, almost linear, increases with time

(up to 23 hours) were found in the kidney, spleen, intestine, skin and legs (bone marrow ?) whilst levels in lungs fell during the same time period. Using the gamma-ray perturbed angular correlation technique in conjunction with these studies, these authors were able to measure intact liposomes in the liver and they found that degradation occured with a half life of 3.5 hours with maximal intrahepatic release of entrapped drug occuring after 8 hours. The entrapment of iodinated BLMs in liposomes and their tissue distribution has not been previously reported.

iii) Asparaginase.

No published data exist concerning the tissue distribution of liposome-entrapped ASPase. This enzyme has, however, been entrapped in homologous red cell ghosts which were slowly cleared by the liver and spleen (Updike et al., 1976) and were able to lower plasma asparagine levels (Ihler, 1979). Other proteins entrapped in liposomes have been studied and these will be used for comparative purposes.

The original reports of Gregoriadis and Ryman (1972a; 1972b) on the entrapment of albumin, amyloglucosidase and beta-fructofuranosidase demonstrated that the liver and spleen were the principal sites of liposomal radioactivity and enzyme activity. These tissues have been repeatedly implicated as the major organs of uptake for liposomes containing proteins by many authors. Gregoriadis and Ryman (1972a) found very little liposomal albumin associated with the kidneys and lungs. Similarly, Gregoriadis and Allison (1974) found entrapped diptheria toxoid not only in the liver and spleen but also in the kidneys of mice.

In man Gregoriadis et al., (1974a) found liposomal radiolabelled albumin in normal and tumorous tissue of the liver, spleen, kidney and colon; the uptake by tumour tissue was consistently higher than the normal tissue in the kidney and colon. These authors also reported negligable radioactivity in the bone marrow. Tyrrell et al., (1976b) attempted to treat a lysosomal storage disease (Pompes disease:type II glycogenosis) with amyloglucosidase entrapped in anionic liposomes. They found that the enzyme had had some activity against the stored glycogen of the liver but not against muscle glycogen. Similarly, Belchetz et al., (1977) and Gregoriadis (1980b) found a reduction in liver size in a patient given entrapped glucocerebroside: beta-glucosi-

dase for the treatment of Gaucher's disease. No other tissues were studied.

Steger and Desnick (1977) studied the fate of beta-glucuronidase entrapped in anionic and cationic liposomes (MLV) following I/V injection into enzyme-deficient mice. The anionic liposomes were taken up by the liver (75 % of injected dose) and the enzyme retained its activity in this organ for up to 8 days. The levels of unentrapped enzyme were always very much lower than the liposomal enzyme after the first hour post-injection. This is not confirmed by results in the present work which do not show persistance of liver ASPase radioactivity nor higher levels of entrapped enzyme after 1 hour. These authors also found a long persistance of entrapped enzyme in the kidneys (> 3 days) and some (lower) levels in the spleen. No enzyme activity was detectable in the brain, heart, lungs, or bone marrow. Unfortunately no comparative studies of unentrapped enzyme were made in any tissues apart from the liver.

Steger and Desnick (1977) also found significant differences between anionic and cationic liposomes when the latter were used to entrap the enzyme. A longer liver retention of enzyme activity and lower and shorter retention of the enzyme in the kidneys was reported. It is unclear if the differences between the two liposomes types are due to charge alone or if size (not controlled) of the populations could account for some of these differences.

The findings of the present study are clearly at variance with the above description even taking into account that a different enzyme and species was used, the reported accumulation and retention of protein-containing liposomes by the liver and spleen is not confirmed with ASPase entrapped in anionic MLVs. The liver uptake of free ASPase, probably results in degradation of the enzyme (total radioactivity was found to be significantly higher than TCA radioactivity) but this is not the case for the entrapped material. It therefore seems that the different liver uptake pattern of entrapped ASPase (as compared to other entrapped drugs) is not due to complete lysis of the circulating liposomes. Indeed, Neerunjun and Gregoriadis (1976) failed to find interactions of antibodies with liposomal ASPase or, at least, not high enough levels of free ASPase to result in anaphylactic shock.

Initially, Neerunjun and Gregoriadis (1976) reported that large doses of liposome entrapped ASPase were able to profoundly decrease the

levels of plasma asparagine despite the fact that the permeability of this amino acid into these liposomes can be expected to be low (Naoi et al., 1977). Later, (Gregoriadis, 1980b) it was reported that liposomal ASPase was cleared so rapidly from the circulation that lower plasma asparagine levels were not responsible for the antitumour effects seen with the entrapped enzyme. It was therefore proposed that ASPase acted in the liver to reduce the output of asparagine. However, it is known that asparagine synthesis and output by the liver is controlled by plasma asparagine levels (Woods and Handschumacher, 1973). The plasma half-life of asparagine has been reported to be less than 40 minutes in humans but reduction of plasma asparagine to 20 % of the normal levels does not reduce tumour growth (Cooney et al., 1970). Moreover, in the absence of an inhibitor of asparagine synthesis, it is not possible to deplete the asparagine pool in the liver (Woods and Handschumacher, 1973) even with asparaginase (Patterson and Orr, 1969). In view of the forgoing it is, therefore, difficult to see how liposomal ASPase acting intracellulary in the liver can account for lower tumour levels of asparagine 1 and 3 hours after injection (Gregoriadis, 1980b); especially since other tissues can donate asparagine to the plasma and to neighbouring cells, e.g. : erythrocytes, which cannot synthesize asparagine, retain considerable amounts of the amino acid for days after the plasma asparagine levels are undetectable following ASPase treatment (Cooney et al., 1970).

The antitumour effect of entrapped ASPase may, therefore, be due, initially at least, to a direct lowering of intracellular asparagine levels following uptake of the liposomes by the tissues rather than indirect effects on the liver ouptput or lower plasma levels of the amino acid. In this respect, the findings, reported here, that the free enzyme concentration was higher than the entrapped in all the tissues examined correlates well with the report of Neerunjun and Gregoriadis (1976) that a greater than three fold higher dose of the liposomal enzyme was required for complete regression of tumours in mice.

The disposition of the entrapped ASPase remains to be fully determined. Two hours after injection, when the liver concentration of the liposomal and free enzyme are the same, 20.9 % of the injected dose of entrapped ASPase was recovered in the plasma plus all the tissues examined whereas the comparative value for the free drug was 44.3 % : a large proportion of the entrapped enzyme and more than half of the free enzyme therefore remain unaccounted for. Tissues and body fluids not

examined for radioactivity must concentrate high quantities of the entrapped enzyme. It is possible that some enzyme and radiolabel could have been excreted by the urine and in the bile but one site of tissue accumulation which may account for the remaining protein is the bone marrow. High levels (up to 10 % of the injected dose at 6 hours in a sample from both tibias) of liposome entrapped drug have previously been reported in this tissue in animals (Rahman et al., 1975) although these high levels were not confirmed by others using smaller liposomes (Segal et al., 1974; Car ide et al., 1976; Kaye et al., 1981 and this work; table 4:3). In man both Segal et al. (1976) and Belchetz et al. (1977) have reported liposomal localization of radioactivity in the legs (bone marrow ?) of patients by means of whole body scanning although this had not been shown previously by Gregoriadis et al (1974a). It seems possible, therefore, that some of the "missing" radioactivity may be concentrated in bone marrow but the extent to which this is determined by the size of the liposome preparation or the presence of ASPase entrapped in the liposome remains to be investigated.

Section 4:4

SUBCELLULAR LOCALIZATION OF LIPOSOME-ENTRAPPED DRUGS

Introduction.

The previous section demonstrated that the major site of uptake of I/V injected liposomes was the liver. In this section the subcellular localization of liposome entrapped drugs in this organ has been studied.

In principle, subcellular fractionation should give evidence as to the mode of uptake of liposome entrapped solutes as well as their final destination within the cell. Thus, the finding of liposome-entrapped drugs localized with lysosomes might be used as evidence that endocytosis of the carrier has taken place. However, care must be taken to compare this localization with the unentrapped material because many foreign molecules (e.g. : proteins) also find their way into lysosomes during their metabolism by cells (Gordon and Cohn, 1973). Localization of entrapped markers (e.g. : cytotoxic drugs) in the nucleus, which may be their site of action depending upon the type of drug involved, might suggest that liposomes can deliver their contents to the correct organelle and that drug degradation during uptake of the carrier is minimized. On the other hand, concentration of drugs in the microsomal fraction may be taken as evidence that the compound is in the process of being metabolized or transformed (Mannering, 1971; Mandel, 1971). Finally, the localization of entrapped material with the cytosol of the cells may suggest a variety of different factors depending upon the timing involved, e.g. : cytosolic localization at times when the plasma concentration of the drug is high implies that the free drug or carrier is entering directly into the cytoplasm by a mechanism which probably does not involve endocytosis. At later times the finding of drug in this compartment suggests that either the compound is not being metabolised or it has been transfered from another site. Without giving definite proof, subcellular localization of drugs, therefore, provides indicators as to the mode of uptake and subsequent fate of the agents in the cell.

Results of subcellular fractionations.

The results in this section are expressed as the amount (percent)

of the whole liver radioactivity which was found to be associated with each subcellular compartment studied. However, in the majority of cases the total hepatic radioactivity was many fold higher for entrapped drugs than the free agent but this will not be reflected in the subcellular fractions.

i) Control values

The fractionation performed was only a crude separation of the liver cells into four components: nuclei, mitochondrial-lysosomal (large granules), microsomal (small granules) and soluble (cytosol). No attempt was made to define these fractions any more precisely apart from the localization of two marker enzymes.

a) Enzyme controls

Tables 4:9A and 4:9B show the activities of two enzymes measured in the subcellular fractions of rat liver. N-acetyl-B-glucosaminidase (NABGase) (Table 4:9A) is known to be a lysosomal enzyme and latent until these organelles are lysed by a detergent. The distribution demonstrates that 46.55 % of the enzyme activity was found in the large granule (ML) fraction. Moreover, in a single experiment to test the latency of the enzyme (not shown), it was found that the enzyme was at least 63.5 % latent in this fraction. Enzyme activity found in other fractions can be accounted for in part by variations in lysosome size. The lysosomal contamination of the nuclear fraction represents large lysosomes and probably also an insufficient washing of the fraction to separate all these larger organelles (latency > 73.8 %). Similarly, microsomal (P) NABGase may represent smaller lysosomes (> 48.2 % latent) and some release of enzyme. Finally, the cytosolic (S) activity represents enzyme derived from broken organelles and adequately accounts for the loss of latency in the large granule fraction.

The mitochondrial enzyme, succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium-reductase (Table 4:9B) was found to have a similar subcellular distribution to that found for the lysosomal enzymes. The large granule (ML) fraction contained, on average, more than 55 % of the total activity in the liver and the majority (> 71.3 %) of this activity was found to be latent (not shown) until the granules were lysed with detergent. Contamination of the other subcellular fractions can be explained by lysis, size differences and inadequate washing as explained previously.

Table 4:9 A.

Activity of N-acetyl-beta-glucosaminidase (NABGase) in whole liver and liver fractions (n = 50).

Fraction*	Mean: ug PNP produced/ mg liver/min. (± st.dev.)	Mean % activity in whole liver (± st.dev.)
		total recovery =
WH	0.3383 ± 0.0573	99.610 ± 16.557
N	0.0627 ± 0.0096	18.547 ± 2.864
ML	0.1575 ± 0.0576	46.554 ± 17.021
P	0.0416 ± 0.0165	12.311 ± 4.876
S	0.0751 ± 0.0282	22.198 ± 8.355

Table 4:9 B.

Activity of Succinate-INT reductase (INTase) in whole liver and liver fractions (n = 50).

Fraction*	Mean adsorbance value (490 nm)/mg liver/min. x 10^3 (± st.dev.)	Mean % activity in whole liver (± st.dev.)
		total recovery =
WH	3.615 ± 0.58	93.76 ± 13.24
N	0.804 ± 0.261	22.24 ± 7.19
ML	2.045 ± 0.663	56.58 ± 18.263
P	0.401 ± 0.134	11.11 ± 3.713
S	0.138 ± 0.141	3.822 ± 3.948

* Abbreviations : WH : whole liver homogenate; N : nuclei; ML : mitochondria/lysosome; P : microbodies; S : cytoplasm; PNP : p-nitrophenol; INT : 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium.

Rat livers were removed, weighed and homogenized in sucrose. Homogenates were fractionated into subcellular components by differential centrifugation. The activities of the two enzymes NABGase and INTase were estimated in the homogenate and each subcellular fraction by published methods (see chap. 2).

Total homogenate activities of the two marker enzymes were found to be similar to that found by others (i.e. NABGase : 0.396 ug PNP/mg liver/min (Borooah, et al., 1961) and INTase : 3.9 $\pm$ 0.14 x 10^3 extinction units/mg liver/min. (Hinton, et al., 1969).

In general the enzyme markers found in the individual drug-treated liver fractionations were not significantly different from the mean results shown in tables 4:9 A and 4:9 B.

In the case of ASPase there was some contamination of the N fraction from the ML. All these fractionations were carried out at the same time but only some of the N fractions (from 4 centrifugal runs at 600 g) were contaminated and these are the values at the time points 0 - 5 hours. Both the enzyme markers were effected and the N fraction showed (on average) 22.3 % higher values for NABGase than the means in table 4:9 A and 25.16 % higher for INTase than the means in table 4:9 B (the tables do not, therefore, contain these higher values). However, when the means from this experiment were compared with those in table 4:9 A and 4:9 B they were not significantly different. The values for the enzyme markers in ML were slightly lower but the other fractions were not effected.

b) Fraction controls

Table 4:10 shows the values obtained in each subcellular fraction when samples of the injected preparations (except ACT-D) were co-homogenised with untreated rat liver which was then fractionated. In most cases the majority of the radiolabelled drug, whether free or entrapped, was found associated with the S fraction. It is unclear if the minor amounts of radioactivity found in other fractions are due to binding of the free drugs to the subcellular structures found in the fractions or, in the case of the liposomal preparations, due to liposomes which intrinsically sediment with these structures. It seems unlikely that all the liposomal radioactivity in N, ML and P is due to drug leakage since, in general, the amounts found are significantly higher than those of the free drugs.

An exception is seen in the case of ASPase, where much more free drug associated with the ML and P fractions and this was probably due to the aggregated nature of the material. In addition, significantly more liposomes were recovered in the ML fraction than were found for

Table 4:10.

Control values for subcellular fractionation studies *

PREPARATION F = free E = entrapped	% Total added to whole liver in each fraction mean (± St. dev.) (n = 3 except ^{111}In-BLM) F R A C T I O N				Total Recovered (%)
	N	ML	P	S	
^{111}In-BLM					
F.	7.24	15.7	12.43	66.14	101.51
E.	10.70	12.41	8.62	65.68	97.43
^{125}I-BLM (ICL)					
F.	9.31 ± 0.85	2.25 ± 0.06	3.70 ± 0.05	83.2 ± 4.62	98.46
E.	9.93 ± 0.7	9.35 ± 0.5	7.51 ± 1.14	75.59 ± 2.91	102.38
p =	N/S	< 0.001	< 0.005	N/S	

continued on following page.

Table 4:10. (continued)

PREPARATION F = free E = entrapped	% Total added to whole liver in each fraction mean (± St. dev.) (n = 3 except ^{111}In-BLM) F R A C T I O N				Total Recovered (%)
	N	ML	P	S	
^{125}I-BLM (B/H)					
F.	7.76 ± 0.5	1.48 ± 0.08	2.78 ± 0.1	83.08 ± 3.41	95.1
E.	4.82 ± 2.2	7.63 ± 1.36	7.58 ± 1.4	76.57 ± 10.4	96.6
p. =	< 0.02	< 0.002	< 0.005	N/S	
^{125}I-ASPase (TCA ppt)					
F.	5.23 ± 3.2	10.42 ± 1.61	25.36 ± 2.58	72.57 ± 25.26	113.58
E.	9.16 ± 1.3	25.52 ± 4.15	14.72 ± 7.5	43.95 ± 9.6	93.35
p. =	N/S	< 0.01	N/S	N/S	

* A sample (0.5 ml) of the injected material was added to a portion (usually 2 g) of liver from an untreated rat. Subcellular fractionation was carried out as in Chapter 2

p = Probability (Students 't' test) that means are the same.

N/S = Not significant.

the other drugs. It is unclear if this is due to a larger population of big MLVs or to entrapped aggregates or to a mixture of MLVs plus free aggregated ASPase. The larger mean in P for the entrapped material was due to one fractionation which contained almost three times more material than the other two experiments.

The control values for ASPase present some difficulties in interpretation at later time points. Aggregated free ASPase will be cleared rapidly from the plasma but some of these aggregates will still be present in the blood during the first hour post-injection; it is therefore appropriate to use this material as a control at this time. However, at later times (> 2 hr) there should be no aggregated material in the plasma so to compare these fractions with the control fractionation is not correct because if the high levels of ASPase associated with the granular subcellular fractions are due to the aggregates they could give falsely high control values at later times.

A similar criticism can be leveled at all the liposomal preparations. At later time points, when only SUVs are available in the plasma for uptake into the liver, the control values which were carried out with a mixed population of MLVs and SUVs will be too high. The solution to this problem will be to use liposomes of defined size.

ii) Results.

The results of the subcellular fractionations are shown in figures 4:9 (ACT-D), 4:10 (^{111}In-BLM), 4:11 (^{125}I-BLM-ICL), 4:12 (^{125}I-BLM-B/H) and 4:13 (ASPase). Each drug will be considered under the overall heading of the individual subcellular fractions. Where possible the means between the free and entrapped drug concentrations in each fraction and between these values and their appropriate controls were compared by Student's 't' test. Comparisons of means between individual BLM preparations in each fraction and at each time point were also made, these comparisons are reported after the B/H-BLM results for each fraction. In the case of ASPase the individual fractions were further subjected to precipitation with trichloroacetic acid (TCA pptable radioactivity) and it is these latter results which are shown in figure 4:13. A comparison of means between the total radioactivity and the TCA pptable radioactivity in each fraction was made for both the free and the entrapped injected preparations.

All the comparison data, however, ought to be treated with some caution, especially where large differences were not found, because the

injected liposome preparations are heterogeneous populations and so cannot be said to be directly comparable in every respect. Moreover, it must be remembered that the liposomes will leak entrapped drug in the plasma (and probably within tissues as well) so that, unless the corresponding free drug can be shown to be excluded from the particular fraction, the liposome data may represent the distribution of leaked free drug as well as entrapped drug.

Nuclear fraction (N)

i) ACT-D.

This fraction contained the major portion of the free drug and this is consistent with the binding of ACT-D to DNA. Binding of the drug to N was very rapid (within 5 minutes) but it then remained relatively constant throughout the 24 hour-period. Much less liposomal drug was found in N throughout the period suggesting that drug leaked from liposomes in the plasma was not a significant factor in the total uptake by the liver. Moreover, liposome derived drug more than doubled in this fraction over the period suggesting that it was being relocated in the nuclei from other sites.

ii) ^{111}In-BLM

The uptake of both the free and the entrapped drug into N was comparatively low (cf.free ACT-D). Differences between the means were significant ($P < 0.05$) at 5 minutes (first time point) post-injection but at all other times up to 48 hours there were no significant differences.

At later times (24 hours and 48 hours : not shown) the entrapped drug maintained the same concentration whilst the free drug showed a slight, but not significant, increase to 23 % at 48 hours. The level of the entrapped drug was always quite close to the control value (table 4:10) whilst that of the free drug was more than double its control value at all times.

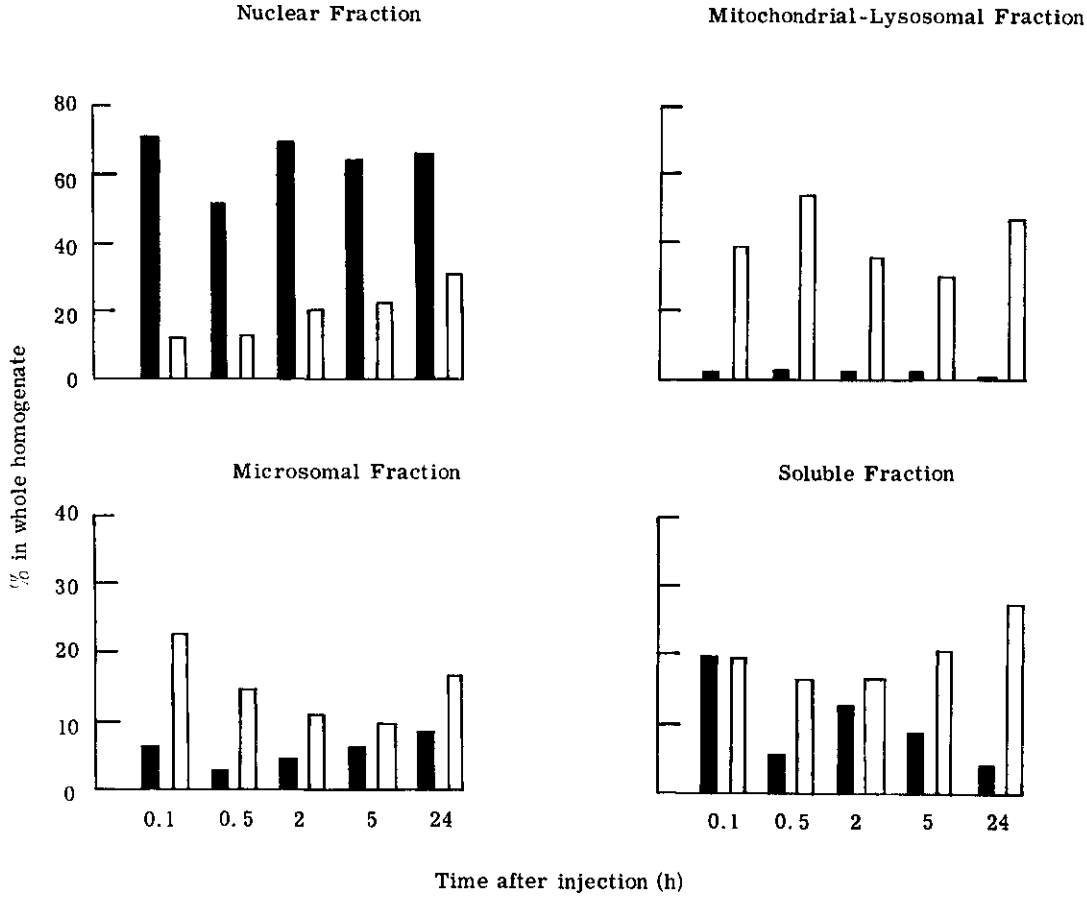
iii) ICL-BLM.

The free ICL-BLM concentration in N at 5 minutes post-injection was not significantly different from the control values (table 4:10) but thereafter the values were significantly higher at all times up to 48 hours. The free drug values were also significantly higher than the

Legend fig. 4:9

Subcellular Distribution of ^{3}H-Actinomycin D.

The histogram shows the percentage of total liver ^{3}H-radioactivity (fig. 4:6) found in each of four subcellular fractions at timed intervals (hrs) after injection. Each bar represents the mean from two animals. Solid bars, free ACT-D : open bars, entrapped ACT-D. Quantities of drug and lipid injected are shown in table 4:1. Total radioactive recoveries ranged from 70-94 %.



entrapped drug values at 30 and 60 minutes post-injection and again at 24 and 48 hours. The increase of free drug radioactivity associated with N during the first hour post-injection probably reflects binding of the drug to the nucleus.

The entrapped ICL-BLM levels in N were marginally higher ($p< 0.05$) than the control value at early time points (5,30 and 60 minutes) later they became very much higher and after 5 hours steadily increased up to 48 hours. As was mentioned before (ACT-D) this is probably a reflection of the slow accumulation of drug following its release from other compartments.

vi) B/H-BLM.

At all times up to 48 hours post-injection both the free and the entrapped B/H-BLM were significantly higher ($p < 0.02 - 0.001$) in N than their comparative control values (table 4:10).

The free radioactivity in N steadily increased to reach a plateau value of around 35 % between 2 and 5 hours post-injection. However, these values (30 min. - 5 hours) were not significantly different from the entrapped drug radioactivity. Nevertheless, at 24 and 48 hours the free drug value in N further increased to reach a peak of 55.5 % at 48 hours; these latter values were significantly higher than the corresponding entrapped radioactivity. These results suggest that the free drug was being slowly accumulated in N at the expence of other fractions.

Liposome entrapped B/H-BLM was always localized in N to a significantly greater extent than either the entrapped ^{111}In-BLM or the ICL-BLM. On the other hand, liposomal ICL-BLM uptake into N was not significantly different from the ^{111}In-BLM at any time. It is unclear if the modification made to BLM by succinylation (B/H) results in increased avidity for DNA but the free drug shows the same effect.

The free B/H-BLM was always significantly higher than the ICL-BLM in N and also higher than free ^{111}In-BLM between 2 and 48 hours. These data are similar to that found for the liposome preparations. At early times (5 minutes) the ^{111}In-BLM was significantly higher than the B/H labelled material but at this time the ^{111}In in the plasma had probably not yet bound to protein and was still partially drug bound. For this reason the early localization of ^{111}In-BLM in N may be due to BLM binding to DNA; at later times the ^{111}In label was significantly lower than the B/H-BLM material.

v) ASPase.

There was no significant difference between the total radioactivity and the trichloroacetic acid precipitatable (TCA ppt) radioactivity for either the free or entrapped drug levels in N.

Both the preparations gave significantly higher radioactivities in N than their corresponding controls at all times studied. Differences between the means of the preparations were not significant at any time during the 48 hour period except at 5 hours when the liposomal radioactivity was significantly ($p < 0.02$) higher.

The marker enzymes in the ASPase experiment were skewed towards the N fraction so that more lysosomes and mitochondria were found here than normal. This result means that a proportion of the radioactivity in N was derived from the ML for both preparations. The inference that ^{125}I counts were being localized in N therefore cannot be made. The fact that the preparations were not different from each other suggests that, whatever the contamination from ML, they were localized in the same compartment.

In general, the free drug results in N are similar to that found for free ^{111}In-BLM where the enzyme markers were good. This similarity of two, essentially, radiolabelled protein preparations may mean that the radioactivity is associated with cell structures which co-sediment with the nuclei.

Mitochondrial-lysosomal fraction (ML)

i) ACT-D.

This fraction contained the major proportion of the entrapped ACT-D. Quite high levels of drug were found within 5 minutes post-injection and this quantity increased further during the next 25 minutes. Thereafter, the quantity of liposomal drug in this fraction started to decrease despite the fact that the total quantity of drug in the liver was constant (fig. 4:6). The increase in ML radioactivity concentration during the first 30 minutes correlated well with the rapid phase of plasma clearance of the entrapped drug and suggests that these liposomes are being concentrated in the lysosomes.

The uptake of the free ^{3}H-ACT-D was minimal so that it can be confidently stated that localization of drug in the ML is entirely due to entrapment within liposomes : liposomal ACT-D levels were much higher than would be expected (table 4:10) from the small population of other liposomes which sediment in this fraction.

ii) ^{111}In-BLM.

Both the free and the entrapped ^{111}In-BLM appeared to be concentrated in ML. Over the five hour period shown, both preparations showed a steady increase (differences not significant) which was maintained in each case up to 24 hours. At 48 hours the quantity of both preparations in ML started to fall. The initial values in this fraction for both preparations were slightly higher than the controls (table 4:10) but thereafter the control values were very much lower at all times.

iii) ICL-BLM.

In contrast to the results found with ^{111}In BLM there was a marked difference between the free and entrapped ICL-BLM in ML. Both preparations were always significantly ($p < 0.02$-0.01) higher than their corresponding controls. The entrapped drug was significantly higher ($p< 0.01$) than the free at all time points up to 48 hours. The free preparation showed a steady increase in the ML over five hours and this continued up to 48 hours post-injection to reach a maximum of 28 % at this time.

The entrapped drug showed a much greater rate of increase to reach a peak in ML at 2 hours. This time corresponds to the end of the rapid plasma clearance phase and to the peak of total liver uptake (Fig 4:7). After 2 hours the ML radioactivity slowly decreased but remained at more than 55 % of the total liver level for up to 48 hours post-injection despite the fact that the total liver radioactivity had fallen to 10 % of the injected dose at 48 hours.

iv) B/H-BLM.

Both the free and the entrapped B/H-labelled BLM were significantly higher than their control values in ML. Moreover, except at 5 hours, the entrapped drug was significantly higher than the free ($p < 0.01$ - 0.001) at all times during the 48 hour period. The free drug slowly increased throughout the 48 hours under study and at no time were the means significantly different from the ICL-labelled material.

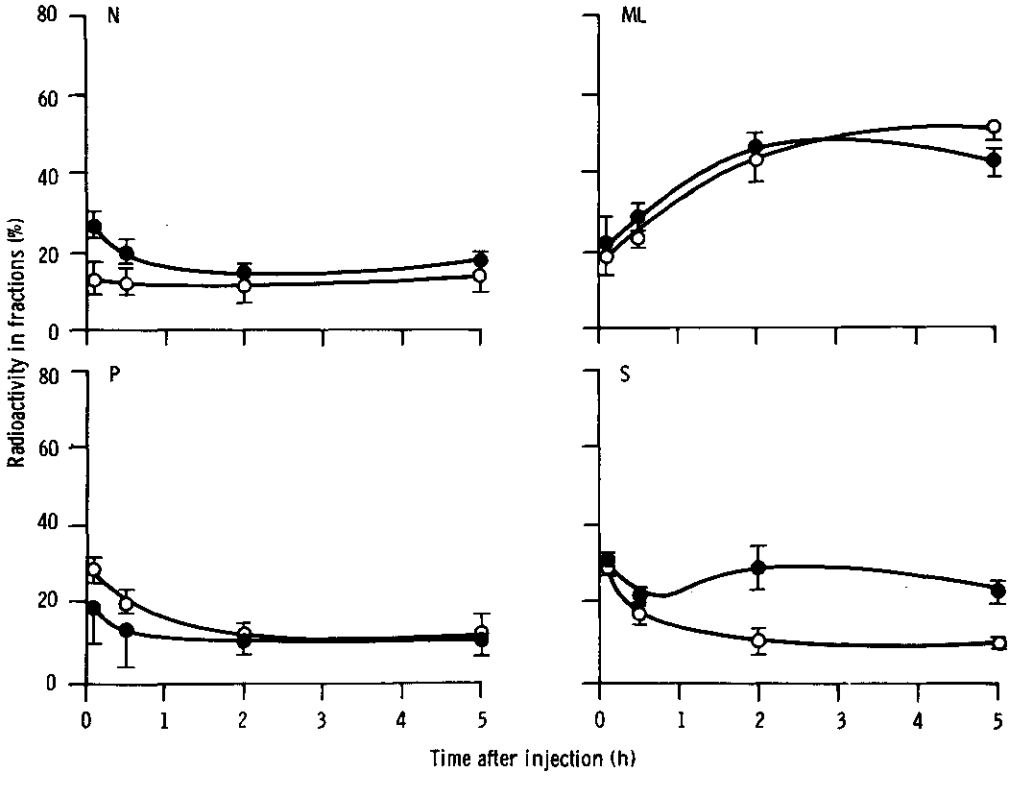
Liposome-entrapped B/H-BLM showed similarities to the entrapped ICL-labelled BLM since the initial rapid increase in radioactivity (up to 1 hour) was not significantly different. However, the values at 2 and 5 hours plateau but thereafter the radioactivities at 24 and 48 hours increase to give a maximum at 48 hours of 46.8 %.

Legend fig. 4:10.

Subcellular Distribution of ^{111}In-bleomycin.

The figure shows the percentage of total liver ^{111}In-radioactivity (fig. 4:7) found in each of four subcellular fractions at timed intervals (hrs) after injection. Each point represents the mean from 3 animals. Closed circles, free ^{111}In-BLM : open circles, entrapped ^{111}In-BLM. Quantities of drug and lipid injected are shown in table 4:1.

Recoveries of radioactivity in the fractions : entrapped 79.8 - 103.3 % (mean ± st.dev. = 85.44 ± 13.21 % (n = 18)) and free 84.7 - 108.9 % (mean ± st.dev. = 99.89 ± 6.97 (n = 18)). The enzyme markers were similar to tables 4:9 A and 4:9 B and the recoveries ranged between 73.5 - 114.91 % of the total hepatic activity.



In general the uptakes of liposomal B/H-BLM and ^{111}In-BLM into ML were not significantly different but the ^{111}In-BLM was significantly higher at 5 hours for unknown reasons. Conversely, the ICL-BLM was significantly higher in ML than both the other BLMs at all times. One explanation for this effect could be that the ICL-BLM preparation contains more of the larger liposomes (see plasma clearance and hepatic uptake data).

Except at 24 hours, when the B/H-labelled BLM showed an anormalously higher value, the distribution in ML of the two iodinated free BLMs were not significantly different. This result suggests that there was little difference in the lysosomal metabolism of the two drugs. However, the free ^{111}In-BLM was significantly higher than either of the iodinated drugs. This is probably due to ^{111}In bound to protein in the lysosomal fraction and these data reinforce the previous results obtained with this radiolabel which suggested that ^{111}In is not a good marker for either BLM tissue localization or for the aqueous phase of liposomes.

v) ASPase.

The free drug was significantly lower than the entrapped drug in ML at all points throughout 48 hrs. Further, the free ASPase was not significantly different from its control value at any time except at 5 minutes when it was higher ($p < 0.02$). At 24 and 48 hours post-injection the ML levels of free ASPase remained constant and not significantly different from the value at 5 hr. There were differences between the total and TCA-ppt free radioactivity in this fraction at 2 and 5 hours post-injection. This suggested that some ML uptake of the free drug had occured but, since the TCA-ppt material was not different from the controls, it is probable that a rapid degradation of the ASPase occured with the release of ^{125}I label.

Entrapped ASPase demonstrated a marked increase in level in ML when compared with the free drug. The slight fall at 30 minutes was not found to be significantly different from the values at either 5 minutes or 1 hour. The concentration of liposomal radioactivity in the ML remained constant, between 42 and 53 %, for 48 hours. There was no significant difference between the total and TCA precipitated radioactivity for the entrapped drug.

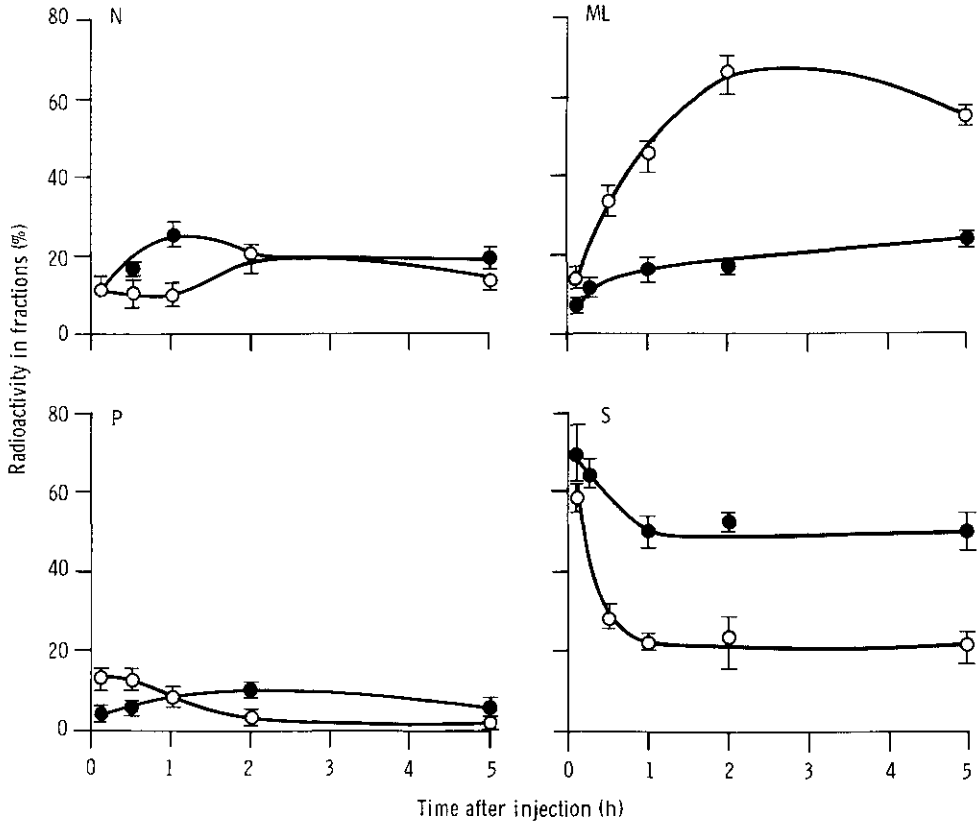
These data show that liposome uptake into this fraction was rapid at 5 minutes (i.e. similar to ACT-D liposomes) with no increase with

Legend fig. 4:11.

Subcellular Distribution of ^{125}I-bleomycin (ICL)

The figure shows the percentage of total liver ^{125}I-radioactivity (fig. 4:7) found in each of four subcellular fractions at timed intervals (hrs) after injection. Each point represents the mean from 3 animals. Closed circles, free ^{125}I-BLM(ICL) ; open circles, entrapped ^{125}I-BLM(ICL). Quantities of drug and lipid injected are shown in table 4:1.

Recoveries of ^{125}I radioactivities ranged between 88.3 - 114.8 % (mean ± St.dev. = 96.55 ± 9.66 (n = 21)) for the entrapped drug and between 82.6 - 110.5 % (mean ± St.dev. = 95.59 ± 9.44 (n = 21)) for the free. Recoveries of marker enzymes were between 82 - 115 % of total hepatic activity similar to the distribution shown in tables 4:9 A and 4:9 B.



time (c.f. ^{125}I-BLMs). This suggests that the ASPase which reaches the ML was actually entrapped because it was significantly higher than the free drug at all times.

Microsomal fraction (P).

i) ACT-D.

Liposome association with P was significantly higher than the free drug at all times. The high association of entrapped ACT-D at 5 and 30 minutes post-injection may have several causes : i) normally about 8 % of liposomes sedimented in this fraction (table 4:10). ii) at early times post-injection the blood levels of liposomes were high and it is know that MLVs can be centrifuged out of plasma using the same g forces (100.000) as is used to sediment this fraction although the majority of these might be expected to be found in S (table 4:10). iii) this radioactivity may represent sedimented liposomes either within vacuoles (small lysosomes ?) or free in the cytoplasm which have not yet fused with lysosomes and were released on liver cell lysis. A combination of these points seems a probable explanation of this data. At later times (2 and 5 hours) ACT-D liposomes were similar to control levels. The decrease in liposomal radioactivity in P may be either a reflection of falling bood contamination or an increase in vacuole-lysosome fusion.

Free ACT-D levels in P were lower than 10 % of the total throughout but higher than the levels found in ML. It may be that these levels represent free drug bound to proteins or nucleic acids in other organelles or fragments of organelles which co-sediment with microsomes at this density. In view of the stability of ACT-D *in vivo* it seems unlikely that these levels represent drug in the process of being metabolised.

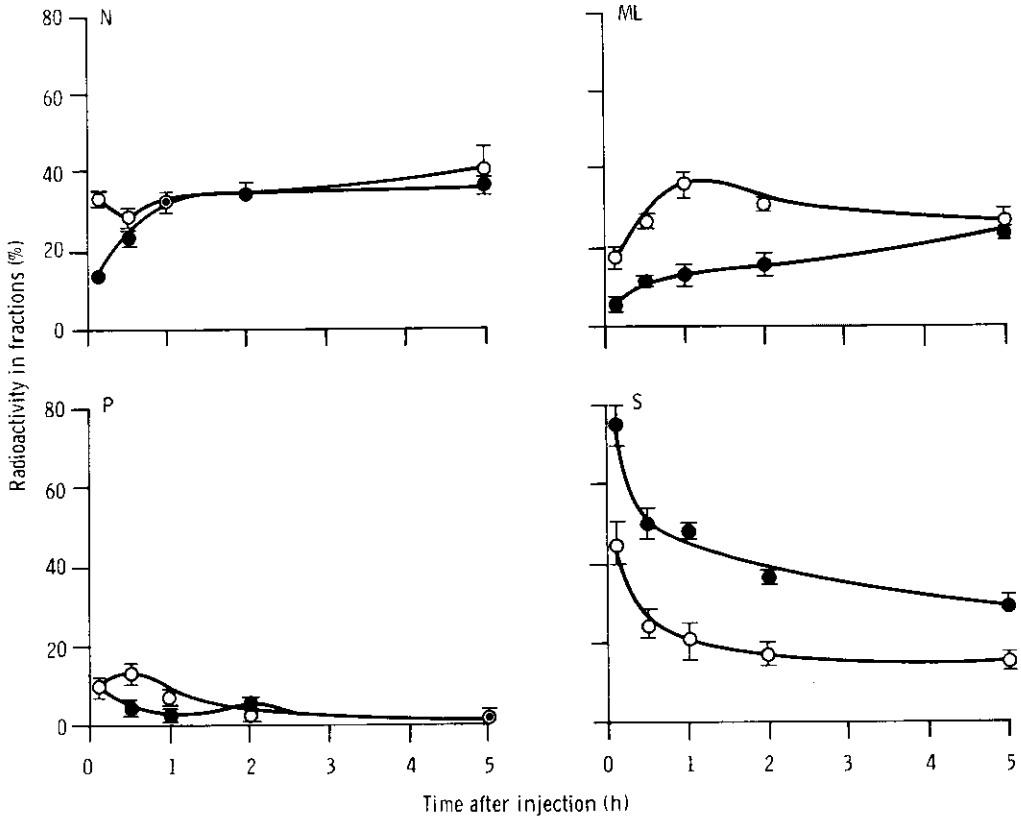
ii) ^{111}In-BLM.

At no time, up to 48 hours, were there significant differences between the free and the entrapped ^{111}In-BLM in P. Initially, (5 and 30 minutes) the uptake was higher, for both preparations, than the corresponding controls. The probable reasons for this have been explained previously (see ACT-D results).

Legend fig. 4:12.

Subcellular Distribution of ^{125}I-bleomycin (B/H)

The figure shows the percentage of total liver ^{125}I-radioactivity (fig. 4:7) found in each of four subcellular fractions at timed intervals (hrs) after injection. Each point represents the mean from 3 animals. Closed circles, free ^{125}I-BLM(B/H); open circles, entrapped ^{125}I-BLM(B/H). Quantities of drug and lipid injected are shown in table 4:1.



The recoveries of ^{125}I-BLM (B/H) radioactivity ranged between 87.9 - 115.3 % (mean ± St.dev. = 94.51 ± 8.54 (n = 21)) for the entrapped drug and between 85.1 - 118.8 % (mean ± st.dev. = 102.3 ± 8.84 (n = 21)) for the free drug. Enzyme marker disposition was similar to tables 4:9 A and 4:9 B.

At time intervals between 2 and 48 hours the values in P for both preparations were unchanged and similar to their control values.

iii) ICL-BLM.

The free ICL-BLM radioactivity at 5 and 30 minutes post-injection was not significantly higher than the control values, thereafter it was higher. At all time points between 2 and 48 hours the free drug in P was higher ($p < 0.02$) than the entrapped preparation.

The initial (5 and 30 minutes) values of the liposomal radioactivity were significantly higher than both the free ICL-BLM and the control values. This latter suggests a localization of released liposomes into this fraction. Later, between 1 and 48 hours, the levels of liposome radioactivity were not significantly different from the controls.

iv) B/H-BLM.

The entrapped drug was significantly higher than both the free drug and the control value at 5 and 30 minutes post-injection thereafter it was significantly lower ($p < 0.002$) than the control at all times : at 24 and 48 hours the free B/H-BLM was higher ($p < 002$) than the entrapped in P. At the early time points entrapped B/H-BLM was not significantly different from the liposomal ICL-BLM levels.

Free B/H-BLM was significantly higher ($p < 0.02$) than the control value at all time points to 48 hours except 5 hours. The level of the free drug in P remained relatively constant (2.6 - 5.6 %) during the period 1 - 24 hours but increased slightly (8.2 %) at 48 hours. In general the free B/H-BLM was significantly lower than the free ICL-BLM at all time points.

Although there was no significant difference between the entrapped iodinated preparations at any time, the entrapped ^{111}In-BLM was significantly higher in P than the iodinated preparations during the first 2 hours. The reasons for this higher ^{111}In-BLM level may be due to a preponderance of smaller liposomes in the preparation which sediment with this fraction. Alternatively, the binding of leaked ^{111}In-radiolabel to protein may also be the cause but this localization did not occur with the free drug.

Free B/H-BLM was not significantly different from the free ^{111}In-BLM at any time in P. This result may, therefore, reflect the back-

ground radioactivity associated with this fraction. However, between 1 and 48 hours the ICL-BLM was significantly higher than either of the other two preparations. The reasons for this result is unclear but it was also found in the S fraction.

v) ASPase.

The free ASPase level was significantly lower ($p < 0.02$) than its control value in P up to 5 hours, thereafter it was not significantly different. The entrapped ASPase was not significantly different from its control throughout the 48 hour period. Except at 5 minutes, when the entrapped radioactivity was significantly higher ($p < 0.05$), the comparison of means between the two preparations in P were not significantly different.

For the entrapped drug after the initial 30 minutes, the level of radioactivity in this fraction remained constant for 48 hours (around 11 % of the total). The free drug levels of radioactivity in P also remained constant for 5 hours but were significantly increased over this value at 48 hours. There was no difference between the total and the TCA precipitatable radioactivity for either the free or the entrapped preparation.

Soluble fraction (S).

i) ACT-D.

During the first half hour post-injection, the entrapped ACT-D content of S was probably due to contamination from whole liposomes in the blood plus whole vesicles released from their adsorbtion to sinusoid lining cells as well as liposomes released from the interior of the cell following homogenisation. The control values for other liposome preparations (table 4:10) uniformly showed that the major part of the uninjected preparation was localized in this fraction.

However, it is clear that as the liver and plasma entrapped drug concentrations fell (i.e.: at 5 and 24 hours) the concentration of ^{3}H-ACT-D from liposomes in S increased. This may be due to the release of the entrapped drug from the lysosomal fraction into the cytosol from where it is able to enter other compartments (e.g.: the nucleus).

For the free drug, the levels in S at 5 minutes post-injection reflected the rapid entry of this agent into liver cells. As the levels of free drug in the plasma fell so, in general, did the level of the drug in S.

Legend fig. 4:13.

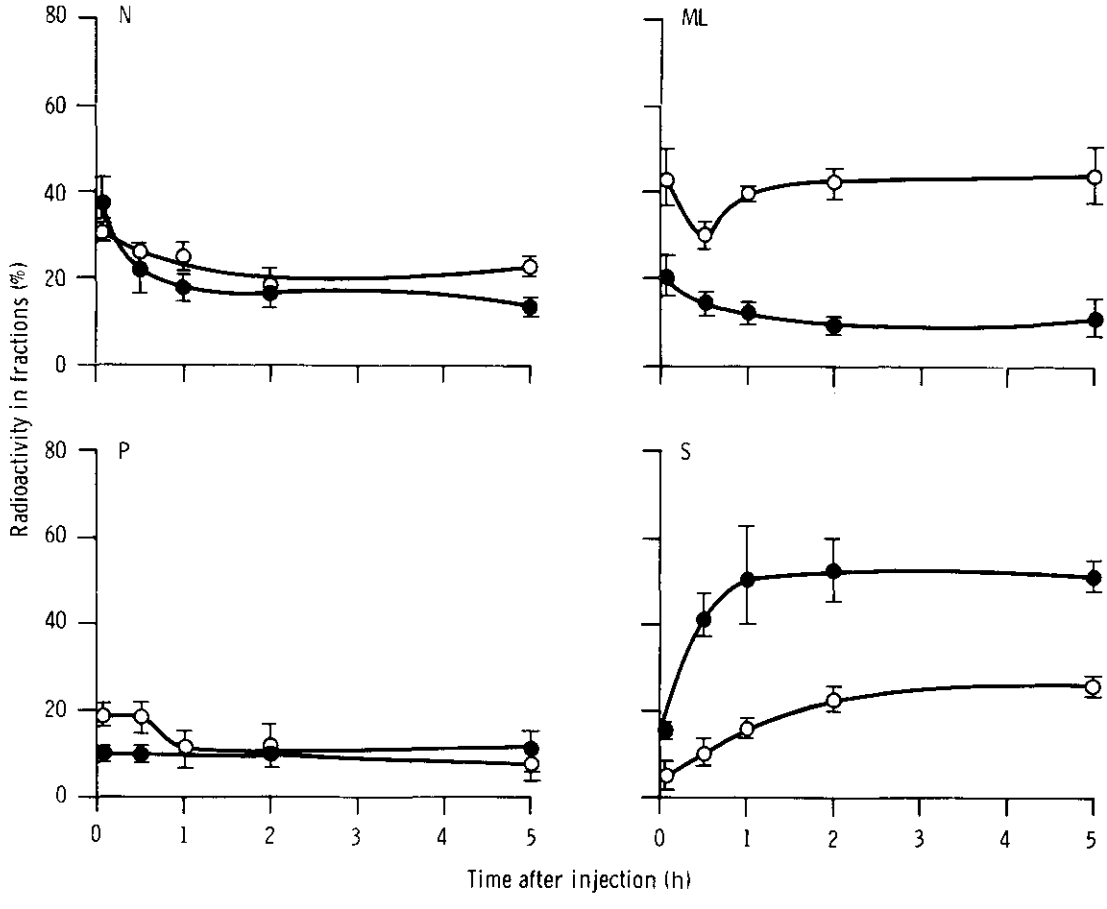
Subcellular Distribution of ^{125}I-Asparaginase

The figure shows the percentage of total liver ^{125}I-radioactivity (TCA precipitatable) (fig. 4:8) found in each of four subcellular fractions at timed intervals (hrs) after injection. Each point represents the mean from 3 animals. Closed circles, free ^{125}I-ASPase; open circles, entrapped ^{125}I-ASPase. Quantities of drug and lipid injected are shown in table 4:1.

Radioactive recoveries were :

TCA-ppt Free - Range : 85.4 - 104.8 % (mean ± St.dev. = 95.22 ± 9.16 %)

TCA-ppt Entrapped - Range : 86.4 - 113.9 % (mean ± St.dev. = 97.67 ± 8.96 %) (number of fractionations = 21 in each case).



ii) ^{111}In-BLM.

The quantity of ^{111}In radioactivity associated with S was lower than the corresponding control levels for both preparations at all time points. During the first half hour there was no significant difference between the means of the two preparations. Thereafter (2-48 hours) the radioactivity due to each injection remained constant with that of the free drug being significantly higher ($p < 0.01$) than the entrapped. This fraction is the only compartment studied where sustained differences between the preparations were found. The fall in entrapped radioactivity (30 minutes) whilst the total hepatic uptake (fig. 4:7) was rapidly increasing means that the S fraction was not primarily involved in liposome uptake. Such a conclusion cannot be drawn for the free drug since the total hepatic uptake was constant over a 24 hour period.

iii) ICL-BLM.

The initial values (5 min.) of both free and entrapped ICL-BLM radioactivity were not significantly different from their corresponding controls. Similarly at 30 minutes the free radioactivity was still the same as the control values but thereafter it fell significantly below the controls. After 5 minutes, the free drug concentration was significantly higher than the entrapped ($p < 0.01$) up to 48 hours. Between 1 and 24 hours the free radioactivity in S remained constant (50-59 % of total hepatic radioactivity) but at 48 hours it had fallen to 26 % and this was accompanied by a slow increase into the ML. If the drug was degraded in S the levels of ^{125}I would be expected to fall over 48 hours. The fact that the level remained constant suggests that either this degradation is incomplete (i.e.: it does not remove the radiolabel) or it does not occur. The entrapped radioactivity in S rapidly fell at 30 minutes post-injection, thereafter it remained constant (around 25 %) for 48 hours and significantly lower than the control values. Since during the initial period (0 - 2 hours) the liver uptake increased (fig. 4:7) this result again suggests that the hepatic uptake of liposomes is not occurring into the S fraction.

iv) B/H-BLM.

The entrapped B/H-BLM radioactivity in S was always significantly lower ($p < 0.005$) than both the free drug and the entrapped control

value. Except at 5 minutes, when it was lower ($p < 0.02$), these entrapped values were not significantly different from the corresponding liposomal ICL-BLM values and so these data may be taken as being similar.

For the free B/H-BLM following the rapid initial (0 - 1 hour) fall, the levels continued to fall slowly up to 48 hrs. This is unlike the free ICL-BLM where the concentration is S remained constant during the period 1 - 24 hrs. The free levels were always lower than the control values ($p < 0.001$) except at 5 minutes.

There was no real difference between any of the entrapped BLM preparations in S. Perhaps this means that the rate of metabolism of BLM is unaffected by liposome entrappment. However, this lack of difference is evidence that the mode of uptake of the liposomes is the same for all the preparations and this lack of localization of liposomes in S suggests that uptake is into another fraction (ML).

The quantity of free ICL-BLM in S was greater than free ^{111}In-BLM throughout the 48 hr period and this suggests that these two drugs may be entering the liver by different mechanisms. However, between 2 and 24 hrs post-injection the free ICL-BLM also significantly higher than the free B/H-BLM. It is possible, therefore, that the ICL material is more resistant to enzyme inactivation in the cytosol and so persists for longer than the B/H-BLM. At early times (0 - 1 hr) the iodinated preparations were not significantly different.

v) ASPase.

A comparison of the means of the free drug values with the control preparation revealed that the two were not significantly different at any time after 5 minutes. A similar comparison for the entrapped drug showed that the radioactivity values in S were always (0 - 48 hr) significantly lower than the controls. Comparisons of means between the preparations were also carried out and showed that, apart from 5 minutes, the free drug was always significantly higher than the entrapped.

The free radioactivity in S showed a rapid increase during the first hour post-injection to reach a peak of 53.7 %. This level was maintained for the rest of the time studied (48 hr; 32.2 % not significantly different from 1 hour value). The results are probably due to the uptake of free ^{125}I-ASPase into the cytoplasmic compartment.

A comparison of means between the total radioactivity and the TCA pptable radioactivity due to the free drug in S revealed that they were not significantly different except at 5 minutes post-injection. This results suggests that either degradation of the ASPase was minimal or that excretion of the ^{125}I radiolabel from the liver was rapid.

The entrapped drug also accumulated in the S fraction but the amount of whole ASPase (i.e. : TCA pptable radioactivity) was significantly lower ($p < 0.01$) than the total radioactivity between 30 minutes and 5 hours. In view of the apparent lack of breakdown of free ASPase in S, this result suggests that ^{125}I radiolabel removed from the labelled entrapped drug may have been transfered to S from other sites (eg. ML). The amount of liposomal ASPase in S reached a peak at 5 hours.

Section 4:5 DISCUSSION OF SUBCELLULAR DISTRIBUTION RESULTS.

i) Subcellular fractions.

In the results section it has been implied that the fractions are pure and contain only the organelles mentioned but this is not the case. An analysis of subcellular components in rat liver and their localization in the crude fractionation performed in this study is beyond the scope of this thesis. The following brief résumé was taken from the work of de Duve and his co-workers and is simply meant to illustrate (non-exhustively) that these fractions contain may more structures which might also account for the subcellular localization of drugs (de Duve et al., 1955; Beaufay et al., 1964; Baudhuin, 1974; Beaufay and Amar-Costesec, 1976; Wibo et al, 1981).

Nuclear fraction.

In addition to nuclei, enzyme markers (table 4:9) confirm that this fraction can become contaminated with both lysosomes and mitochondria. Since this fraction is the first "cut" from the homogenate it will also contain whole (unbroken) or partially broken cells and all the cell debris e.g.: large fragments of cytoskeletal components as well as any larger material which has a tendency to aggregate.

Mitochondrial lysosomal fraction (ML).

Although these two organelles are mostly found in this fraction, enzyme latency experiments confirm some overlap with N and P. Mitochondria account most of the protein content of the ML but this fraction contains about equal proportions (70 % of each) of the total liver content of : - mitochondria, lysosomes and peroxisomes. These latter organelles are derived primarily from the PCMC in the liver.

Microsomal fraction (P).

This fraction is the most complex in terms of the number of cell components which localize in it. As well as some lysosomes the fraction contains : - Mitochondrial membranes, plasma membranes, phagolysosomes, endocytic vacuoles, perhaps nuclear membranes, the Golgi complex and associated membranes and the rough and smooth parts of the endoplasmic reticulum, which will include the drug-metabolising cytochrome P-450 enzymes, as well as ribosomes and RNA. This heterogeneic mixture, containing almost all the membrane components, has proved to be difficult to resolve into pure fractions because of the difficulty in finding marker enymes to uniquely define each component.

Soluble fraction (S).

This fraction should not contain any cell organelles but, in addition to its normal enzyme constituents, it will contain enzymes and other molecules released from fractured particles. So, for example, broken nuclei or lysosomes which contained radiolabelled drug might be expected to contribute to S. Other molecules stored in or released from subcellular structures will also be found in S e.g. : insoluble fats and fatty acids, in which lipophilic drugs may dissolve either before or during fractionation, can be located floating on the surface of S. However, lipophilic drugs released either during fractionation or which are in the process of uptake by the cell could equally well be found in the lipid of membrane components in P.

ii) Free drugs.

Actinomycin-D.

The subcellular distribution of free ACT-D in the liver has been studied by Dingman and Sporn (1965) and Weissbach et al. (1966) amongst others. The results of these studies indicate that the free drug specifically localized in the nucleus. However, since the drug will bind to isolated nuclei and DNA (Refs above and Schwartz et al., 1968a, b) it is unclear if this is due to true subcellular localization or to artifactual redistribution during fractionation. Typically 85 % - 90 % of I/V administered drug binds to nuclear DNA in rat liver at 30 minutes post-injection, a further 3 % binds to the nucleoli (Ro, 1967). However, Dingman and Sporn (1965) reported that 70 minutes after injection only 55 % of the injected dose was in N with a further 10 % in ML, 3 % in P and 25 % in S. In general, uptake was independent of the amount of ACT-D injected. The ACT-D associated with P was not bound and could be dialysed out of the fraction; similar treatment of the S fraction revealed that a third of the ACT-D had interacted with a non-dialysable component (DNA from lysed nuclei ?). These authors suggested that the ACT-D in ML was due to contamination. The high level found in S at 5 minutes can be accounted for by drug in the cytoplasm en route for the nucleus. Other workers (Kessel, 1967) reported that ACT-D would also bind to lipid and since cell lipid floats on the surface of S after fractionation it is possible that this accounts for some of the S localization of the drug. Ro (1967) also reported that, at low doses of ACT-D, extra nuclear DNA was saturated before binding to the nucleus occured. This result might account for the small ACT-D levels in the

ML fraction. Finally, as pointed out above, the P fraction contains a variety of cellular materials in addition to the microbodies, and includes most of the membrane fragments; it is possible that lipid soluble ACT-D is associated with this fraction (but c.f. Dingman and Sporn (1965) above).

In agreement with the work of others, in this work the free ACT-D is obviously highly concentrated in the nuclei with, overall, decreasing amounts in S over time. The cationic charge on ACT-D in aqueous solution will probably preclude its permeability through membranes. However, since ACT-D is also lipid soluble it seems probable that a substantial amount of the drug will be in the uncharged form which would allow it to penetrate membranes (Goldman, 1976). Once inside the drug could become trapped by protonation (see below). It seems reasonable, therefore, to suppose that free ACT-D could easily become lysosomally localized; perhaps competition from the nucleus prevents this. Binding to the nucleus is apparently complete at 5 minutes and does not increase with increasing liver uptake or time. These data suggest that the nuclear fraction is artificially high and probably reflects ACT-D redistribution during homogenization and fractionation : it is unclear if the low ML levels are the result of active exclusion from lysosomes. A further mechanism of free drug entry comes from the work of Kaye and Ryman (1980) who found free ^{3}H-ACT-D bound to albumin *in vivo*; it is possible that the drug/ albumin complex could be taken in whole by pinocytosis or into vacuoles prior to subsequent lysosomal localization. Alternatively the drug-protein may bind to membranes and contribute to the P fraction.

Bleomycins.

The subcellular localization of free BLM has not, to this authors knowledge, been previously studied.

The lysosomal capture of free BLMs is not an unexpected finding; other antibiotics containing aminosugars (e.g. : Steptomycin) are known to be lysosomotrophic (Tulkens *et al.*, 1975) which may account for their ineffectiveness against intracellular infections. Iodinated BLMs had an increases localization in the ML fraction suggesting that there is some lysosomal uptake of the free drug although this was not so marked as the localization in S. From theoretical considerations (de Duve *et al.*, 1974), the lysosomal capture of free BLM can be predicted

since at physiological pH the drug is weakly basic. The non-protonized form (i.e. lipid soluble) of the drug could permeate plasma and other membranes, however, in vivo the terminal groups probably prevent the drug from existing in an uncharged form. In the acid milieu of the lysosomes BLM could be trapped by protonation and so no longer escape. As de Duve et al. (1974) have pointed out, drugs with pK s near to 8 are most likely to be so concentrated, however, as the pK increases the rate of lysosomal uptake and trapping will be reduced by a factor of ten for every unit increase of pK; bases with pK s below 8 never achieve a maximum lysosomal concentration ratio with respect to S. Conversely, weak acids are kept out or driven out of lysosomes with the maximum effects being with compounds of pK's around 6.

The effects of this proton trapping in lysosomes on free BLM are uncertain. The bulk of the drug, which contains the chelated cations (e.g. $^{111}In^{3+}$), will probably be effectively uncharged in the cytosol at pH 7.0 but the ionized guanido groups will still prevent its free permeability through membranes. However, as discussed previously (chapter 2), lowering the pH removes the chelate so that in the lysosomes both drug and radiolabel will be trapped but the chelate might later escape. The enzyme inactivation of BLM in the cytosol occurs at the amine group on the beta-aminoalamine moiety changing its apparent pKa from 7.3 to 9.4 (Umezawa, 1974) but this same group has been proposed as one of the sites where chelates are bound (fig. 2:1b). It is uncertain if the presence of ^{111}In at this site will inhibit the action of the enzyme but released $^{111}In^{3+}$ would bind to any anionic structure. The ML localization of ^{111}In will, in addition, be complicated by the presence of ^{111}In labelled proteins from the plasma. The extent to which ^{111}In-labelled transferrin or other proteins contribute to the total uptake of both the free and entrapped drug is uncertain. Free $^{111}InCl_3$ at pH 6.5 is almost totally cleared from the plasma by mouse liver (Konikowski et al., 1975) but this preparation is reported to be a colloidal suspension. Non-colloidal $^{111}InCl_3$ (pH 1.5) was also reported to be cleared by the liver to give higher amounts than ^{111}In-BLM. It seems possible that this $^{111}InCl_3$ was protein bound and so may account for the ML localization of the radiolabel. Uptake of transferrin labelled with iron (^{59}Fe) has been demonstrated to be due to receptor mediated endocytosis in rat hepatocytes (Octave et al., 1982 and personal communication). However, since the rate of accumulation of labelled iron into lysosomes is 3 times higher than the accumulation of

labelled protein it has been proposed that transferrin remains bound to the receptosome and is recycled back to the plasma membrane whilst the ^{59}Fe is preferentially donated to lysosomes. A similar mechanism could be operating with ^{111}In-labelled transferrin.

Iodinated BLMs demonstrate much higher S and much lower ML localization than the ^{111}In-BLM. The ICL-BLM species has been reported to be labelled on the imadazole group (Eckelman et al., 1976) whilst the N-succinimidyl-3(4-hydroxyphenyl)proprionate ester is known to react with free amino groups (Bolton and Hunter, 1973) and alter the charge. The free drug ML localization does not appear to be affected by the method of iodination but in S, although the initial capture (first hour) was similar for both iodinated species, the persistance of ICL-BLM was higher than the B/H-BLM. Radiolabel in B/H-BLM may be attached to the beta-aminoalanine amino group and so removeable by enzyme action, alternatively the terminal amino groups, which might also be iodinated, could become more susceptible to attack by other enzymes. In any event, the fact that iodination by this method changes the immunoreactivity of some proteins (Bolton and Hunter, 1973) suggests that the two iodinated BLM drugs might be treated differently by cellular enzymes. Such a change in structure may effect the B/H-BLM since it appears to become localized in N to a greater extent than either ICL-BLM or ^{111}In-BLM. Interestingly, the terminal amine groups of BLM have been implicated in the binding of this drug to DNA (Kasai et al., 1978).

Microsomal (P) localization of free BLMs also appears to be determined by the method of radiolabelling, and this is further reinforced by the finding that the difference between the iodinated species disappears when they are entrapped in liposomes. Whilst there is no difference between free B/H-BLM and ^{111}In-BLM, the ICL labelled drug concentrates here to a greater extent. It seems possible that this is a genuine difference which cannot be explained by ML contamination of P because in the former fraction the two iodinated BLMs are not different. The cause of ICL-BLM localization in P is unclear but it may be due to an increased association with membranes.

Asparaginase.

Free ASPase from E. Carotovora is also weakly cationic at neutral pH although it is uncertain if, following radiolabelling and interactions with plasma and/or other proteins, this enzyme remains completely unchanged. Immunological and enzyme activity studies (Patterson,

1975) suggest that substantial changes to the protein do not occur following injection, however, Blazek and Benbough (1981) have recently demonstrated that even slight chemical modification of the amino groups of ASPase changes not only the enzyme activity but also the pI and thus the plasma clearance rate. Only the zwitterionic protein could be expected to freely permeate the cell membranes and it therefore seems unlikely that, with pI = 8.8 (Miller et al., 1971), ASPase can enter organelles or cells by permeation. Since the E. carotovora enzyme does not appear to contain carbohydrate groups it also appears unlikely, at present, that ASPase enters cells by methods other than pinocytosis. However, aggregated material, which was implicated in the initial rapid plasma clearance, may well be taken up by phagocytosis and so account for ML localization of the free drug at 5 minutes. Thereafter, the level of uptake by S is the predominant feature.

The levels of free ASPase in the N and ML fractions, which fall with time, probably reflect degradation of the protein since in the ML, at least, total radioactivity was higher than TCA radioactivity at 2 and 5 hours and this degradation was further reflected in the S level of non-precipitable ^{125}I at 5 hours. If the uptake of the free enzyme had been simply into the ML fraction free ^{125}I could have been expected in S throughout the period under study. The constant levels of precipitatable radioactivity in S indicate that little degradation of the enzyme has occured in this fraction. These results are consistent, therefore, with the hypothesis that the aggregated enzyme is cleared via endocytosis whereas the non-aggregated material enters by a mechanism which involves direct localization in the cytosol.

iii) Entrapped Drugs.

The subcellular localization of entrapped drugs was originally studied by Gregoriadis and Ryman (1972a and b). Liposomes containing entrapped amyloglucosidase or invertase were shown to be preferentially located in the ML fraction at time intervals up to 21 hrs post-injection. For amyloglucosidase the level of the ML-localized radioactivity fell from 62 % to 47 % of the whole liver level during a 30 minute period, while that of invertase remained constant (about 42 %) for 6 hours before falling to 34 % at 21 hours. Both enzymes had high initial levels of radioactivity in P which fell over time whilst the levels in S remained relatively constant.

Localization of entrapped invertase in the lysosomes of ML was

made more definitive by the use of density gradient centrifugation to separate lysosomes from mitochondria (Gregoriadis and Ryman, 1972b) in rats pretreated with Triton WR-1339. In addition, an ML sample from rats previously injected with ^{131}I-albumin-containing liposomes was found to release non TCA-pptable ^{131}I radioactivity on incubation *in vitro* suggesting that ML was capable of degrading the entrapped material.

Lysosomal localization of entrapped neuraminidase (Gregoriadis *et al.*, 1974b) and ^{111}In-BLM (Gregoriadis and Neerunjun, 1975b) has also been found. Moreover, Segal *et al.* (1976) found ^{111}In radioactivity distribution was very similar to a lysosomal enzyme marker following isopycnic centrifugation of a post-nuclear superatant from a liver biopsy of a patient 90 minutes after I/V injection of ^{111}In-BLM entrapped in liposomes.

Steger and Desnick (1977) also found liver lysosomal localization of anionic MLVs containing beta-glucuronidase into mice deficient in this enzyme. However, the same enzyme entrapped in cationic liposomes was equidistributed in both the ML and S fraction at early times post-injection (up to 1 hour). At later times whilst the level of enzyme in ML decreased the amount in S increased up to a plateau level (24-72 hrs) of about 70 % of the total hepatic activity, thereafter the level in S started to fall and this was parallelled by an increase again in ML and N. Moreover, the uptake of the enzyme into S was proportional to the amount of stearylamine incorporated into the liposomes. These cationic liposomes were also capable of increasing the release of some (but not all) lysosomal enzymes into the S fraction particularly between 1 and 3 days post-injection and this corresponded to the plateau level of the entrapped enzyme in ML. Since this is the only published study of the subcellular distribution of cationic liposomes it is impossible to assess if these differences are due solely to charge. No control was made of the size of the two populations which might account for some differences in subcellular distribution patterns, however, it is tempting to speculate that the increase in S location of the entrapped enzyme was due to the uptake of SUVs in the population perhaps into the cytosol of the PCMC via an intracellular fusion mechanism (see later).

Finally, Rahman (1980) has reported that ^{14}C-EDTA entrapped in large neutral MLVs was located mainly in the S fraction, although, as she points out, it need not necessarily have been taken up into this

compartment but may have escaped there from the lysosomes. Since this is the drug's location at all times between 1 and 24 hr post-injection it is difficult to equate these findings with all the other published work. It is unlikely that the uptake into S was due to free leaked drug since hepatic uptake of free EDTA was reported to be very low. It may be that these liposomes are too big to be instantly delivered to lysosomes of the KC and so the drug is released into S by a process of phagosome stasis followed by leakage through the membranes. Previous work by this group (Jonah et al., 1975) tends to support the work of Steger and Desnick (1977), mentioned above, since the former authors also reported longer liver EDTA retention times following cationic liposome administration although whether this is due to S fraction accumulation is unknown because fractionation studies were not done.

The present work is, therefore, in agreement with the majority of published data using anionic liposomes. All the entrapped drugs were concentrated into the ML fraction initially, moreover, all showed substantial and significantly higher levels in this fraction than the comparable free drugs and usually this increase was maintained for periods up to 1 or 2 days post-injection. Unfortunately, few of the published papers have compared the distribution of both the entrapped and the free compounds so it is difficult to assess if others would also have found high levels of the free drug in the ML. Gregoriadis et al. (1974b) found that uptake of free enzyme (neuraminidase) was undetectable in ML 30 minutes post-injection.

Section 4:6.

ACTIVITY OF DRUGS AGAINST RNA AND DNA SYNTHESIS IN THE REGENERATING LIVER.

Introduction.

In the previous section it has been shown that liposome-entrapped drugs reach higher levels in the liver than the corresponding free drug and that the major portion of the entrapped material can be found in the lysosomal fraction of this organ. It is possible that the lysosomes might be able to digest, or at least inactivate, the entrapped drug. The question must, therefore, be asked whether liposome-entrapped anti-tumour drugs retain their activity.

Many studies have reported that liposome entrapped agents are active, or exhibit enhanced activity when compared to the unentrapped drug, against tumours (Rahman et al., 1974; Gregoriadis and Neerunjun, 1975a; Mayhew et al., 1976; Neerunjun and Gregoriadis, 1976; Rutman et al., 1976; Kobayashi et al., 1977; Kosloski et al., 1978; Rustum et al., 1979). The mode of action of these entrapped drugs is not well understood. Several reports (see sections 4:1 and 4:3) have demonstrated that tumour tissue can preferentially take up liposomes, however, other workers have suggested that in vivo liposome-entrapped cytotoxic drugs act by slow release of the agent from a depot site (Kimelberg and Mayhew, 1978; Mayhew et al., 1978, 1979; Kotaoka and Kobayashi, 1978; Rustum et al., 1979; Kaye et al., 1981).

Since the liver is the major site of accumulation of the entrapped drug it was decided to test the activity of the agents against regenerating liver tissue following partial hepatectomy (66 % removal of the liver) in rats. To minimize the possible effects of leakage of entrapped agents in the circulation the drug was administered immediately after the operation and an assessment of RNA and DNA synthesis made at sometime later.

The regeneration of rodent liver is a well defined system which was subjected to much research in the 1960's (Bucher and Malt, 1971). The following non-exhaustive description summarizes the major morphological and biochemical events which occur during the first 24-48 hours after the operation.

Partial hepatectomy sets in motion an extraordinary active burst of cell proliferation which reaches its peak near the end of the first

day. The parenchymal cells (90-95 % of total hepatic cell volume) respond first, showing infiltration with lipid and loss of glycogen within a few hours. The whole cell, the nuclei, nucleoli and the smooth endoplasmic reticulum enlarge, lysosomes increase in number and some autophagocytosis can be seen. By 6 hours RNA synthesis has commenced and DNA synthesis is activated by 16-18 hours although it does not reach a peak until 4-6 hours later. Nucleic acid synthesis starts in the cells at the periphery of the surviving liver lobule and spreads towards the centre. Mitosis follows the same pattern 6-8 hours later, i.e.: about 32 hours post-hepatectomy.

The non-parenchymal cells (KC and EC) lag about 1 day behind the PCMC in initiating DNA synthesis and mitosis. It is uncertain if the KC multiply in situ or if they are replaced from the bone marrow.

Whatever the origin of the non-PCMC, it is clear that during the first 24 hours synthesis of nucleic acids is confined principally to the hepatocytes. If liposomes are only delivered to KC lysosomes, the entrapped agent must, therefore, not only escape lysosome digestion but also be capable of crossing membrane barriers into adjacent PCMC, and perhaps, of traversing PCMC to reach all parts of the lobule.

During regeneration, the normal liver tissue architecture is found. The restoration of normal liver weight is extre mly rapid; e.g.: the remaining liver weight doubles in 48 hours and increases a further 50 % in the next 24 hours. The surviving lobe of the liver enlarges and later other lobes arise from this. The restoration process is complete within 2 weeks.

Experiments in vitro on regenerating liver cells have demonstrated that RNA transcribed in the nucleus subsequently appears in the cytoplasm as t-RNA, r-RNA and m-RNA. The overall rate of RNA synthesis rises by about two-fold within 3-6 hours and this is associated with an increase in the number of ribosomes. The bulk of the RNA synthesised appears to remain in the nucleus where it has a very rapid turnover. Twelve hours after hepatectomy protein synthesis can be detected and this corresponds to the maximum rate of entry of m-RNA into the cytoplasm. Protein synthesis continues at elevated rates for a further 24 hours but it appears that some normal liver functions (e.g.: protein excretion) may be supressed in favour of enzymes participating in amino acid, nucleotide, DNA and RNA synthesis.

Replication of DNA may start as early as 12 hours post-hepatectomy but reaches a peak at 20-24 hours. At this time about 35 % of all the

cells are undergoing replication. The enzymes involved in DNA synthesis (e.g.: Ribonucleotide reductase, DNA polymerase) which are present in very small amounts in the normal liver, are increased many-fold during regeneration probably by de-novo synthesis. These elevated enzyme levels are maintained even after the peak of DNA synthesis has passed.

A) Results

i) Preparation of drugs.

Liposomes were prepared in the usual manner except that where necessary the quantity of drug entrapped was increased to enable an effective dose be given.

Actinomycin-D. For liposomal ATC-D, 8 mgs (25 uCi ^{3}H/mg) ACT-D was added to four times the standard quantity of lipids. The final preparation contained 26.4 ug ACT-D and 3 umol EPC per ml PBS. Immediately post-hepatectomy each animal received either 1 ml free ACT-D, 1 ml liposomal ACT-D or 1 ml 0.145 M NaCl by I/V injection. Some animals were sham operated (i.e. the peritoneal cavity was opened but the liver was not touched) and these also received the saline injection.

At timed intervals after injection the animals were killed but each animal received ^{14}C-orotic acid I/P exactly one hour before death. The concentration of ^{3}H-ACT-D (corrected for ^{14}C counts) in the plasma was measured (fig. 4 : 14) and the livers were subjected to sub-cellular fractionation. The concentration of ^{3}H-ACT-D was measured in the whole liver (fig. 4:15.) and in the subcellular fractions (fig.4:16).

Nucleic acids were extracted from the whole liver as outlined in chapter 2. The ^{14}C-orotate incorporation (per hour) (fig.4:17) was measured in hepatic RNA extracted from rats treated with saline, entrapped ACT-D or free ACT-D at timed intervals after injection. The total amount of RNA per gm liver is shown in table 4:11. The incorporation of ^{14}C-orotate into hepatic DNA is shown in table 4:12.

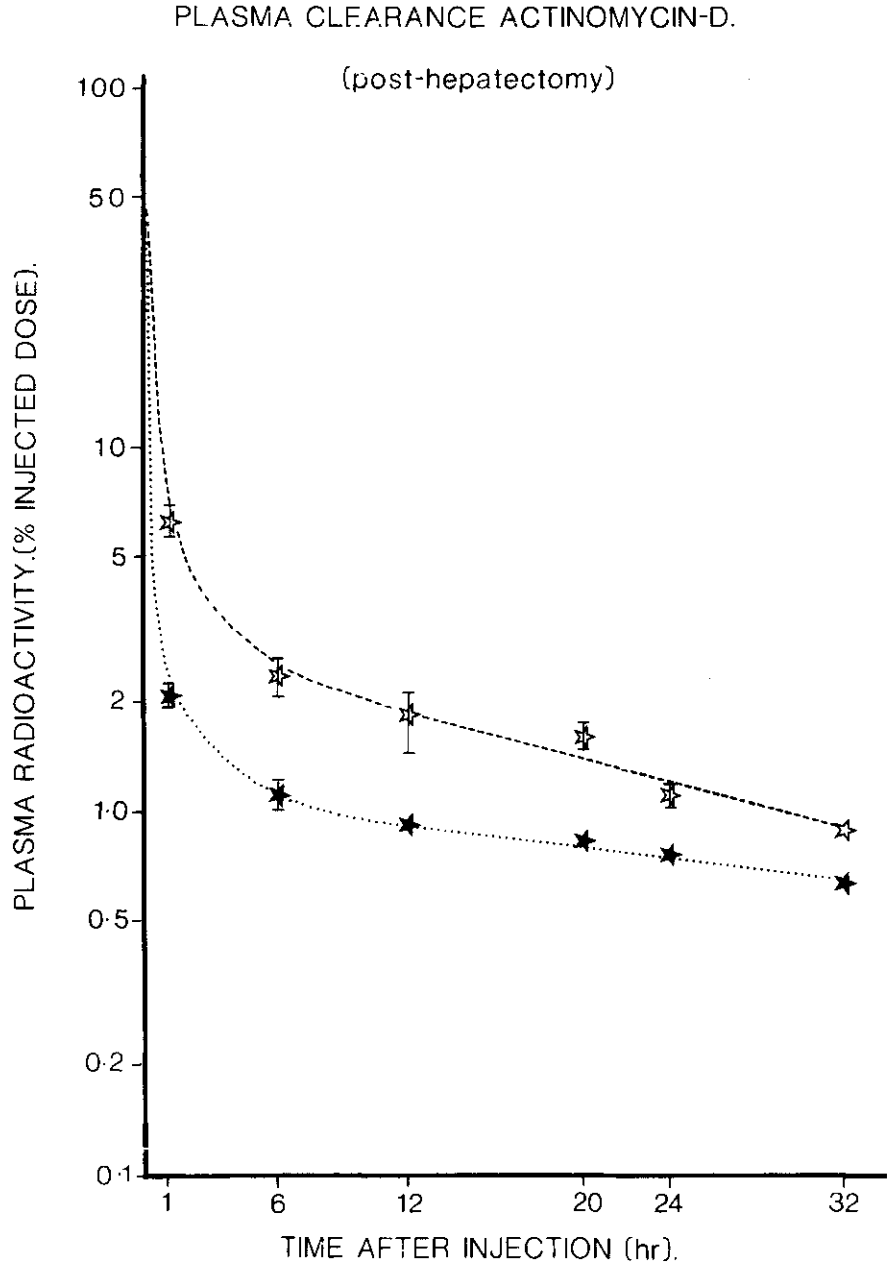
Bleomycin.

Bleomycin liposomes were made from four times the standard lipid quantities dispersed with 15 mg bleomycin sulphate dissolved in 2 ml PBS and containing a trace amount of ^{111}In-BLM to measure the entrapment. The final solution contained, typically, 0.71 mg

Figure 4:14.

Plasma clearance of ^{3}H-actinomycin-D in partially hepatectomised rats.

Rats (110-120 g body wt) were partially hepatectomised (67 % removal of the liver) and immediately injected I/V with free ACT-D (closed stars) or entrapped ACT-D (open stars) in anionic liposomes (7:2:1, EPC:CHOL: PA). The animals (3 to each time point (mean ± S.E.)) were killed at timed intervals post-injection and the amount (% injected dose) of radioactivity remaining in the plasma was measured. Doses : - free; 232 ug ACT-D/kg (10 uCi ^{3}H-ACT-D/mg), entrapped; 220 ug ACT-D and 18.75 mg EPC/kg (25 uCi ^{3}H-ACT-D/mg ACT-D).



BLM and 5.33 umol EPC/ml PBS. To achieve higher amounts of BLM some preparations were concentrated by centrifugation (100.000 g x 1 hour) to give concentrations of up to 3 mg BLM/ml; the amount of lipid in these solutions was not measured. Free BLM was made up at concentrations of 5 mg/ml bleomycin sulphate and a trace of ^{111}In-BLM in PBS; this was diluted in PBS to match the liposome preparation.

No previous experiments have been performed using BLM to inhibit DNA synthesis in partially hepatectomized rats so initial experiments were performed with free BLM to estimate the dose required for this effect. In addition, free BLM was given at different periods post-operatively to assess whether the timing of the injection was a critical factor. These results are summarized in table 4:13. The effect of entrapped BLM against DNA synthesis is shown in table 4:14.

Asparaginase.

Liposomes were prepared from 4 times the standard lipid mixture and dispersed with the 10 ml of ASPase solution (see methods) The final, post-column, preparation contained 270 units and 4 umols EPC/ml. This preparation was concentrated to 672.5 units/ml (0.516 uCi ^{125}I/ml) by centrigugation.

This liposome-ASPase preparation and free preparation (matched for radioactivity) was injected at concentrations of 300 or 600 units per animal (860 units/mg protein) to inhibit DNA synthesis in partially hepatectomized rats (table 4:15).

ii) Plasma clearance ACT-D.

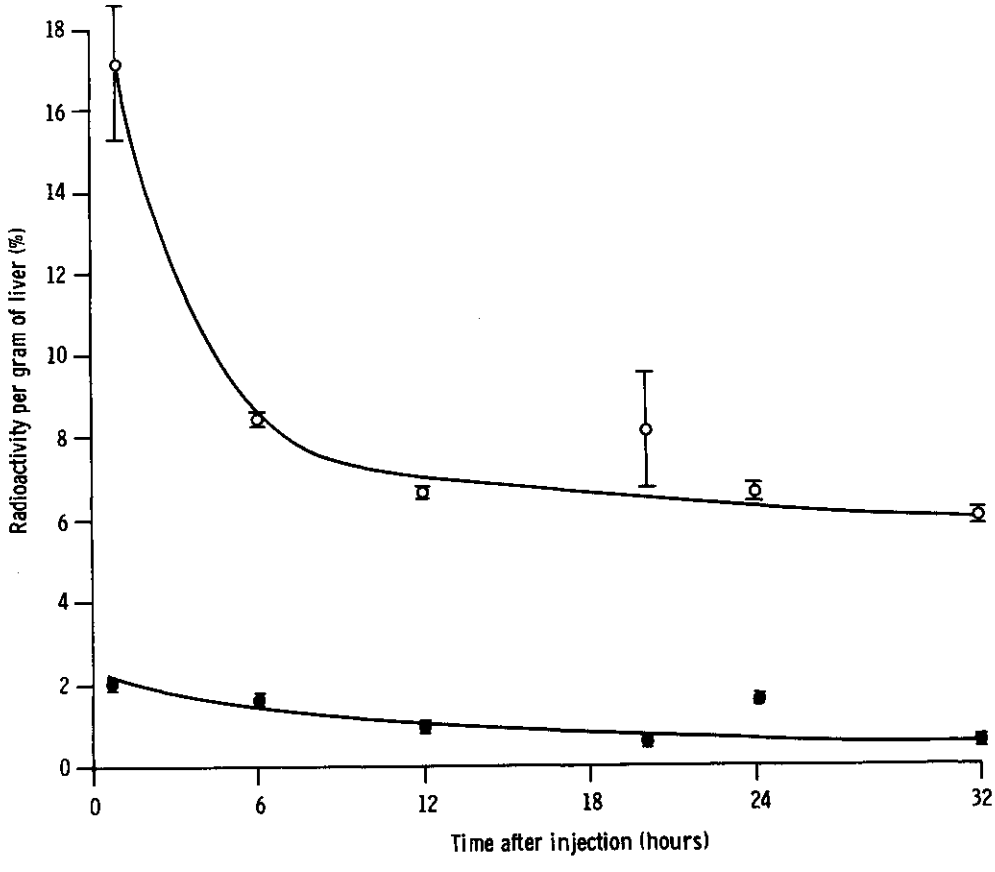
Figure 4:14 represents the plasma concentrations (% dose remaining in plasma) of free and entrapped ACT-D at timed intervals after injection; i.e.: timed intervals post-hepatectomy.

Despite the removal of about 67 % of the liver mass, the plasma clearance of the entrapped drug was very similar to that found previously in normal animals (fig. 3:1C) but significantly different ($p < 0.05$) from the free drug up to 32 hours post-injection. However, although the free drug plasma clearance was not initially (0 - 6 hours) significantly different from that of free drug found previously in intact animals (fig. 3:1E), the plasma

Figure 4:15.

Liver uptake of ^{3}H-actinomycin-D in partially hepatectomised rats.

Rats (110-120 g body wt) were partially hepatectomised and immediately injected I/V with free ACT-D (closed circles) or entrapped ACT-D (open circles) (details are the same as fig. 4:14). Animals were killed at timed intervals after injection. Each point represents the mean (± SE) of the % of the injected dose associated with each gram of liver from 3 animals. Remaining liver weights were 1.5 -2 g depending upon body weight.



concentration at later time intervals (12 - 32 hours) was very much higher in the hepatectomised animals. This latter result suggests that the operation interferes with the plasma clearance, perhaps by reducing the excretion of the drug.

iii) Uptake of free and entrapped ACT-D by the liver.

The hepatic uptake of ACT-D by the hepatectomised liver, is shown in fig. 4:15. A comparison of these results with table 4:8 (% uptake/gm normal liver) reveals that initially (0 - 6 hours) the amount of liposomes taken up by the partial liver was 2.5 - 3.0 times higher than in the total liver and at later times (12 - 32 hours) about 4 times higher. The amount of radioactivity due to the free drug in the hepatectomised liver was about 2- fold higher than in the normal liver at later times although initially these values were similar. The hepatic uptake of the entrapped drug was always significantly higher ($p < 0.001$) than the free drug.

iv) Subcellular fractionation of hepatectomised liver.

A 20 % homogenate of the remaining liver was made and this was separated into the component fractions as before. The results are shown in figure 4:16.

i) Nuclear fraction (N).

The quantities of both free and entrapped drug in N were not significantly different from each other at anytime post-injection. Levels at 24 and 32 hours (not shown) were not significantly different from the levels at 20 hours. The control levels (table 4:10) from other preparations suggest that the concentrations of liposome drug in N is about twice as high as might be expected from localization being due to sedimented liposomes.

Comparison of fig. 4:16 with fig. 4:9 (normal liver sub-cellular distribution) showed only small differences between the entrapped drug levels. At one hour the N level of entrapped drug in the partial liver was sightly higher than in the whole liver. At later times there was a slight increase in the entrapped drug levels as was found in the whole liver.

The free drug levels in N were lower in the partial livers at all times; levels were about a quarter of those found in the whole liver preparation. The reasons for this difference will be discussed later but the reduction may be due to the damage caused by the operation.

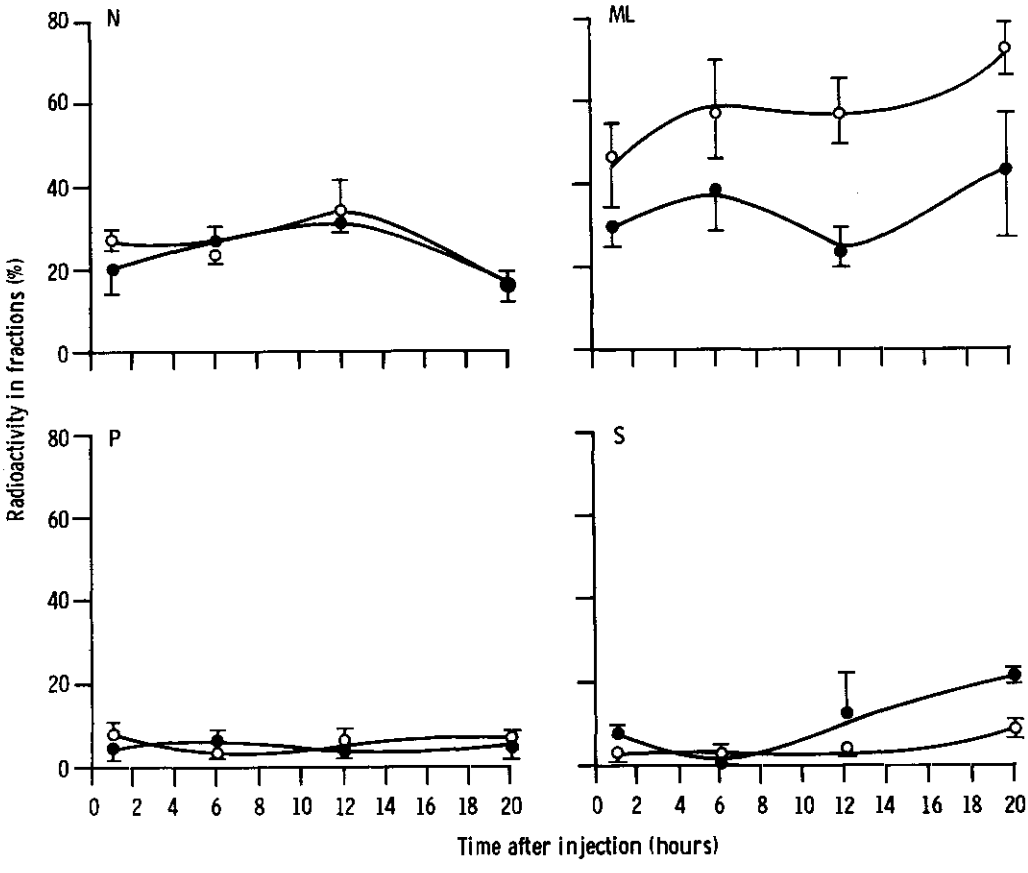
Figure 4:16.

Subcellular distribution of ^{3}H-actinomycin-D in partially hepatectomised rat livers.

The liver remnants from partially hepatectomised rats were subjected to differential centrifugation to separate their component fractions. The percentage of total liver ^{3}H radioactivity found in each of the four fractions at timed intervals (hours) post-injection is shown. Details may be found in figure 4:15. Each point represents the mean (± S.E.) from 3 animals. Closed circles; free ACT-D: open circles; entrapped ACT-D.

Radioactive recoveries (mean ± S.E.; n = 12) were : -

90.35 ± 7.05 % entrapped ACT-D and 73.58 ± 15.13 % free ACT-D.



Mitochondrial/lysosomal fraction (ML).

Both the free and the entrapped drug accumulated in ML (cf. ML fig. 4:9). A comparison between the free and entrapped levels revealed that the means were not significantly different at any time between 1 and 32 hours except at 12 hours ($p < 0.02$).

The entrapped drug attained a steady concentration of 57 % at 6 hours; despite apparent fluctuations in the curve, this value was not significantly different from the subsequent entrapped levels at 12, 20 or 24 hours.

The free drug reached a constant level at one hour of around 27 %. This level was maintained throughout the 32 hour period: the level of drug at one hour was not significantly different from any subsequent value. This result is obviously very different from the free drug levels in ML in the non-hepatectomised liver (fig. 4:9) and cannot be explained by a poor separation of N from ML since the marker enzymes were similar to those discussed previously.

Microsomal fraction (P).

Levels of both free and entrapped drug in P were not significantly different at any time up to 32 hours. The concentrations of both preparations were very similar to those of the controls (table 4:10) and suggests that neither localizes in this fraction.

Neither of the preparations were very different from those found in the whole liver (fig. 4:9).

Soluble fraction (S).

The levels of both preparations were low in S and lower than those in the unoperated liver (fig.4:9). At early time points (up to 12 hours) there was no significant difference between the means of the free and entrapped drugs but at 20, 24 and 32 hours the free drug slowly increased and was significantly higher than the entrapped.

Controls.

In general the marker enzyme distribution was found to be the same as discussed previously (tables 4:9A, 4:9B). The lysosomal enzyme content in ML was found to be at the upper limit of the range shown in table 4:9A (but not significantly different) in all the operated livers at more than 6 hours post-operation. The mitochondrial enzymes were not

increased. Lysosomal enzymes in P and S were reduced, again not significantly. Total recoveries of liver enzymes ranged between 83.5 and 104.2 % (free) and 82.9 and 112.3 % (entrapped).

The ^{3}H recovery of the free ACT-D was consistently lower than the entrapped drug (fig. 4:16). The reasons for this low value are unclear (it was not found with the enzyme recoveries) but it appears to be only associated with the free drug in the partially hepatectomised liver.

B) INHIBITION OF NUCLEIC ACID SYNTHESIS BY DRUGS.

i) Actinomycin-D.

The incorporation of 6-^{14}C-orotic acid per hour into RNA extracted from the liver remnant of partially hepatectomised rats is shown in fig. 4:17. The saline treated animals showed a peak of radioactive incorporation at 6 hours but thereafter the rate of incorporation fell. However as shown in table 4:11, the total amount of RNA present in the remaining liver did not fall after 6 hours but remained elevated (maximum at 12 hrs) throughout the following 24 hours. The drug treated animals exhibited reduced ^{14}C-orotate incorporation into RNA at all times (fig. 4:17) when compared to the saline treated animals ($p < 0.001$). However, there was no difference between the means of the free treated animals and those of the entrapped treated animals at anytime. The means of both drug preparations were very similar to the sham-operated controls (one animal per time point : not shown). The drug treated animals probably represent maximal inihibition of the RNA synthesis. In table 4:11 it may be seen that the total amount of RNA was significantly reduced ($p < 0.01 - 0.001$) in the drug treated animals when compared to the saline controls. Comparisons between the means of free and entrapped drug treated animals revealed that the liposomal drug reduced the amount of RNA more than the free drug at 6 and 32 hrs ($p < 0.02$) whereas at 20 hr these RNA levels were lower in the free drug treated animals ($p < 0.02$).

The incorporation of ^{14}C-orotate into DNA (table 4:12) demonstrates the low incorporation of this precursor even around the time of maximum DNA synthesis (24 hr). The low level of inhibition of precursor incorporation in animals treated with both drug preparations is consistent with the action of ACT-D at the level of transcription by the inhibition of DNA-dependant RNA polymerase. Both drug preparations produced a significantly lower incorporation rate than the saline controls at 24 hours.

Legend figure 4:17.

Incorporation of ^{14}C-orotic acid into RNA extracted from regenerating livers of rats treated with actinomycin-D.

The histogram shows the incorporation (dpm/mg RNA x 10^{-3}/hour) of ^{14}C-orotic acid into hepatic RNA at timed intervals (hours) after partial hepatectomy. Rats were injected I/V with 1 ml of either 0.145 M NaCl (black bars) or 220 ug/kg entrapped ACT-D (open bars) or 232 ug/kg free ACT-D (striped bars) immediately after partial hepatectomy. One hour before death each animal received 1.5 uCi 6-^{14}C-orotic acid in 0.5 ml saline by I/P injection. RNA was extracted from the liver homogenates by published methods. Each bar represents the mean radioactivity from 3 animals.

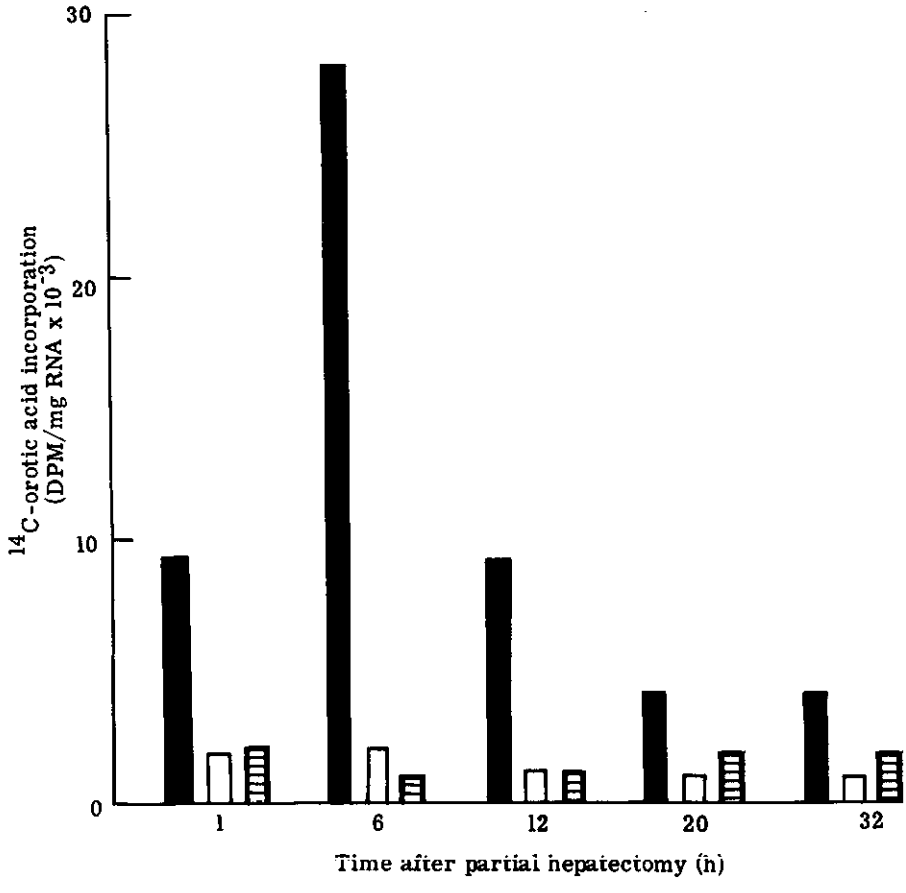


Table 4:11. Total RNA content in remaining liver at timed intervals after partial hepatectomy : effect of treatment with actinomycin-D.

Time post-injection (hrs)	TREATMENT		
	Saline	Entrapped ACT-D	Free ACT-D
	mg RNA/g liver (wet weight) (mean ± SE : n = 3)		
1	7.21 ± 0.25	4.61 ± 0.44	4.67 ± 0.17
6	7.38 ± 0.35	3.98 ± 0.26	5.72 ± 0.41
12	9.37 ± 0.78	4.77 ± 0.36	4.65 ± 0.28
20	8.08 ± 0.46	5.07 ± 0.17	3.79 ± 0.22
32	8.63 ± 0.35	3.93 ± 0.24	6.29 ± 0.48

RNA was extracted from the liver remnant of partially hepatectomised rats at timed intervals after treatment (I/V) with either saline, entrapped ACT-D (220 ug/kg) or free ACT-D (232 ug/kg).

Table 4:12.

Incorporation of 6-^{14}C-Orotic acid into DNA at timed intervals after partial hepatectomy : effect of treatment with actinomycin-D.

Time post-injection (hrs)	TREATMENT		
	Saline	Entrapped ACT-D	Free ACT-D
	Incorporation dpm/mg DNA/hr. (mean ± SE) n = 3		
1	154.61 ± 14.57	175.08 ± 41.41	199.75 ± 16.74
6	292.99 ± 47.41	108.85 ± 27.78	206.62 ± 30.63
12	118.54 ± 51.85	112.82 ± 58.12	149.17 ± 12.41
20	234.81 ± 37.14	143.11 ± 20.78	100.45 ± 11.47
24	415.94 ± 56.79	136.69 ± 16.67	166.78 ± 69.24
32	125.35 ± 17.57	225.33 ± 73.37	82.97 ± 8.70

The incorporation of ^{14}C-orotic acid into DNA extracted from the liver remnant of partially hepatectomised rats was measured at timed intervals following treatment (I/V) with either saline, entrapped ACT-D (220 ug/kg) or free ACT-D (232 ug/kg).

Table 4:13.

Effect of free bleomycin on the incorporation of ^{3}H-thymidine into DNA from regenerating livers.

A. DOSE RESPONSE

Dose (mg BLM/kg)	Incorporation dpm/mg DNA/hr ($x10^{-3}$) (mean ± S.E. : n = 3)	probability vs. saline
30	25.32 ± 2.73	< 0.02
10	33.36 ± 13.31	< 0.05
2	39.43 ± 5.70	< 0.05
0.4	56.81 ± 9.41	N/S
Saline control (n=7)	73.21 ± 9.82	-
Sham control (n=3)	14.68 ± 4.38	< 0.01

B. TIME RESPONSE (10 mg/kg BLM)

Time (hr) of injection before death	Incorporation dpm/mg DNA/hr ($x10^{-3}$) (mean ± S.E n = 3)	probability vs. saline
24	27.65 ± 2.69	< 0.02
12	34.26 ± 9.61	< 0.05
6	55.94 ± 12.13	N/S
2	75.63 ± 8.34	N/S

The incorporation (dpm/mg DNA/hour) of ^{3}H-thymidine into DNA extracted from the liver remnant of partially hepatectomised rats was measured 24 hours post-operatively. In A : - rats were injected immediately post-operatively with saline or various doses of free BLM and killed 24 hours later. In B : - rats were injected with 10 mg/kg free BLM at timed intervals before being killed 24 hours post-operatively. One hour before being killed all the animals received 0.5 ml saline containing 10 uCi 6-^{3}H-thymidine by I/P injection. Sham control animals were operated upon but no liver was excised.

ii) Bleomycin.

The dose response data (table 4:13A) for the free drug shows that the incorporation of ^{3}H-thymidine into DNA (24 hr post-operation) in the regenerating rat liver was inhibited by doses of 2-30 mg BLM/kg when compared to saline treated controls. A dose of 0.4 mg BLM/kg did not inhibit the incorporation of the precursor. The sham operated animals also incorporated ^{3}H-thymidine but this was significantly lower than the saline control level. Comparisons of means of 30 mg/kg and 10 mg/kg treated animals with the sham controls revealed no significant difference suggesting that the BLM-induced inhibition was close to the maximum.

When partially hepatectomised animals were treated with 10 mg/kg of free BLM at timed intervals before death (24 hr post-operation) (table 4:13B) the administration of the drug was found to be time dependent. Thus, injection 12 and 24 hours before death inhibited DNA synthesis but injection at 2 and 6 hours prior to death did not. These results may, therefore, mean either that modification of the BLM has to occur before inhibition takes place or that accumulation of BLM at its site of activity (presumably the nucleus) takes some time.

Using the data obtained above, liposome entrapped BLM was used in attempts to inhibit ^{3}H-thymidine incorporation into DNA. The results (table 4:14) show that, like the free drug, doses of 2 - 30 mg/kg of liposomal BLM inhibit DNA synthesis whereas a dose of 0.35 mg/kg did not. A dose of entrapped BLM (8.5 mg/kg) administered 6 hours before death failed to inhibit DNA synthesis.

A comparison of the means of similar BLM doses between table 4:13A and table 4:14 was made. At no dose were the means significantly different. Moreover, in the single group of animals treated with 8.5 mg/kg entrapped BLM 6 hours prior to death, the incorporation of ^{3}H into DNA was not significantly different from animals treated with 10 mg/kg free BLM at 6 hours prior to death. The saline control values were not significantly different.

These results suggest that liposome-entrapped BLM does not offer any chemotherapeutic advantage over the free drug in this system.

iii) Asparaginase.

The doses of free or entrapped ASPase used were similar to those already found by others to be effective in inhibiting DNA synthesis in

Table 4:14.

Effect of entrapped bleomycin on the incorporation of ^{3}H-thymidine into DNA from regenerating livers.

Dose mg BLM/kg	Incorporation dpm/mg DNA/hr ($x10^{-3}$) (mean ± SE) n=3	probability vs. saline
28.57	31.46 ± 7.49	< 0.01
8.33	33.21 ± 5.87	< 0.01
8.51 (6 hrs)	45.63 ± 11.22	N/S
1.76	41.75 ± 10.31	< 0.05
0.35	56.44 ± 15.017	N/S
Saline control (n=4)	76.491 ± 7.74	

The incorporation (dpm/mg DNA/hour) of ^{3}H-thymidine into DNA extracted from the liver remnant of partially hepatectomised rats was measured 24 hours post-operatively. Rats were treated immediately post-operatively with saline or BLM entrapped in anionic MLVs (7:2:1 EPC:CHOL:PA) (approximately 7.5 umol EPC per mg BLM) at the doses shown except for one group of animals which received 8.5 mg/kg BLM 6 hours before death. All the animals also received 10 uCi ^{3}H-thymidine in 0.5 ml saline by I/P injection one hour before being killed.

regenerating rat liver.

Table 4:15 shows the effects of two doses of either free or entrapped ASPase on the incorporation of ^{3}H-thymidine into DNA. The drugs were administered at the time of hepatectomy. Both preparations were effective in inhibiting DNA synthesis : there was no significant difference between the effects of any of the doses of ASPase probably because of the high standard deviations. The two preparations appear, therefore, to be equipotent and in this system there does not appear to be any advantage for either the free or the entrapped preparation.

In a single experiment (not shown) liposome entrapped ASPase (600 units/animal) was administered to two animals 12 hours after the operation. No inhibition of the incorporation of ^{3}H-thymidine into DNA from these liver remnants was found.

iv) Controls.

Preliminary experiments (not shown) confirmed that in the regenerating livers of untreated rats the incorporation of radioactive precursors in nucleic acids was time dependent. For ^{14}C-orotic acid, maximum RNA incorporation occurred at 6 hours post-operation ($6.6 \pm 1.42 \times 10^{4}$ dpm/mg RNA/hr (mean $\pm$ st.dev.: n = 3)) whereas the maximum for DNA occurred at 24 hours and was several orders of magnitute lower (415.9 ± 56.8 dpm/mg DNA/hr). For ^{3}H-thymidine, incorporation into RNA was low at both 6 hours and 24 hours post-operation (6 hr; $0.43 \pm 0.28 \times 10^{3}$ dpm/mg RNA/hr : 24 hr; $6.13 \pm 2.54 \times 10^{3}$ dpm/mg RNA/hr) when compared to that of DNA (6 hr; $8.44 \pm 1.53 \times 10^{3}$ dpm/mg DNA/hr : 24 hr; $78.31 \pm 8.95 \times 10^{3}$ dpm/mg DNA/hr). In much of the published work (e.g. Schwartz et al., 1965 and Becker et al., 1970) the incorporation of ^{14}C-orotate or ^{3}H-thymidine was expressed as radioactivity per nucleus or per cell so that comparison between these values and those obtained here for the inhibition of RNA or DNA synthesis cannot be directly made. However, for the control values, i.e. : saline and sham operations, experiments on the incorporation of dpm per mg DNA (or RNA) have been performed by others (Fujioka et al., 1963; Miyamoto et al., 1973). The levels of incorporation of the precursors (per mg nucleic acid) found in this study are very similar to those found by these authors. The quantities of total RNA and DNA (not shown) found in unoperated rats and in sham operated controls were similar to published normal values (1.92 mg DNA and 5.88 mg RNA/g liver wet weight : Altman and Dittmer, 1964).

Table 4:15.

Effect of free and entrapped asparaginase on the incorporation of ^{3}H-thymidine into DNA from regenerating livers.

Dose ASPase units/kg	Incorporation dpm/mg DNA/hr ($x10^{-3}$) (mean ± SE) n=3	probability vs. saline
Free		
5350	20.07 ± 7.53	< 0.005
2803	29.77 ± 9.42	< 0.02
Entrapped.		
5825	13.91 ± 2.62	< 0.002
2725	31.51 ± 12.93	< 0.05
Control.		
Saline (n=4)	76.49 ± 7.74	

The incorporation (dpm/mg DNA/hour) of ^{3}H-thymidine into DNA extracted from the liver remnant of partially hepatectomised rats was measured. Rats were treated immediately post operatively with either saline (control), or free ASPase (860 units/mg protein : 0.66 uCi ^{125}I/mg) or ASPase entrapped in anionic MLVs (7:2:1, EPC:CHOL:PA) (approximately 67 units/umol EPC : 860 units/mg protein : 0.66 uCi ^{125}I/mg protein) and killed 24 hours later. One hour before death each animal received 10 uCi ^{3}H-thymidine in 0.5 ml saline by I/P injection.

C) DISCUSSION.

i) Actinomycin-D.

a) Plasma Clearance.

Despite partial hepatectomy of the animals, the plasma clearance of liposomal ACT-D (fig. 4:14) was similar to that in whole animals (fig. 3:1c). However, as has been previously discussed (chapter 3), the plasma clearance of liposomes can be very variable depending upon their size and lipid content. The liposomal ACT-D used in these experiments was not injected into non-operated animals so that it is not possible to conclude that partial hepatectomy did not change the plasma clearance of liposomes.

If future experiments show that there is, genuinely, no effect on plasma clearance following partial hepatectomy it may be because of a large residual capacity of the liver Küpffer cells. It will be recalled that a blockade of murine liver uptake by high lipid doses of MLVs (500 mg/kg) could not be detected (Souhami et al., 1981). Nevertheless, Saba (1970) using doses of 500 mg/kg of a lipid emulsion, showed that in hepatectomized rats the plasma $\overline{T}_{1/2}$ was increased 3-fold. Although such differences can be explained by species variations (Saba, 1970), it is clear that, at the lipid doses used in this study, removal of 67 % of the liver would probably not result in blockade of the remaining KC even when the volume and velocity of liver blood flow is so drastically changed.

The plasma clearance of free ACT-D was altered by partial hepatectomy. Again no comparison of the dose used here was made in non-operated rats but these differences are probably not due to the dose since no difference was found between doses of 10 ug/ rat and 25 ug/rat in normal animals (fig. 3:1 E); moreover, the latter dose is very similar to that used in these partial-hepatectomy experiments (232 ug/kg). The plateau of free ACT-D in the plasma of hepatectomised animals is, therefore, probably due to saturation or alteration of normal mechanism of excretion of the drug. In this respect these data are consistant with the findings of Schwartz et al (1968a) that the major part of ACT-D is excreted in the bile. A reduction of biliary output following 67 % removal of the liver can be anticipated whilst the uptake per gm liver (see following section) is not changed to compensate for the loss of tissue.

b) Hepatic Uptake.

The capture of liposomes by the liver was increased 2.5 - 4 fold (% dose/gm) following hepatectomy (c.f. table 4:8 and fig. 4:15). Taken with the results of the plasma clearance (fig. 4:14) these data suggest a spare capacity in the uptake mechanism for liposomes but not for the free drug. These results are suggestive of different modes of uptake for the two preparations, i.e. : KC for liposomes and PCMC for free drug.

The uptake of liposomal ACT-D confirms the data of Saba (1970) that, even at high lipid doses, the capture (per gm liver) is increased following this operation. This author also showed that lipid emulsion uptake by other organs of the RES (lungs and spleen) was increased following hepatectomy which probably contributes to the maintainance of the plasma clearance curve of liposomes at levels similar to non-operated animals.

c) Subcellular Distribution.

The major differences in the sub-cellular localization between the whole and the partially-removed liver concern the distribution of free ACT-D in the N and ML fractions. It is tempting to speculate that these changes are associated with the higher plasma concentration and also perhaps with the lack of bilary excretion. In addition, the entrapped drug appears to concentrate to a lower extent in P and S than in the unoperated liver and this was associated with less lysosomal enzyme localization in these fractions.

Given the well known binding of free ACT-D to nuclei (Dingman and Sporn, 1965; Ro, 1967) discussed previously, it is apparent that the free ACT-D within the regenerating liver is handled differently from that in the unoperated liver. The reasons for this may due to : i) alterations in the nucleus which prevents binding ii) lysosomal alterations which retard ACT-D escape.

The morphological changes which occur in the nucleus within hours of partial hepatectomy include enlargement of the nucleus and especially in the nuclear volume (Bucher and Malt, 1971) and presumably the unwinding of DNA and the induction of enzymes preparatory to RNA synthesis. However, from figure 4:17 it is clear that ACT-D inhibits RNA synthesis as soon as one hour post-operation so that these changes may not affect the binding of the

drug. Studies on ACT-D binding to DNA (Sobell, 1974) have shown that the drug binds to double-helical DNA and that the interaction of ACT-D between one base pair promotes interaction in the opposite strand. If it is assumed that in the normal liver the DNA is in its normal "supercoiled" structure then ACT-D may bind to a greater extent than in the regenerating liver where the DNA is undergoing re-organisation.

On the other hand, as has already been discussed, the very high levels of free ACT-D in N (fig. 4:9) could have been artificially produced during homogenization and fractionation. This may mean that the levels of free ACT-D in fig. 4:16 represent the "normal" levels of ACT-D binding and the increase concentration above this level in the whole liver are due to the release of free ACT-D during the preparation. One possible source of this contamination may be the common bile duct . failure to remove this structure prior to homogenization of the unoperated liver might release its contents into the homogenate. Partial hepatectomy, which probably excises or fractures the bile duct , would remove this contaminating source.

Even if the bile duct remains intact the excretion of ACT-D into the bile is likely to be delayed or arrested following partial hepatectomy. In addition to the effect on the plasma clearance, this delay may cause an intracellular redistribution of the free drug in an attempt to "store" the ACT-D prior to the resumption of normal excretion. It seems possible that this storage occurs in the lysosomes which therefore trap more ACT-D than in the normal liver. Against this latter hypothesis are the data in fig. 4:15 which do not show an increased uptake of the free drug when compared to the unoperated liver. In addition, the binding of ACT-D to N in the whole liver may possibly represent a saturation level; it is difficult to see why saturation of N in the partial liver does not occur before storage of the excess drug in lysosomes unless the nuclear rearrangement prevents saturation. The increase in the S fraction at 20 hrs, and thereafter, may be due to either the release of ACT-D from an intracellular site (although both N and ML remain constant at these times), or additional capture from extracellular sites (although the plasma levels also remain constant).

Liposome-entrapped ACT-D appears to have lowers levels in P and S in the partial liver than in the whole liver. Since these lower levels may also be associated with lower lysosomal enzyme content of these fractions it is possible that these results reflect increased phagocytosis. If the drug concentration in P is due, in part, to liposomes in unfused phagocytic vacuoles, the lower levels could be due to increased vacuole/lysosome fusion either because of an increase in the number of lysosomes or because the activity of the lysosomes is increased (Bucher and Malt, 1971) in response to injury. These effects may also contribute to the lysosomal accumulation of free ACT-D. In addition, the lower levels of entrapped ACT-D in S may be a reflection of intracellular fusion because of a lower number of phagocytic vacuoles awaiting lysosome processing. Alternatively, the escape of both free and entrapped drug from the lysosomes may be retarded because of alterations to the lysosomal (and perhaps other) membranes in response to the operation.

d) RNA Synthesis.

Despite the fact that a 3-4 fold higher concentration of liposomal ACT-D was found in the liver, there was no apparent increase in the entrapped drug's ability to inhibit the incorporation of ^{14}C-orotic acid into RNA. In addition, the same percentage of the total liver radioactivity of both preparations was present in N. These data and those of the sham-operated contols suggest that, at the doses used, inhibition of RNA synthesis was maximal. A dose response curve (not done for ACT-D) might demonstrate if the liver drug concentration is proportional to the inhibition of RNA synthesis and whether there is any therapeutic advantage for the liposome preparation. However, these results do demonstrate that liposome entrapped drugs are active in vivo in a cellular compartment removed from the lysosomes. The central question, which cannot be answered by these experiments, is whether liposomes are being transfered from Küpffer cells (KC) to hepatocytes (PCMC) or whether the activity of liposomal ACT-D against RNA synthesis is due to leaked ACT-D in the plasma or leaked ACT-D in the KC entering the PCMC or, finally due to the uptake of SUVs into the PCMC.

Schwartz et al. (1965, 1968a) have shown inhibition by free

ACT-D of ^{14}C-orotate incorporation into rat partial liver RNA at doses of 1 mg/kg although the I/V LD_{50} in intact rats is 0.60 mg/kg. These authors also reported that inhibition of RNA synthesis was reversible i.e. : incorporation of orotate resumed after a 16 hour delay induced by the drug. This result was not confirmed in these studies where both the free and the entrapped drug supressed RNA synthesis for at least 32 hours post-operation. The reasons for this difference are not clear.

Papahadjopoulos et al (1976) reported that liposome-entrapped ACT-D could overcome the resistance of cells to free ACT-D, probably by modifying the mode of entry of the drug. But inhibition of RNA synthesis was not different from that produced by an equivalent dose of free drug in the parent cell line (non ACT-D resistant) : i.e. : there was no therapeutic advantage for the liposome preparation at the level of nucleic acid synthesis.

Inhibition of liver DNA synthesis (as measured by ^{3}H-thymidine incorporation) was found to be sensitive to ACT-D (Schwartz et al., 1965) but only at dose levels of between 5 and 10 fold higher than the LD_{50} of the drug. Inhibition of protein synthesis was also found to be partially effected by these ACT-D doses. The data from this present study failed to show any differential effect of the liposome drug on DNA synthesis except at 24 hours.

In studies on liposomal ACT-D toxicity, Rahman et al. (1978) showed that ^{3}H-thymidine incorporation into DNA in proliferating intestinal cells was inhibited four-fold less by the entrapped drug despite the fact that the drug uptake into the intestine was similar for both preparations. These data suggest that the two preparations are handled differently intracellularly. Yet, Schwartz et al. (1968a) have shown that intestinal synthesis of DNA is only inhibited at much higher doses of free ACT-D than used by Rahman et al.; again it is unclear why these differences exist.

ii) Bleomycin.

Disappointingly the entrapped BLM was no more effective than the free drug in its action on DNA synthesis in spite of a much higher liver capture, (at least in the intact liver). These results suggest that only a small proportion of the intracellular entrapped BLM is active. Several reasons can be advanced for this finding : 1) entrapped BLM

accumulated in the lysosomes may not be able to escape from this organelle, ii) entrapped BLM in the cytoplasm will be inactivated but this should be the case for free BLM as well, iii) co-factors involved in BLM action may only react with free BLM, iv) the cell type involved in drug accumumation in the liver may be different. This last point also applies to other entrapped agents and will be discussed later.

In the previous section lysosomal accumulation and degradation of BLM has already been mentioned. In addition, some of the factors discussed in relation to ACT-D may also be applicable to BLM eg. : increased phagocytosis and perhaps a decreased premeability of membranes in the damaged liver.

One area of note in these BLM results is the large variability (eg. standard errors of means) in the results. Such variability has been found by others working on the binding of BLM to DNA (reviewed Pietsch, 1975); it is probably dependent on the stage of cell cycle during which BLM will bind to DNA. Although BLM acts to arrest the cycle in both G_2 and mitosis (Hill,1978) its ability to bind to DNA alters during the cell cycle so that if the receptive binding period is missed no increase in dose can bring about the inhibition of DNA synthesis. Such variability has been shown in animal cells in culture and in tumours *in vivo* (Pietsch, 1975) and is most notably seen in bacterial cultures which continue to multiply in medium which contains a thousand-fold excess of antibiotic above the dose that inhibited growth during the receptive period (Pietsch and Clapper, 1969). These data probably explain the time effects seen with free BLM where only the doses given within 12 hours post-hepatectomy had any effect on precursor incorporation into DNA.

More recent studies on the binding to, and action of, BLM at the level of DNA have been made by Horwitz and co-workers. They have shown that the bithiazole rings and the terminal dimethyl sulphonium groups (fig. 2:1B) participate in the binding of BLM to DNA (Chien *et al*, 1977). They have further shown that Fe^{2+} forms an integral part of this complex, binding BLM in a 1:1 ratio (Sausville *et al*., 1978a). Early work established that BLM was able to produce breaks in single stranded DNA (Suzuki *et al*., 1968), a process which was enhanced by the presence of sulphydryl compounds and hydrogen perioxide (Nagai *et al*., 1969). Sausville *et al*. (1978b) have shown that *in vitro* Fe^{2+} plays an important role in the DNA breakage since substitution of other metals or the addition of the iron chelator, desferioxamine, leads to loss of the

strand-breaking ability. These authors have proposed that oxidation of BLM-bound Fe^{2+} to Fe^{3+} produces free radicals which attack the DNA; such a hypothesis is consistent with the findings of Haidle (1971) that molecular oxygen is needed for BLM activity and that DNA strand-breakage is inhibited by superoxide dismutase. However, the addition of ionizing radiation to BLM treatment, which should enhance the effects of free radical production, has proved to be inconsistent (Byefield et al., 1976).

The binding data outlined above raise an important question in terms of the inhibition of DNA synthesis by entrapped BLM; if iron is needed for BLM action, will liposome entrapped BLM be able to acquire the metal ? Thakur et al. (1973) have shown that the indium in BLM can be displaced by iron from transferrin in vivo and it is, presumably, this chelated iron which generates the free radical species for breaking the DNA strands. However, it seems probable that truly entrapped BLM cannot remove chelated iron from transferrin through the liposome membrane; it may therefore be inactive intracellularly unless it is able to pick up iron after its escape from the liposome and prior to its binding to DNA. If this proves to be correct, liposome entrapped BLM chelated to both radiolabelled iron and Fe^{2+} might prove to be superior to free BLM in subsequent experiments in partially hepatectomised animals.

In the present experiments, it may be that only the BLM on the external surface of the liposome or the BLM which has escaped/leaked from the liposomes in the plasma or within the cell is able to inhibit DNA synthesis. However, the dose response curves for both free and entrapped BLM are similar so that perhaps all the liposomal BLM is available in the PCMC or else only a small proportion of the free BLM (ie: that which escapes cytoplasmic inactivation) reaches the nucleus in an active form.

iii) Asparaginase.

Becker et al. (1970) have investigated the effects of ASPase on DNA synthesis in regenerating rat liver. A dose of 600 units/animal (as used in this study) was effective in delaying DNA synthesis (^{3}H-thymidine incorporation into nucleii). In contrast, other dividing cells (intestinal crypt cells, lymphoid tissue, renal tubular cells, skin basal epidermal cells and salivary cells) were not affected by this dose of free ASPase. Moreover, these authors also reported that the

enzyme had to be administered soon after the operation (0-6 hours) since an increase in asparagine levels, due to enhanced asparagine synthetase activity (Patterson and Orr, 1969), occurs about 12 hours post-operation and nullifies the later therapeutic effects of ASPase. Liposome entrapment of ASPase did not overcome this effect (experiment not shown).

As in the previous experiments with ACT-D and BLM, there was no therapeutic advantage in using ASPase entrapped in liposomes : many of the general points made previously probably also apply to this lack of enhanced liposomal-ASPase activity. In addition, the poor liver uptake of entrapped ASPase probably contributes to this lack of enhancement especially if the hepatectomised liver behaves in a similar manner to the whole organ and the drug leaks from the liposomes.

iv) General Discussion.

The lack of any increased effect against nucleic acid synthesis using liposome entrapped drugs when compared to free drugs has already be discussed in respect to the properties of the individual agents. In general, liposomes are taken up by the KC of the liver and there is no reason to suppose that this will be markedly changed in the undamaged lobe of the liver which remains post-hepatectomy. Yet, it is principally the PCMC which undergo nucleic acid synthesis during the time period considered in these experiments (Grisham, 1962; Bucher and Malt, 1971). It is, therefore, perhaps not surprising that only a small amount of the liposomal drug appears to be active. Entrapment of drugs in SUVs, which would probably enhance the PCMC uptake, might increase their pharmacological activity in the hepatectomised liver when compared to larger liposomes or the free drug; such an experiment would be relatively easy to perform. Indeed, using high enough doses of precusors, it is possible to study nucleic acid synthesis in the unoperated liver (Bucher and Malt, 1971) and such an experiment might help solve the question of the amount of the injected dose of liposomes accumulated by each cell type providing that the liposomes carried an appropriate drug.

Such experiments assume that there is no transfer of drug from KC to PCMC. This may not, however, be the case. There is evidence for the transfer of lysosomes from activated macrophages into the cytoplasm of other cells (Hibbs, 1974; Bucana et al., 1976) and macrophages have been proposed as "second carriers" to transport phagocytosed liposomes

to other tissues (Fidler et al., 1981). In addition, it is clear that many processes involved in tissue injury, whether induced by infection, surgery, accident or perhaps iatrogenically, could be responsible for the leakage of drugs from one cell type to another (Canonico et al., 1979). Thus, for example, tissue damage can result in increased capillary permeability by the actions of histamine and 5-hydroxytryptamine liberated from mast cells; activation of macrophages and leucocytes may lead to infiltration of the damaged area and increased phagocytosis of liposomes found therein as well as the release of cellular and lysosomal enzymes which could digest liposomes (and perhaps drugs) extracellularly. The release of hormones and proteins in response to injury (e.g. : glucocorticoids, insulin, glucagon, thryroxine, actute phase proteins, alpha-2-macroglobulin) as well as changes in plasma ion levels may all have unknown effects on drug uptake, disposition and metabolism.

Section 4:7. : General Discussion

A) Tissue Distribution Studies.

From these present studies and from the many reports of other workers in the field it is clear that the predominant tissues which control the distribution of liposomes *in vivo* are the cells of the reticuloendothelial system. This involvement was recognised from the outset (Gregoriadis and Ryman, 1972a, 1972b; Segal *et al.*, 1974).

In an oft-quoted review, Saba (1970) has outlined the historical aspects of RES research and stressed the role of these cells in the plasma clearance of all types of particulate matter as well as their physiological functions in the area of host defense, i.e.: the immunology, detoxification, biotransformation and excretion of foreign and endogenous matter. The sessile macrophages which are primarily, but not exclusively, responsible for the clearance of colloidal and particulate material are to be found lining the sinuses of the liver, spleen, lung, bone marrow and, often forgotten, in the capillaries of the adrenal and pituitary glands. In addition macrophages compose the reticular cell populations of the spleen, lymph nodes, bone marrow and of the central nervous system but these are not as closely in contact with the blood as with other body fluids e.g.: lymph and cerebrospinal fluid. Finally, macrophages can be found in all organs and in connective tissues (histiocytes) but these are derived from the motile blood macrophages (monocytes).

Using the other colloidal systems, it has been shown (Saba, 1970) that the stationary macrophages of the liver and spleen are the major areas of uptake of injected particles and that uptake is primarily by endocytosis (Silverstein *et al.*, 1977). Liposomes are no exception to this general rule (Segal *et al.*, 1974), however, the finding in this and many other studies of liposomal solutes localized in many tissues other than those with substantial macrophage populations raises two important questions which are the subject of continued debate :

i) is tissue uptake of liposomes entirely mediated by the macrophages or are other cells involved ?

ii) is endocytosis the only mechanism by which liposomes can gain access to the interiors of the cells ?

It will be appreciated that, in terms of drug delivery, the first question is of paramount importance since if liposomes can only be

"targetted" to macrophages their usefulness will be limited.The second question is important in terms of drug stability and effect since, except in very exceptional cases, the metabolic sequal to endocytosis is digestion of the material in the lysosomes of the cells. Thus drugs which are unstable at the lower pH found in lysosomes or which contain moieties capable of being attacked by enzymes may be ineffective when liposome entrapped although the lysosomal milieu can be used to promote drug activity and stability (de Duve et al, 1974).An addition question might also be asked which is based on factors involved in the previous two : is it possible to alter the disposition of liposome entrapped agent by blockade of the RES ?

i) Factors effecting cell uptake of liposomes.

Tissues other than the liver will be discussed here. The special case of the liver will be considered later.

a) Interpretational problems. (Poste and Papahadjopoulos, 1978; Poste, 1980).

The tissue disposition of entrapped material is beset by several different problems of interpretation. These problems revolve around the use of radioactive markers to describe the location of the liposomes, e.g.: mention has already been made of the leakage of entrapped material from liposomes, in the case of ^{111}In, protein bound radioactivity not only changes the apparent plasma clearance of the radiolabel but may also contribute to the tissue disposition. The uptake of the protein-^{111}In complex may be very different from the liposomal material not only in the mode of entry into cells but also in the kinetics.

Further problems arise when exchangeable lipid markers are used to assess the integrity of liposomes, exchange can take place not only between carrier and lipoproteins but also between carrier and cell membranes. Other problems involve the mode of uptake of liposomes by cells, e.g.: adsorbtion of liposomes to cell surfaces followed by leakage of the entrapped solute through both liposome and cell membranes has been shown to be a major route of solute uptake by cells in vitro (Blumenthal et al., 1977; Weinstein et al., 1978); in such a system, providing that the adsorbtion is stable, an apparent uptake of both liposome and solute will be found even though the vesicle might, in vivo, be removed at some later time by mechanical factors such as blood flow or collision with blood cells. In the example above, interpretation of the

results might become even more confused if, during adsorbtion, exchange of liposomal lipid for membrane lipid occured (Zilversmit, 1978; Wirtz et al., 1980).

Uptake by endocytosis may also cause interpretational problems depending upon the species of the entrapped solute, e.g.: the rapid removal of radiolabel from drugs or proteins by lysosomal digestion followed by translocation of either the label or drug out of the lysosomes back to the blood or into neighbouring cells might easily be wrongly taken as specific uptake by a particular tissue or cell type.

b) Liposome size.

The plasma clearance of liposomes according to size is of the order LUV > MLV > SUV (Juliano and Stamp, 1975; Sharma et al., 1977; Hinkle et al., 1978; Abra and Hunt, 1981). The uptake into spleen, lung, brain and bone marrow was the highest when larger liposomes were used (Jonah et al., 1975; Rahman et al., 1975; Kimelberg et al., 1976; Gregoriadis, 1977a; Sharma et al., 1977; Richardson et al., 1978; Ryman et al., 1978; Hunt et al., 1979; Jackson, 1980; Abra and Hunt, 1981), however, Gregoriadis et al. (1977a) found higher levels in the lungs of mice when smaller liposomes were used, contrary to most published data. Both Kimelberg et al. (1976) and Richardson et al. (1978) found higher levels of the larger liposomes in the kidneys but this latter group (Sharma et al., 1977) had previously shown almost no differences between the sizes in this organ. However, this last data was obtained using ^{14}C-cholesterol-containing liposomes which are known to exchange the radiolabel with lipoproteins (chapter 3) and labelled HDL will concentrate in the kidneys to a certain extent (Gotfredsen, 1982). Uptake into myocardial muscle has been reported to be independent of size (Sharma et al., 1977) but in the ischaemic heart MLVs are preferentially accumulated (Caride and Zaret, 1977) whilst in skeletal muscle the smaller liposomes are more highly concentrated (Kimelberg et al., 1976; Gregoriadis et al., 1977a).

In general tissues which contain large capillary networks or sinuses seem to accumulate the large liposomes probably because they can trap them in their terminal branches(Ryman and Tyrrell, 1980). Tissue uptake consists of a balance between the rapid clearance of large liposomes by the RES and the longer circulatory

time of the smaller liposomes which allows these vesicles a better chance to interact with cells of limited or slow uptake capacity. For this reason tissues such as skeletal muscle should be more susceptible to therapy with the smaller liposomes. Such effects were reported by Dapergolas et al. (1976) in tumour tissue and Richardson et al. (1978) found that rat tumour tissue was better visualized by gamma-camera when smaller liposomes containing ^{99m}Tc were injected intravenously. However, similar studies in humans failed to provide visualisation of a variety of tumour tissues (Segal et al., 1976; Richardson et al., 1979).

c) Liposome composition.

Manipulation of the composition of liposomes (usually the charge) has been used by several authors in attempts to change the tissue disposition of liposomes. However, as shown in chapter 3, in vivo the final charge on the liposomes, after interaction with plasma proteins, does not necessarily reflect the charge of the injected preparation. Indeed, anionic liposomes may be less electronegative than cationic liposomes. Nevertheless, Jonah et al. (1975) reported that the tissue distribution of unsonicated MLVs containing EDTA could be altered by changes in charge; cationic liposomes produced the highest levels of EDTA in lung and brain tissue whilst anionic liposomes gave the highest levels in the spleen and bone marrow. No control of size or drug leakage was made in these preparations so that it is unclear how much of the differences are due to these factors.

Richardson et al. (1978) studied the tissue uptake of heterogenous small MLVs in rats and found that neutral liposomes were taken up less well into the kidney than either cationic or anionic vesicles which were about equal. In agreement with Jonah et al, (1975) these authors also reported higher spleen levels of anionic liposomes but unlike them they found that the liver uptake was higher for anionic and neutral liposomes than for cationic ones. Richardson et al., (1978) were also able to confirm the findings of Gregoriadis et al., (1977a) that small anionic liposomes localize the best in solid animal tumour tissue. Yet previously, others (Mc Dougall et al., 1974) using ganglioside-containing vesicles failed to alter the in vivo disposition by the addition of an anionic charge and this is consistent with the fact that ganglioside vesicles have a similar plasma clearance to anionic liposomes

(Gregoriadis and Neerunjun, 1974). These last authors also reported that the levels of liposomes in the spleen were almost uneffected by the presence of charge whereas the initial liver levels showed that anionic liposomes were accumulated to a higher extent than neutral or cationic liposomes. Steger and Desnick (1977) reported lower and less prolonged levels of an entrapped enzyme in both kidney and spleen when cationic, as opposed to anionic, liposomes were injected I/V. In the damaged mouse heart Caride and Zaret (1977) reported that neutral and cationic liposomes accumulate in the endocardium to a higher extent than anionic MLVs. This accumulation is probably due to capillary and cell damage coupled with changes in blood flow.

The incorporation of cholesterol into liposomes has been discussed previously and until recently it was assumed that its presence in the liposome membrane changed the distribution of entrapped material by a reduction in either the leakage of entrapped solutes or in lipid exchange with lipoproteins. However, recently Senior and Gregoriadis (1982a, b) have suggested that the porosity of SUVs, as effected by CHOL, controls the plasma half-life of the carrier. Wharton and Green (1978) studied the tissue distribution of ^{3}H-PC in liposomes containing different sterols. Two derivatives (3-hydroxycholest-3-en-2-one and 1-methyl-10-norcholesta-1, 3, 5 (10)-tirene-3-ol) tended to aggregate the liposomes and were cleared more rapidly from the plasma to give high tissue concentrations in the liver, spleen, intestine and kidneys. Two other derivatives (3-hydroxycholest-5-en-7 one and cholesta-4, 6 - dien -3-one) were cleared at the same rate as normal cholesterol-containing liposomes but gave lower tissue levels than the normal. It is unclear if these data reflect a true direction of liposomes or if the novel cholesterol derivatives are binding to PC in the liposome membrane in a different manner and so perhaps preventing PC exchange with lipoproteins or cells or, finally, if the liposome surface has been altered in such a manner that interaction with plasma proteins or cell "receptors" is changed.

Recognition that cells contain surface membrane receptors specific for carbohydrate groups prompted Mauk and Gamble (1979) to investigate sugar derivatives of cholesterol incorporated into SUV membranes. They found that the presence of either fucose or galactose on the surface of cationic SUVs caused no statistically

significant alteration in the tissue distribution of the vesicles irrespective of the route of administration (I/V, I/P, Sub-Cut, Oral).

Replacement of liposomal PC by sphingomyelin (Gregoriadis and Senior, 1980; Hwang et al., 1980; Ellens et al., 1981) changes the plasma $T_{1/2}$ of the liposomes to make it slower and more linear. According to Gregoriadis and Senior (1980) increases in plasma circulation times reduce the liver uptake of sphingomyelin SUVs but Hwang et al;, (1980) and Ellens et al., (1981) consider these effects to be due to a change in the kinetics of uptake rather than in the total amount of liposomes captured. Furthermore, since they appear to be antigenic (Strejan et al., 1979) it would appear that the use of sphingomyelin liposomes may be limited. Reduction of liposome fluidity by the replacement of EPC by DPPC failed to show significant changes in the uptake of liposome entrapped ^{111}In-BLM into the lungs, spleen, liver or tumour tissue of rats (Gregoriadis et al., 1977a). However, recently Senior and Gregoriadis (1982b) have produced distearoyl-PC (DSPC) liposomes with 20 hour half-lives and low hepatic capture although it remains to be seen if the total amount of liposomal lipid finally taken up by the RES is reduced. Gotfredsen (1982) found little difference between the hepatic capture of PC:PS vesicles and DSPC:CHOL vesicles. In contrast, mouse tumour tissue took up at least twice the amount of fluid liposomes (EPC with cis-dichlorobiscylopentylamine platinum (II) entrapped) as similar DPPC liposomes but uptake by the liver was highest with this latter preparation. (Deliconstantinos, et al., 1977).

d) Liposome dose

Gregoriadis and Neerunjun (1974) demonstrated that the initial rapid plasma clearance of MLVs did not occur when large amounts of lipid were injected into rats. They attributed this change to immediate saturation of uptake sites. This data was later confirmed by Tanaka et al., (1975) and the effect is also apparent with SUVs (Gregoriadis and Senior 1980, Senior and Gregoriadis 1982a). The dosage effect has been more thoroughly studied by Abra and Hunt (1981), Souhami et al., (1981) and Gotfredsen (1982). As Gregoriadis and Neerunjun (1974) correctly sumised these effects are connected with blockade of the RES and this subject will be discussed in more detail in connection with lipo-

some uptake by the liver. However, RES blockade theoretically allows a greater time for liposome uptake by other tissues. Unfortunately, blockade prior to subsequent liposome challenge has not resulted in the expected relocation of liposomes to organs other than the liver and spleen. At the most only modest increases in the kidneys and bone marrow have been reported (Caride et al., 1976; Kao and Juliano, 1981; Souhami et al., 1981). In the lungs, Kao and Juliano (1981) using LUVs and Caride et al., (1976) (MLVs) have reported an increase uptake of their challenge preparations but Souhami et al., (1981) failed to find an increased lung concentration with either MLVs or SUVs. This descrepancy is probably due to the relative sizes of the populations. However, Abra et al. (1980) reported that blocking doses of liposomes administered 5 and 24 hours prior to the challenge dose could depress the latter doses' uptake into tissues other than the liver and spleen. This delayed blockade of other tissues might be the same effect that Kao and Juliano (1981) observed using SUVs to block the liver uptake of LUVs. If this is so, it is possible that a case could be made for uptake into other tissues being mediated by saturatable mechanisms associated with phagocytosis by cells of the RES in these tissues. On the other hand, these data might also imply that sites of stable adsorbtion of liposomes are also saturatable. Souhami et al., (1981) have suggested that these adsorbtion sites can account for the 50 % or so of "lost" liposomes which cannot be found in the liver and spleen after injection. These sites may be expected to contribute to the "apparent" distribution of liposomes in tissues.

e) Foreignness of the surface of liposomes.

An extensive review of the role of liposomes as immunogens and their role in immune reactions is beyond the scope of this thesis (Tom and Six, 1980; Nicolau and Paraf, 1981). However, as already stated liposomes can act as immunological adjuvants and certain compositions result in the formation of antibodies. Most liposomes used to date were made from natural lipids which are exchangeable (discussed chapter 3) or biodegradable (Fowler and de Duve, 1969): liposome degradation has been demonstrated in vivo (Zirenberg and Betzing, 1979; Gotfredsen, 1982). Such evidence does not suggest that liposomes are recognised as non-self in the same way as might be expected for other particles (e.g.: bacte-

ria). The adsorbtion of proteins to the surface of liposomes in vitro (chapter 3) further suggest that any "foreignness" might be covered by these molecules. However, there is evidence (Gregoriadis, 1980 a,b,c; Gregoriadis et al., 1981b; Gregoriadis and Meehan, 1981) that entrapped solutes are detectable in or on the surface of liposomes. The extent to which entrapped drugs or proteins contribute to the foreignness of the liposome remains to be established.

vi) Opsonins in normal serum.

Although this subject has been considered in chapter 3, the role of proteins (e.g.: alpha-2-M) adsorbed to liposomes in the removal or uptake of these carriers into cells has not been determined. Opsoninization has been proposed (Black and Gregoriadis, 1976; Souhami et al., 1981) as a mechanism by which liposomes are directed to the RES. However, in a recent publication, Kao and Juliano (1981) have shown that opsonin depletion is probably not the cause of the liposomal blockade of the RES. This does not, however, rule out the possibility that proteins adsorbed to liposomes can determine the fate of the vesicle in vivo.

Uptake of liposomes by the liver.

There is no doubt that the liver is the principal organ involved in the removal of particulate and colloidal material from the circulation (Saba, 1970). Numerous reports of liposome concentration in this organ following I/V Sub-Cut, I/P and I/M injection have led to speculation as to the factors with control liposome removal, the cell type involved in uptake, the final destination of entrapped solutes and how liver uptake can be manipulated or modified to enhance uptake by other tissues.

The liver consists of three major cell types: i) the parenchymal cells (PCMC) ii) Küpffer cells (KC) and iii) endothelial cells (EC), but other types of cell (e.g.: fat cells, blood cells: Emeis and Planqué, 1976) may also be present in the sinusoids. The basic architecture of the liver sinusoids has been extensively studied by Wisse (1977). In brief, the liver sinusoids and capillaries are lined with endothelial cells between which extend long protoplasmic processes containing fenestrae of about 100nm diameter. The fenestrae lead, via the space of Diss to the parenchymal cells. Interspersed between the EC and covering some of their surface are the Küpffer cells which may

extend out into the sinusoidal lumen. The sinusoidal capillaries of the liver do not contain a continuous basement membrane beneath the EC so particulate matter can pass out of the capillary via the fenestrae and come directly into contact with PCMC membranes. In other tissues containing fenestrated endothelial cell linings (e.g.: kidney and intestinal lumen) a continuous basement membrane is present. Yet other tissues such as the brain, skin and muscle contain tight junctions between adjacent endothelial cells with little or no fenestration (although pore formation does occur) so that uptake of particulate matter into tissue supplied by these capillaries involves the traversal of the endothelial cells, interstital space, basement membranes and finally the cell membrane in order to reach the interior of the cell.

Factors effecting liver uptake of liposomes.

It is often impossible to separate the various factors responsible for the uptake of liposomes into this organ. For this reason such factors as charge, size, dose and RES blockage will be considered together. Other compositional changes will be considered separately.

a) Cell type involved.

It is still unclear, despite ten years work, what is the relative contribution of the three major liver cell types to the uptake of liposomes in vivo. Other colloids and particles are almost exclusively concentrated in the phagocytic cells of the spleen and liver (KC) (Saba 1970). Apparently liposomes are different since these organs rarely account for more than 50-60 % of the injected dose at any time after injection irrespective of size or liposome composition.

In the original description of the in vivo fate of injected liposomes Gregoriadis and Ryman (1972a, 1972b) demonstrated uptake of radiolabelled protein and cholesterol into both PCMC and KC; moreover, they suggested that the mechanism of uptake was by endocytosis because most of the entrapped protein was localised in the lysosome fraction of whole liver. Other workers have confirmed morphologically that liposomes of a variety of compositions and entrapped solutes are localized in these two cell types (Rahman and Wright, 1975; Tanaka et al., 1975; de Barsy et al., 1976). However, Wisse et al., (1976) have emphasized the kinetics of cell uptake since they demonstrated that horseradish peroxidase (HRPase) entrapped in liposomes was found associated with the KC and EC during the first half hour post-injection but at 3 hours

all the cell types appeared to take up the enzyme. This correlates quite well with the biphasic plasma clearance of liposomes and suggests that the initial uptake (KC and EC) is due to the rapidly cleared large MLVs whereas the PCMC uptake (> 3 hours) may be due to SUVs in the injection which are cleared more slowly and are small enough to pass the fenestrae to interact with these cells.

Once again problems of interpretation of data play a leading role in this controversy. Much of this early work has been carried out with liposomes which are now recognised to be unstable in the plasma (i.e.: low CHOL: except Tanaka et al., (1975) who used so much CHOL that it seems probable that not all the sterol was localized in the liposome membrane). Exchange of radiolabelled liposomal lipid with lipoproteins and/ or cell membranes and leakage of entrapped solute can easily account for many of the observations (Roerdink et al., 1977). Moreover, the finding of "liposome-like" multilamella structures in cells untreated with liposomes is by no means an uncommon feature (Wisse et al. 1976; Scherphof et al., 1980); such structures have been attributed to fixation artifacts but they may also be formed by many cationic compounds, particularly amphiphilic drugs, as has been amply demonstrated in electronmicrographs by Lüllmann-Rauch (1979). These structures are consistent with a drug-induced polar lipid storage disease of lysosomes particularly in the liver and this suggests that caution be used not only in the equation of intracellular "liposome-like" strutures with injected liposomes but also in the therapeutic use of cationic amphiphiles entrapped in liposomes made from polar lipids.

Further problems arise when certain entrapped solutes (e.g.: proteins like albumin or HRPase) are used as markers for the internal aqueous spaces of the liposome because uptake of free foreign proteins by liver cells is a well recognised phenomenon (Dean, 1975; Gregoriadis, 1975; Wisse et al., 1976); ideally an entrapped solute should not concentrate in the liver at all in its free form so that discrimination of liposomal material from leaked/released free solute can be made. Using such a discriminating solute Segal et al., (1974) demonstrated that a heterogeneous population of liposomes localized entirely in phagocytic cells of the liver and spleen. In contrast, Gregoriadis et al., (1974a) found entrapped radiolabelled albumin exclusively in the PCMC following injection of MLVs into a patient but since the time needed to digest both liposomes and protein is not known it is possible that the

KC had already digested and removed the liposomal material; this emphasises the need for the solute to be relatively inert for true localization to be evaluated.

Separation of the livers of injected animals into different cell types has further added to the controversy of hepatic liposome uptake. Scherphof et al. (1981) have reported that stable liposomes containing the inert polymer polyvinyl pyrrolidone (PVP) are not taken up by endothelial cells. Furthermore, using unstable SUVs, which donate PC to HDL, these authors have demonstrated that the uptake of radiolabelled PC by PCMC can be accounted for by the capture of the radiolabelled HDL. However, the finding of MLV-derived PC in hepatocytes cannot be explained by this process because these liposomes are more stable. These authors explain this PCMC radiolabelling by assuming transfer of PC to PCMC from liposomes digested in the KC. Coupled with their previous in vitro findings of low liposome uptake and phospholipid exchange to hepatocytes in the presence of serum, (Hoekstra and Scherphof, 1979) these authors conclude that stable MLVs are removed only by endocytosis into the KC (Roerdink et al., 1981).

Gotfredsen (1982) does not completely agree with this. Using smaller unilamella vesicles of sizes below 100 nm he found that uptake into KC/EC was 3 - 5 times more (per mg protein) than into PCMC but that PCMC uptake was still substantial. On kinetic grounds, Gotfredsen suggested that the uptake of radiolabelled PC by PCMC was not only mediated by a slow donation from HDL but could also be accounted for by a rapid, and mostly stable, adsorbtion of liposomes to the cell surface. Such adsorbtion results in the release of entrapped solute into the plasma so that radiolabelled lipid is apparently taken up to a greater extent. In addition, Gotfredsen(1982) found that aggregated liposomes were more highly concentrated in non-parenchymal cells suggesting that size is an important parameter. In terms of therapy this is certainly true since intracellular parasitic diseases of the RES are more effectively treated with the larger liposomes (Black - unpublished observations).

In rats Freise et al., (1980, 1981) have studied the uptake of exchangeable ^{14}C-CHOL and leaky ^{3}H-methotrexate (AP) from MLVs into PCMC and KC. One hour after injection mostly whole liposomes were found in KC but at 6 hours the PCMC account for most of the cholesterol and drug. Results such as this are not surprising since others have shown that both lipid (Scherphof et al., 1981) and drugs (section 4:6) can be

transfered from PCMC to KC. In addition, one of the final destinations of HDL (containing ^{14}C-CHOL:chapter 3) is known to be hepatocytes and high levels of free methotrexate can be found in liver tissue at this time (Colley and Ryman, 1975; Kimelberg and Atchison, 1978).When rats received a portacaval shunt, which markedly reduces the activity of KC, the uptake of both radiolabels in the liver was not reduced (Freise et al., 1980, 1981) which prompted these authors to suggest that on a cell to cell basis the PCMC are responsible for the most of the hepatic uptake of liposomes. Incidentally the portacaval shut operation in rats does not alter the plasma clearance or tissue distribution of the liposomes. The liposomes used in this study were, overall, too big to pass through the endothelial fenestrae. Such an operation would not be expected to reduce the uptake of non-liposomal CHOL or methotrexate indeed it might increase their PCMC localization in the liver.

The cell type involved in the uptake of liposomes by the liver remains to be firmly established. Neither electron microscopy nor fractionation of the liver into its component cells have provided irrefutable evidence of liposome localization in PCMC. As a current working hypothesis it would seem that large MLVs or LUVs are taken up by KC. Smaller MLVs and SUVs may be found in both PCMC and KC. Uptake into EC remains to be established although larger vesicles probably do not locate there.

The endothelial sieve plates appear to act exactly like a sieve but their cut off, at 100nm, will not apply to all liposomes of diameter larger than this because vesicles are deformable structures. One wonders if the hepatic- portal vein blood pressure is high enough to force quite large liposomes through the fenestrae (in an analagous manner to the filter sizing method of Olson et al., 1979). This mechanism might account for the finding of large multilamellar structures associated with parenchymal cells (Rahman and Wright, 1975; de Barsy et al., 1976).

b) Liposomal size and dose : RES blockade.

Apart from the effects of liposome size on their relative uptake by the liver cell types, the size of the liposome has an effect on the total liver uptake. Gregoriadis et al., (1977a) demonstrated that the total hepatic uptake of large liposomes (0.5 min sonicated) was higher in mice than smaller (3 min sonicated) liposomes. However, in rats the uptake was not apparently size dependent. Also using rats, Sharma et al., (1977) failed to find differences between SUVs and MLVs or MLVs

and LUVs 2 hours post-injection although calculation of the differences between SUVs and LUVs suggests that the larger (LUV) preparation gives the higher liver uptake. In mice, and using liposomes of defined size, Abra and Hunt (1981) demonstrated that the total uptake (irrespective of dose) was always lower with the smallest liposomes. Souhami et al., (1981) have also demonstrated a lower hepatic uptake of SUVs, irrespective of charge, compared to larger MLVs.

The dose and size effects of liposome disposition in vivo have been studied by Abra and Hunt (1981). These authors have found that the numbers of vesicles of three different sizes taken up by the liver cannot account for the difference in size uptake, however, when surface area was considered similar uptakes were observed irrespective of size. Using doses of 10mg total lipid/mouse, Souhami et al., (1981) were unable to show liver blockade with a mixed population of MLVs but blockade of the RES may be dependent upon uptake sites which are themselves size dependent and low CHOL-containing liposomes give anomolous results (Abra and Hunt, 1981). The effects of predosing animals with liposomes or other particulate materials are seen in the plasma clearance of the liposomes injected at a later time. In general, the fast initial phase of clearance of MLVs in most effected by the blockade (Gregoriadis and Neerunjun, 1974; Souhami et al., 1981) irrespective of the agent used to create the blockade (dextran sulphate polymer or colloidal carbon). There appears to be a slight difference in the manner in which charged liposomes are cleared from the circulation since neutral MLVs only have their initial clearance rate blocked whereas anionic MLVs have both the initial and final rates blocked. This effect may be due to a homologous charge effect (dextran sulphate is highly anionic) since cationic liposomes appeared to associate with the blocking agent in vivo (Souhami et al., 1981). Using carbon particles as the blocking agent an increase in both phases of the plasma concentration with neutral and positive liposomes was found. Blockade of the RES effected the plasma clearance of both MLVs and SUVs. However, Souhami et al., (1981) have also emphasised the need to establish that blocking agents really do block the RES before attributing this process to changes in liposome clearance and uptake.

Predosing animals with liposomes also slows the plasma clearance of a subsequent dose of radiolabelled liposomes. Abra et al., (1980) found that the time of blockade prior to dosing was an important factor, blockade at 1 hour and 5 hours result in a good or partial (respective-

ly) blockade whereas predosing with liposomes 24 hours before a second challenge had little effect. Kao and Juliano (1981) reported that predosing with LUVs, latex particles, xenogeneic red cells and dextran sulphate could delay the plasma clearance of LUVs injected one hour later. Predosing animals with SUVs did not effect the plasma clearance of LUVs if the blocking dose was given at 1 or 4 hours before challenge, however, predosing at 24 hours before challenge was effective. These authors explain this by assuming that the uptake of SUVs by the RES is so slow that 24 hours is needed to establish the blockade. Others (Abra et al., 1980) would not agree; several groups of workers believe that digestion of lipids in KC is quite rapid (e.g.: Hwang et al., 1980) so that it is difficult to see how the slow accumulation of SUVs in KC is totally responsible for blockade at 24 hours. The lack of effect of SUVs on LUV clearance at 1 and 4 hours can be explained by the predominant cell uptake site being different (PCMC for SUVs; KC for REVs) as well as differences in clearance time. Increasing the SUV loading dose prior to LUV challenge might clarify the issue.

In the liver and spleen liposome uptake is, effected by prior blockade. Mention has already been made of the work of Gotfredsen (1982) who showed that high doses of liposomes prefentially blocked KC and not PCMC. Gregoriadis and Neerunjun (1974) demonstrated that concurrent administration of carbon particles and liposomes actually increased the liposome concentration in the liver perhaps by causing more liposomes to be directed towards the PCMC. Blockade by latex particles (Kao and Juliano, 1981) caused a reduced level of challenging LUVs in the spleen (where normally the large liposomes have an increased localization: see above) but not in the liver. Blockade with carbon particles or dextran sulphate caused lower levels in the liver for MLVs but not SUVs (Souhami et al., 1981) whereas blockade with carbon particles produced an increased spleen concentration for MLVs but not SUVs. Using liposomes to block subsequent liposome uptake resulted in blockade of MLV uptake by the liver but an increase in spleen uptake (Caride et al., 1976; Abra et al., 1980). These data suggest that the liver KC are the primary site of blockade; for MLVs blockade at this site results in transfer of vesicles to the spleen whilst for SUVs, with longer plasma $T_{1/2}$, blockade here probably results in increased PCMC localization. Pretreatment of animals with methyl palmitate, which has been reported to inhibit phagocytosis (Saba, 1970), also blocks liposomes uptake into the liver but not into

the spleen (Tanaka et al., 1975); a slight increase in lung uptake was also found following this treatment.

c) Liposome composition.

Much of the published work in this area is overshadowed by the effects of size and dose on the liver uptake. Without adequate control of the size of the injected population it is impossible to verify that charge or other compositional changes make a real difference to hepatic uptake. Other factors, already mentioned, will include the stability of the liposomal lipids and entrapped solute in contact with the plasma and the in vivo adsorbtion of proteins to the liposome surface with its associated charge reversal phenomenon. Finally, no publication to date has verified that the entrappment of compounds in either AP or LP of liposomes does not effect the charge of the injected material, this is especially important where proteins or other charged molecules are being used. This procedure has been carried out in this study (section 2:2). Given these limitations and the fact that charge effects may depend not only on the species of charge but also on the degree of charge within the species, it is apparent that charge effects may be responsible for some alteration of liver uptake. However, it must be remembered that charge also effects the plasma clearance of the material (cationic liposomes are cleared more slowly than anionic) so that the timing of the observations will also be important.

Gregoriadis and Neerunjun (1974) showed that anionic liposomes (MLV) are more rapidly concentrated in the rat liver and to a higher amount than neutral or cationic liposomes. Moreover, cationic liposomes persisted at higher levels in the liver than the others. These effects are probably due to the longer plasma life of the cationic species with a consequent slower hepatic uptake. Steger and Desnick (1977) also used MLVs but these authors found similar initial (up to 4 days) levels of both cationic and anionic liposomes, containing an enzyme, in the liver. However, at times long after the plasma concentration had fallen to zero, these authors reported that the liver concentration of the cationic liposomes persisted for substantially longer (3 days) than the anionic carrier suggesting cationic liposomes cannot be metabolised as fast. Anionic liposomes are also less stable than the cationic when administered locally (Segal et al., 1975; Mauk et al., 1980a).

The liver uptake of large (hand shaken) MLVs of different charges has been studied by Jonah et al. (1975). These authors failed to find

differences between neutral, anionic or cationic liposomes at short periods after I/V injection. However, it is evident from their data that 24 hours post-injection, when the plasma levels are zero, cationic liposomes were more persistent than the others. By decreasing the fluidity these authors showed that there was little longterm difference between DPPC neutral liposomes and EPC ones although initially higher liver levels were attained with the fluid EPC-containing preparation.

However, Richardson et al. (1978) and Souhami et al. (1981) did not find very much difference in the liver levels of charged MLVs although at 24 hours post-injection the former authors found less cationic liposomes than anionic or neutral ones but it is unclear if these differences were significant. Souhami et al. (1981) also studied the tissue uptake of homogeneous SUV preparations. One hour after injection the liver concentrations (per cent of injected dose) of the entrapped solute were : - anionic : 52.5 %, neutral : 27.5 % and cationic:16.5 %. This hepatic result, whose ranking order is similar to that found with MLV preparations (Gregoriadis and Neerunjun, 1974), again suggests that cationic liposomes may be taken up less well by the liver. Interestingly, after incubation with serum the final charge on the cationic liposomes is the most electronegative (section 3:3). However, Mauk and Gamble (1979), who have made very stable liposomes, reported that cationic SUVs were taken up to a much greater extent (cationic >> neutral > anionic). Liposomes containing galactose or fucose derivatives of cholesterol were localized to about the same extent as the neutral vesicles.

Other compositional changes have been reported to determine the hepatic uptake of liposomes. Deliconstantinos et al. (1977) reported that DPPC-containing liposomes were localized in mouse liver to a greater extent than the fluid EPC-liposomes (c.f. Jonah et al., 1975 and above). Deshmukh et al. (1978) reasoned that circulating or cellular phospholipases were responsible for determining the plasma half-lives of liposomes and so they made SUVs from dialkyl PC which is almost completely resistant to phospholipase attack. Using these vesicles these authors found higher liver concentrations of entrapped solute and longer plasma half-lives than EPC-liposomes.

In the field of parasitology it is well known that malaria sporozoites undergo a primary multiplication phase in liver PCMC. Alving et al. (1979) have reported that glycolipid-containing MLVs (glucosyl, galactosyl or lactosyl ceramides) prevent the appearance of the ery-

throcytic phase of the malaria infection. The suggestion is that the glycolipids interact specifically with hepatocytes to prevent the uptake and/ or release of the parasites perhaps by competition for the same carbohydrate receptor. That such receptors exist on mammalian hepatocytes has been known for some time (Ashwell and Morell, 1974; Sly, 1980), however, they are not unique to the PCMC; other carbohydrate receptors exist for KC and macrophages (Stahl and Schlesinger, 1980) and it is by no means certain that the specificity of all the receptors has been described. Furthermore, the exact mode of uptake of sporozoites by the liver (Carter and Diggs, 1977) is a controversial subject well beyond the scope of this thesis but recent evidence that their preliminary uptake from the circulation could be mediated by the KC (Pirson -personal communication) might also explain the results of Alving et al. (1979). Finally, Surolia and Bachhawat (1977) have demonstrated that a "PCMC-specific" glycolipid (GM_1-ganglioside) entrapped in liposomes could increase their uptake by the liver, presumably by augmenting the PCMC fraction, but Alving et al (1979) found this glycolipid ineffective as a liposomal antagonist to malaria infection.

Gregoriadis and Neerunjun (1975b) have demonstrated liposome homing to target cells by the use of liposome entrapped desialylated fetuin. This protein was found to be expressed on the liposome surface where its sialic acid group was removeable by treatment with neuraminidase, such a treatment resulted in an increased liver uptake of these liposomes when compared to the same, untreated, vesicles. Moreover, the hepatic uptake of the treated liposomes could be partially blocked by free asialofetuin which did not block the untreated preparation. These liposomes were, therefore, the forerunners of other carbohydrate-containing liposomes (Mauk and Gamble, 1979; Mauk et al., 1980 a,b).

Finally mention must be made of the recent work by Fidlers group (Fidler et al., 1980, 1981; Hart et al., 1981 and references therein) which has some relevance to the uptake of liposomes by macrophages. These authors have found that liposomes (anionic MLV) containing natural or synthetic macrophage activating factors can activate lung macrophages in vivo rendering them tumouricidal. Moreover, this tumouricidal function extended to sites which were far removed from the site of macrophage activation. These are important data which may have a profound effect on the use of liposomes as drug carriers. The implication is that macrophages either stationary (e.g.: lungs) or wandering

(circulation) may become "loaded" with therapeutic substances and then allowed to roam throughout the circulation and tissues seeking target sites.

iii) General conclusions on tissue distributions.

How, then may the three questions posed at the start of this section be answered ?

i) Is tissue uptake entirely mediated by macrophages or are other cells involved ?

Data from many different groups suggest that some liposomes (as much as 50 % of the injected dose) are not found associated with the liver and spleen where other colloidal material accumulates. The other sites involved are predominately the lungs, bone marrow and perhaps the intestine and kidneys. In addition, the liver parenchymal cells, because of their special architecture, may also taken up smaller liposomes. Unfortunately, most other cells and tissues do not appear to concentrate liposomes.In tissues which have a low macrophage content (e.g.: muscles) the poor liposomal localization does not encourage the use of these carriers as transporters of e.g.: enzymes for the therapy of storage diseases, unless extensive manipulation of the vesicle can be accomplished. Simple manipulation of size or composition would not appear to be enough. In addition, there is no conclusive evidence that entrapped compounds are actually located inside the relevant cell type apart from the RES. Stable adsorbtion of vesicles to blood vessel walls could account for much of the published data on tissue distribution especially in highly vascular tissues (e.g. : the kidneys). If the aim is to introduce a therapeutic substance into a specific subcellular compartment or into a specific cell type then stable adsorbtion, even if this accompanied by local drug leakage, will be of little more use than a local depot of the drug.

It seems to this author, therefore, that, apart from the liver parenchymal cells, the uptake of the anionic MLVs used in this study can be accounted for by : i) localization in tissue macrophages, ii) adsorbtion to capillary beds or iii) leakage of liposome-entrapped material.

ii) Is endocytosis the only means by which liposomes can gain entry into cells?

The short answer to this question is probably : - Yes, if macrophages are the only cell type involved in the true uptake of whole liposomes. However, if other cell types are involved then there are several other mechanisms which could operate none of which have been conclusively demonstrated to occur in vivo : there are many problems of interpretation which make it difficult to establish if liposomes are taken in whole. It is not, of course, always necessary to have the carrier gain entry into the cell or tissue for a pharmacological effect to occur but, in terms of specificity and toxicity, this would be preferable to a general increase in the local drug concentration. The mode of entry of liposomes into cells will be considered in the following section.

iii) Is it possible to alter liposome distribution by blockade of the RES ?

As has been shown the liver and spleen present the biggest obstacle to intravenous therapy with liposomes. No matter how long the circulation time of the liposomes, these organs finally capture the major part of the injected dose and this argues in favor of there being only limited types of cells which can remove liposomes from the circulation. Further evidence comes from the fact that at high lipid doses or following blockade of the liver KC the only organs which show any substantial change in the uptake of liposome entrapped markers are those (e.g. : spleen and lungs) which contain elements of the RES.

It could be argued that blockade of the liver would also automatically enhance the uptake by other tissues (if this uptake was mediated solely by tissue macrophages) and, since this does not occur, therefore tissue uptake is not macrophage dependant. However, there is no evidence that other macrophages cannot be blocked, especially by liposomes, and it may well be possible to saturate adsorbtion sites with liposomes too, which might contribute to the longer circulation time of the vesicles. Hence, the lack of increased liposome uptake by other tissues following liver blockade does not necessarily mean that the proportion of the dose unaccounted for in the liver and spleen would normally be taken up by tissue cells other than the macrophages.

B) Mechanisms of liposome uptake.

Several questions arise from the subcellular fraction data and the published data on liposome uptake by the liver : -

i) Is the measured radioactivity still entrapped in liposomes, i.e.: are the liposomes still intact ?

ii) What conclusions can be drawn about the mechanisms involved in liposome uptake by the liver ?

iii) Are the lysosomes the only organelle involved in the uptake of liposomal radioactivity ? and

iv) Can lysosomally localized drugs escape from the organelle and be found in other compartments ?

These questions are relevant not only to the uptake and disposition of both free and entrapped drugs but also to the ways in which these agents are active in vivo against nucleic acid synthesis.

i) Are liposomes taken up intact ?

A definitive answer to this question cannot be given from this work. A comparison of the plasma clearance and tissue distribution of free and entrapped drugs in previous sections has shown that : -

a) Liposomes are not stable in the plasma but may lose both lipid and entrapped agents during their time in the circulation and

b) several of the drugs in their unentrapped from will concentrate at least temporarily, in the liver.

It, therefore, seems possible that, at least some of the liver radioactivity could be due to free (i.e. : released or leaked) drug. It must be remembered also that some liposomes will be destroyed during the homogenization procedure whether they are loosely bound to the outer surface of the liver (i.e. adsorbed to capillaries or membranes : Gotfredsen, 1982) or are already within the cell. Control values for the extent of liposome destruction during homogenization cannot be used since the major part of both the free and the entrapped drugs are localized in S. On the other hand, the marked differences in some of the liver fractions (e.g. : free ACT-D in N versus entrapped in ML or free ^{125}I-BLMs in S versus entrapped in ML) strongly suggests that the entrapped drug is handled in a different way from the free. In addition, the well known localization of particulate material in rat liver lysosomes is totally compatible with the finding of liposome-derived radioactivity in this fraction. In general, therefore, it may be stated

with confidence that at least some of the liposomes are taken up into the liver as whole structures. What cannot be ascertained from these or any other in vivo studies is the extent to which free (i.e. : leaked) drug, formerly entrapped, contributes to the radioactivity found in the ML or any other fraction. In this respect the ML results with free ^{111}In-BLM serve as a warning against the easy assumption that lysosomal localization can be equated with entrapment of the drug.

Several investigations in vitro (with or without serum in the culture medium) have concluded that liposomes do not need to be taken into cells whole to account for all of the radioactivity found in sub-cellular fractions. Whilst, in this author's view, a distinction has to be made between cells which will normally take up particulate matter whole (e.g. macrophages) and other cell types, it is clear that the capacity to accumulate particulate material is not a unique function of "professional endocytosers" but can be found and/or induced in most cells in culture (facultative endocytosers) (Silversein et al., 1977).

ii) Mechanisms of uptake.

The mechanisms of uptake of liposomes by the liver can be divided into at least four categories (see chapter 1) and the initial localization of the entrapped solute and liposomal lipid will be different in most cases. Since the uptake mechanism is intimately connected with the subsequent fate of the liposomes, questions, ii, iii and iv (see above) will be considered here.

a) Endocytosis.

It must be stated at the outset that endocytosis does not lead automatically to lysosomal localization of the vacuolated material. In this respect the work of Siverstein et al (1977), Goldstein et al. (1979), Hart (1979), Jacques (e.g. 1981), Pastan and Willingham (1981) and Willingham et al. (1981) prompts the addition question : "what type of endocytosis results in liposome uptake" ?

aa) Pinocytosis.

In this mechanism extra-cellular fluid is taken into a cell non-selectively so that the intracellular concentration is linearly related to the extra-cellular concentration and to the incubation time provided that intracellular digestion and/or release of labelled material does not occur (Silverstein et al, 1977).

Fluid pinocytosis can be divided into two types, micropinocytosis and macropinocytosis, which may be descriminated by the

quantity (volume) of fluid captured and therefore the size of the initial pinocytic vesicle formed. This recognises that pinocytic vesicles may fuse together or break up into smaller structures at any time (Jacques, 1981). The micropinocytic vesicles are considered by Pastan and Willingham (1981) to be formed from caveolae which are present on the surface of cells. These small membrane invaginations appear to be about 40 nm. in diameter (calculated from Pastan and Willingham, 1981) but they have a "neck" at the surface very much smaller (perhaps 10 nm). Poste and Papahadjopoulos (1976b, 1978) consider that SUVs are too large to enter these structures and the resulting intracellular vacuole too small to accomodate these liposomes, yet Pastan and Willingham (1981) claim that the micropinosomes have a diameter of 80 nm. which could accomodate a 25 nm. diameter SUV. It may well be that the constricted "neck" region will exclude SUVs from entry.

Macropinocytosis (Willingham and Yamada, 1978) occurs when a cell membrane ruffle falls back onto the surface of the cell trapping extra-cellular fluid and injecting it into the cell interior inside a macropinosome (300 - 500 nm diameter). Liposomes adsorbed onto the cell membrane could become trapped beneath falling membrane ruffles and so interiorised whole even in cells which have minimal endocytotic ability.

Pinocytosis in general is not impared by agents which inhibit phagocytosis (Poste and Papahadjopoulos, 1978) but it is arrested at 4° C (Silverstein et al., 1977) although adsorbtion of material to cell surfaces will continue at this temperature.

The fate of the micro or macropinosomes remains unclear. Pastan and Willingham (1981) claim that these vacuoles fuse rapidly with lysosomes in vitro. However, in certain cell types (e.g. vascular endothelium and epithelial cells) it has been shown that these pinosomes can shuttle materials from the capillary lumen to the tissue spaces and across the walls of the foetal and neonatal gut (reviewed Silverstein et al., 1977). In addition, vesicles containing newly synthesised proteins can traverse hepatocyte cell interiors to the outside of the cell without fusing with the lysosomes (Davis and Tai, 1980). The factors which regulate this vesicular transport remain unknown : the vesicles may be "marked" in some way (owing to either their contents or the membrane which surrounds them) which prevents lysosomal recognition.

Some of the free drug or liposomes within pinosomes could, theoretically, escape digestion and be released into the cytoplasm. The capture of free drugs from the plasma is probably mediated by the pinocytosis system as well as by their lipid solubility which allows them to traverse cell membranes. Is this the explanation of the presence of free drugs in the S fraction ? In the case of ASPase, it is known that other proteins are endocytosed and broken down in the lysosomes (Gordon and Cohn, 1973; Ryman and Gregoriadis, 1972b; Gotfredsen, 1982). Moreover, the lysosomal contents are rarely if even regurgitated into the cytoplasm and undigestible material stays within the lysosomes which may continue to function as secondary lysosomes (Silverstein et al., 1977) or loose their enzymes (Jacques, 1981). It seems unlikely, therefore, that ASPase can escape intact from lysosomes.

Equally, it seems that ASPase will probably not be able to penetrate through cell membranes directly. However, it is known that both liver and serum ASPase will associate with membranes (Suld, 1976) and can be found in S in the guinea pig liver possibly associated with small non-sedimentable vesicles. Uptake of membrane pieces during endocytosis may therefore transport ASPase bound to its surface into the cell (i.e. into P or S). In this present study cytosolic localization of free ASPase could be due to its entrapment in pinosomes which may account for the fact that most of the of the enzyme is TCA-pptable. It might be asked therefore if this S fraction ASPase is biologically active i.e. : could asparagine enter the vesicle and ammonia and aspartate escape? If this is possible, as has demonstrated for liposome-entrapped ASPase (Fishman and Citri, 1975; Neerunjun and Gregoriadis, 1976), it would appear that this method offers a good intracellular depot of enzyme.

Similar reasoning could explain the S-fraction localization of ACT-D and BLMs. However, in the case of ACT-D its lipid solubility may allow a rapid diffusion from the plasma into the cell and escape from the pinosomes. The fall in S levels of iodinated BLMs may be explained by the fact that the vacuolated drug does not effect the membrane in the same way as ASPase and so the pinosomes containing these drugs slowly fuse with lysosomes resulting in a steady increase in drug radioactivity in ML.

Alternatively, the inability of lysosomes to inacativate BLM

(Umezawa, 1974) may induce an pseudo-storage condition in the organelle which might delay the further fusion of these lysosomes with pinosomes. It is also possible that the free drug escapes from the pinosome since it has been shown that inactivation in S occurs in vivo as well as following the incubation of BLM with tissue cytosol extracts (Ohnuma et al., 1974).

In summary, the initial concentrations of free drugs in S may be the result of drug sequestered in pinosomes. At later times the BLMs probably escape into the cytoplasm and whilst such an escape may also occur with ASPase this enzyme could also remain membrane bound. Actinomycin-D probably escapes from the pinosomes as a result of its lipid solubility which also aids its entry into the cell. The cells involved may be both PCMC and KC.

Liposomes (primarily SUVs) within pinocytic vacuoles may well be handled in a similar manner to the free drugs so that some of the S fraction could contain vesicles within vacuoles. If, however, only the larger macropinosomes contain liposomes, the chances are that the S fraction will not contain vacuoled SUVs because these larger pinosomes may be sedimentable at 100.000g and will therefore be found in the P fraction along with other (phagocytic) vacuoles and membrane-bound free or entrapped drug.

ab) Receptor-mediated endocytosis (RME).

The study of this mechanism of endocytosis of ligands has recently received much attention (Goldstein et al., 1979; Pastan and Willingham, 1981; Sly, 1981; Willingham et al., 1981) and only a general outline of the proposed mechanisms will be given.

The basic features involve cell membrane receptors specific for certain ligands (e.g. LDL, Hormones, alpha-2-M, certain carbohydrates and the Fc portion of IgG). The density of these receptors can be very great, e.g. : fibroblasts have been reported to contain 200,000 receptors specific for alpha-2-M per cell, and they are diffusely distributed over the cell surface. Binding of a ligand to its specific receptor appears to result in a lateral movement of the ligand/receptor complex to cluster in specialized structures on the membrane termed bristle coated pits. The bristle coat is composed of the protein clathrin. The whole pit is closed and interiorized to form a specialized intracellular vesicle called a "receptosome" which contains the ligand bound to the receptor on its interior surface but not still surrounded by a

basket of clathrin. Receptosomes do not immediately fuse with lysosomes but move towards the GERL complex (GERL : Golgi-endoplasmic reticulum-lysosome) close to the nucleus. Eventually fusion between the receptosome and newly synthesised lysosomes occurs but at some stage the ligand becomes separated from its receptor; possibly by changes in ionic or pH conditions rather than by the action of degrative enzymes.

The receptosome membrane appears to be able to escape lysosomal degradation so that the membrane material and receptors are not lost to the cell, which would necessitate wasteful resynthesis, but are able to recycle back to the plasma membrane (Schneider et al., 1979, 1981). It seems clear that the ligand bound within the receptosome and/or the specificity of the receptor can determine the fate of the receptosome. Thus LDL, alpha-2-M, lysosomal enzymes and glycoproteins, containing terminal residues of asialo, mannose or N-acetylglucosamine, interact with receptors at the cell surface, are endocytosed and end up inside the lysosomes to be digested. Immunoglobulins (IgG and polymeric IgA) can escape lysosomal digestion and can be exported from the cell or later found back on the cell membrane (Schneider et al., 1981).

The possibility that the digestion of endocytosed material is delayed or prevented from occuring has obvious and exciting implications for drug delivery systems. Liposomes (SUV) bearing specific antibody have already been shown to enter cells by RME (Leseman et al., 1980a) and it seems likely from the work of Gregoriadis (reviewed Gregoriadis, 1980 a, b, c ; Gregoriadis and Meehan, 1981; Gregoriadis et al., 1981b) and Heath et al. (1980), using IgG in or on liposomes, that they also use this pathway. However, the manufacture of liposomes bearing ligands on their surfaces may only alter the mode of uptake (RME vs phagocytosis) whilst the intracellular handling of the vacuole may depend upon the presence or absence of the specific markers.

Facets of RME which are of interest are those associated with all receptor-ligand systems, namely; its saturability at high ligand concentration and the ability of structurally similar ligands to compete with or block uptake. The claim that liposomes are taken up by RME is verifiable by experimentation. Blockade of uptake by competing IgG or other ligands (which might be co-in-

teriorized in the same coated pit) should be demonstrable and saturation ought to occur at high liposome doses.

For RME there are some other properties which may have bearing upon possible free or entrapped drug endocytosis. The effect of charge and in particular cationic amines has already been alluded to in relation to their ability to induce lipid storage diseases (Lüllmann - Rauch, 1979). Some amines (methylamine but not chloroquine) have the ability to inhibit the clustering of the ligand/receptor complex in coated pits (Maxfield et al., 1979) which effectively blocks RME. Other amines (e.g.: chloroquine) are known to accumulate in lysosomes by the method of proton trapping (de Duve et al., 1974) where they raise the intralysosomal pH (Ohkuma and Poole, 1978) and thereby inhibit lysosomal enzyme function (Wilbo and Poole, 1974). Chloroquine and other lipophilic amines not only promote the lysosomal uptake of some (but not all) materials from pinosomes and receptosomes but may also inhibit the release of both the digested material and vacuolar membranes which cannot recycle back as fast to the plasma membrane (Schneider et al., 1981). The mode of action of chloroquine has not been firmly established in all cases but it may be similar to that found for other cationic compounds which promote lysosome-phagocytic vacuole fusion (Hart, 1981). It is appropriate to mention here also that in true endocytic vacuoles anionic and cationic compounds also effect the closeness of the vacuole membranes to their contents (Hart, 1981). In cells pretreated with anionic compounds there appears to be a gap between the vacuole contents and the membrane whereas cationic compounds produce vacuoles whose membrane tightly surrounds the inclusion.

Without a detailed knowledge of the receptor repertoire of liver cells it is difficult to know whether any of the free drugs are taken up by RME. In view of the possible harmful damage that could result from excess circulating ASPase it could be postulated that a recognition system exists to remove this enzyme from the plasma but to-date no data is available to prove this. Similarly, it is possible that BLM or ACT-D might cross-react with some physiological receptor and so gain entry into the cell's interior by RME. Mention has already been made of the possibility that ^{111}In accumulation in the ML is mediated by RME of transferrin.

For the liposome entrapped drugs the situation is somewhat

different. In chapter 3 it was shown that MLVs will interact with proteins and in this respect the interactions with LDL and alpha-2-M may be crucial. Receptors for LDL and alpha-2-M have been demonstrated on the membranes of cells in vitro (Goldstein et al., 1979; Pastan and Willingham, 1981; Willingham and Pastan, 1980) and more importantly, it has been demonstrated that colloidal material (gold) adsorbed to alpha-2-M is taken up by RME in fibroblasts (Dickson et al., 1981). The gold particules were 100-200 nm diameter and each contained about 400 molecules of alpha-2-M. This demonstration therefore suggests that in vivo smaller liposomes (100-200 nm) with alpha-2-M on their surface could enter cells by RME. Although alpha-2-M was only found associated with MLVs in this study it seems reasonable to suppose that SUVs would also interact with this protein so that any liposome up to 200 nm might enter cells in this way (receptosome : 200-400 diameter, Pastan and Willingham, 1981). However, the larger MLVs are probably too big to enter the coated pit and it is not yet known if either the coated pit or the receptosome can enlarge to accomodate bulky ligands.

In the liver it might be possible, temporarily, to block (saturate) RME with excess alpha-2-M or other ligands preadministered or co-admistered with liposomes. Such an experiment might determine, by comparison with the unblocked state, the extent of RME. However, since blockade of KC phagocytosis is probably impossible with liposomes (see previous section), the liposomes which might normally be taken up by RME could simply be redirected to phagocytosis.

Uptake of LDL-liposome complexes by RME is a more uncertain process when it is recalled that little or no lipoprotein was found associated with the MLVs. Exchange of cholesterol between liposomes and LDL or HDL in the vicinity of cell surface receptors for these lipoproteins might result in RME of lipoprotein-liposome complexes. Yet it must be remembered that LDL uptake results in the loss of the membrane LDL receptors (Goldstein et al., 1979). This process is therefore less plausible than that for alpha-2-M but it could be explored experimentally using LDL and/or HDL to block RME uptake in the presence of SUVs.

a c) Phagocytosis.

The uptake of particulate material into cells by the process

of phagocytosis has been widely reviewed (for a review of reviews see Jacques, 1981) and this will not be repeated here. However, several features of the process are of interest in relation to liposome uptake and will be discussed in more detail.

The basic features of phagocytosis do not greatly differ from pinocytosis and include the following steps : attachment, engulfment, fusion and degredation. Two differences can distinguish pinocytosis from phagocytosis: i) the size of the endocytosed material, and of the endocytic vacuole, are orders of magnitude larger in phagocytosis and ii) phagocytosis appears to be a discrete process whereas pinocytosis is continuous.

The uptake of entire liposomes in vivo is probably due, in large part, to phagocytosis because of their size (for MLVs at least), particulate nature and phagolysosomal localization. The regulation of their uptake during the four stages outlined above remains unexplored.

i) Attachment.

It has been shown; both in vivo and in vitro that macrophages possess receptors which recognise certain proteins or protein domains (Kaplan, 1981). These receptors are distinct from those, present on most cells which recognise specific ligands (i.e. in RME). These receptors are those which bind the Fc portion of IgG and the C_3 component of the complement cascade. Normally these receptors are removeable by protease treatment but some Fc receptors are resistant to this process. In addition, a variety of "non-specific" receptors have been reported for such things as: bacteria, zymosan, latex, denatured protein aggregates, DNA and dead or damaged cells. Phagocytosis of these agents is not dependent upon the presence of IgG or C_3 but it is unlikely that a "specific" receptor exists for each agent. Some common binding sites (e.g.: for mannose (bacteria) or galactose (red blood cells)) may account for some particle phagocytosis.

It will be recalled that IgG was not found on the surface of liposomes following their incubation in plasma (chapter 3) although Juliano and Lin (1980) found immunoglobin heavy and light chains bound. In addition, Weissmann et al., (1974, 1975) found aggregated IgG and IgM would interact with liposomes and bind them (via Fc fragments) to macrophages.

However, in vivo liposomes coated with aggregated IgG did not show any significantly different uptake by the liver (or other tissues) from similar, uncoated, MLVs following I.P. injection into rabbits (Weissmann et al., 1978). Moreover, the findings of several authors (e.g.: Poste and Papahadjopoulos, 1976a,b) that liposomes can be endocytosed in the absence of serum suggest that although Fc-receptor mediated uptake can occur it is not an absolute requirement for sucessful phagocytosis of liposomes. Yet some such receptor may exist since Pagano and Takeichi (1977) found that vesicles adsorbed to cell surfaces could not be removed by washing but were rapidly detached when the cells were trypsinized, implicating a cell surface protein; others do not agree with these findings (Magee et al., 1974; Poste and Papahadjopoulos, 1978).

Attempts to further increase the binding of liposomes to cells with combinations of glycolipids and lectins, while successful, do not enhance the intracellular delivery of the vesicle contents (Juliano and Stamp, 1976; Szoka et al., 1981) although this may depend upon the type of cell used (Suriola and Bachhawat, 1977; Alving et al., 1979).

The function of charge in mediating the attachment of liposomes to cell surfaces has been suggested as a means by which phagocytosis is stimulated (Magee et al., 1974) or membrane fusion is promoted (Poste et al., 1976; Stendahl and Tagesson, 1977). However, such interactions are strongly inhibited by serum (Tyrrell et al., 1977; Mayhew et al., 1980). Moreover, in the light of the acquisition of a net negative charge by particles (Wilkins and Meyers, 1966) and liposomes (Black and Gregoriadis, 1976) when in contact with plasma it seems unlikely that charge interaction is the principal stimulus.

In summary, the factor(s) responsible for the attachment of liposomes to the cell surface in vivo remain unknown. Some sort of receptor may well exist but this is unlike to be specific for liposomes and so it may prove to be blockable by other materials. Whatever its nature, the receptor must not only bind the liposome but also transmit a signal to the cell which either starts the engulfment process or which is some-

how overidden and allows for the stable adsorbtion of vesicles to the cell surface.

ii) Engulfment.

The exact mechanism whereby a cell is induced to extend its cell membrane (pseudopods) to surround an attached particle remains unknown. Currently, the "Zipper" hypothesis (Silverstein et al., 1977, 1981) has proved to be the best explanation: following binding of the particle to a macrophage membrane, pseudopods are induced to extend possibly by lateral movements of similar membrane receptors into the initial area of binding. As more receptors bind the pseudopods advance bearing empty receptors seeking a binding site. Eventually, the advancing lips of the invagination meet and fuse having completely surrounded the particle.

Silverstein et al., (1977, 1981) have suggested that clustering of cell receptors also results in clustering and assembly of contractile proteins in the area beneath the membrane where the receptors have bound to the particle surface. Using the "muscle-like" properties of these proteins (actin and perhaps myosin) the vacuole is pulled into the cytoplasm of the cell. Cytochalasins at low concentrations (10 μM) inhibit actin polymerization and phagocytosis by macrophages. Colchicine, which depolymerizes cytoplasmic microtubules, does not inhibit phagocytosis but it has a maginal inhibitory effect on pinocytosis. The lack of inhibition by colchicine also implies that phagocytic vacuoles are not guided to lysosomes by microtubules. Although Ca^{2+} has been implicated in the binding of surface receptors to the underlying actin fibres (Silverstein et al., 1981) extracellular calcium is not required for phagocytosis. Depending upon the liposome composition, Poste and Papahadjopoulos (1976a) found that cytochalasin B inhibited the uptake of liposomes by endocytosis. Similarly, inhibitors of respiration and glycolysis, used in tandem, were found to block endocytosis but not if they were used separately.

The fate of the phagocytic vacuole in the cytoplasm has been described by Hart (1979) as "the hinge" which determines the destination of the contents. It is noteworthy that almost all parasites which survive and multiply intracellularly are

able to subvert the normal course of events (i.e.: fusion of the vacuole with and digestion in lysosomes) at this stage. Whilst part of this subversion is undoubtedly due to parasite-derived factors, it suggests that phagocytic vacuole is a vulnerable structure and the question must be asked if liposomes are also capable of altering the vacuole in some way.

As has been mentioned previously, endocytic vacuoles need not interact immediately with lysosomes. Indeed, studies on particle endocytosis (Munthe-Kaas, 1976), liposomes (Segal et al., 1974; Rahman and Wright, 1975; de Barsy et al., 1976) and receptosomes (Willingham and Pastan, 1980) have demonstrated that vacuoles can be found in the cytoplasm for hours or even days after phagocytosis. For a drug which leaks from liposomes and can traverse membranes, these sub-cellular deposits may well account for the slow release of materials into the S fraction. It must be stressed that these are not terminal phagosomes (Jacques, 1981) in the same sense that parasitophorous vacuoles are a surviving, semipermanent, sub-cellular structure. Further, interactions between phagocytic vacuoles often occur prior to their fusion with lysosomes (Jacques, 1981) and this may result in large endosomes containing the contents of several smaller phagocytic vacuoles.

A second interaction which may occur between the entrapped liposome and the vacuole is fusion. (Poste and Papahadjopoulos, 1978). Evidence that fusion between liposome and cell membranes occurs will be presented below, here the concern is the consequences of this interaction. It has already been mentioned that, according to the zipper hypothesis, the psuedopods of the forming vacuole closely follow the shape of the particle and form a tight seal with its surface (Silverstein et al., 1977). Moreover, macrophage uptake of extracellular fluid is not increased during particle phagocytosis (quoted in Silverstein et al., 1977) suggesting that fluid is actively "squeezed" out of the vacuole. Therefore, inside the vacuole the membranes of the liposome and the phagosome are in very close contact; extracellular fluid (e.g.: serum), which is know to inhibit fusion, has been

removed so that the only barrier to fusion would be proteins adsorbed onto the liposome surface (some of which may have been mechanically removed by the advancing psuedopods) and cell membrane proteins which, in the absence of serum, do not appear to present a barrier to fusion (Pagano and Weinstein, 1978). If fusion is a valid method of internalization of liposomes and their contents then it seems to this author that the conditions within a phagosome for this to occur may be more favorable than those which are found when liposomes interact with cell surfaces. The factors which influence liposome fusion to cell membranes (e.g.: fluidity of the liposome phospholipid acylchains and charge on the membrane : Poste and Papahadjopoulos, 1978; Papahadjopoulos, 1978b) will also be active within the vacuole.

The consequences of such fusion are interesting since unilamella vesicles (LUV or SUV) will discharge their contents into the cytoplasm whilst MLVs will be found whole in the cytoplasm but minus their outermost bilayer. The hypothesis predicts that all of the LUV and SUV membrane lipids will be found associated with phagocytic vacular membranes. It also predicts that some MLVs will be found which are not surrounded by vacuolar membrane. The fate of these MLVs is unknown but they may be endocytosed by lysosomes as has been reported for other particles; (Glaumann and Marzella, 1981). If the phagosome membrane is able to recycle in a similar fashion to that proposed for receptosomes and pinosomes (Schneider et al., 1979) the liposome membrane could be re-incorporated into the plasma membrane at some later time. In this case the overall picture would be exactly equivalent to that seen when fusion of liposomes with cell membranes occurs. Conversely, if the phagosome membrane is digested by the lysosomes (i.e.: it does not recycle) the distribution will be similar to that seen during lysosomal breakdown of the liposomes followed by partial release of the entrapped solute (radiolabel) into the cytoplasm. Since none of these mechanisms are mutually exclusive a careful kinetic study of lipososme lipids and entrapped markers in vacuolar and membrane sub-cellular fractions will be necessary to prove the hypothesis.

Evidence against the fusion of liposomes with endocytic vacuoles comes from attempts to inhibit phagocytosis (Poste and Papahadjopoulos, 1976, 1978) in which membrane associated lipid and cytoplasmic localization of liposome-entrapped agents was found following Cytochalasin B treatment. This suggests that some liposome/plasma membrane fusion had occured although whether this will happen in the presence of serum remains in question.

This intracellular fusion hypothesis may explain the persistence of MLVs in the PCMC cytoplasm for prolonged periods (Rahman and Wright, 1975). In addition, bearing in mind that cationic liposomes appear to fuse better than others (Poste and Papahadjopoulos, 1976a, 1978), this mechanism may also explain the findings of Steger and Desnick (1977) that enzyme entrapped in cationic liposomes persists in active form for longer in the cytoplasm than that from anionic liposomes. If the escape of the liposome from the vacuole occured during phagosome-lysosome fusion this hypothesis might also explain the increased release of lysosomal enzymes found by these authors.

iii) Vacuole-lysosome fusion

The exact mechanism underlying this process are not well understood. The process of fusion between membranes is an area of active research (Poste and Papahadjopoulos, 1976b; Lucy, 1978; Papahadjopoulos, 1978; Poste and Pasternak, 1978) which has resulted in the implication of a variety of factors (e.g.: fatty acids and lysolecithin) in the event. One must, however, question whether model systems of cell-cell, cell-vesicle or virus-cell fusion have direct application to intracellular fusion. The two surfaces in contact in vacuole-lysosome fusion are the inner membranes of the cell which are known to be different from the external membrane surfaces not only in their protein composition (de Duve and Wattiaux, 1966) but also in their phospholipids (Papahadjopoulos, 1978).

Two other factors which are important for the fate of the vacuole contents have not received much attention: i) Location; how do the vacuoles find the lysosomes(or vice versa) since it is possible that they are not guided to each other

by microtubules (Colchicine and related alkaloids do not effect particle phagocytosis) (Silverstein et al., 1977) and ii) Recognition; how do vacuoles know that they are in contact with lysosomes? One mechanism, involving carbohydrate moieties, has been recently proposed following the discovery that phagosome-lysosome aggregation in vitro was inhibited by mannose, fucose, lactose, N-acetylglucosamine and fetuin (Amano and Mizuno, 1981).

The studies of Hart (1979, 1981) have demonstrated that pretreatment of macrophages with anionic or cationic drugs can modulate the fusion of vacuoles with lysosomes. Polyanionic compounds (dextran sulphate, suramin, polu-D-glutamic acid) apparently enter the lysosomes by endocytosis and effect changes which reduces the fusion of these organelles with vacuoles; secondary or tertiary lipophilic amines (chloroquine, tributylamine and some local anaesthetics) also accumulate in lysosomes, perhaps by permiation, pretreatment of macrophages with these compounds results in increased phagosome-lysosome fusion. It seems possible that charged liposomes might influence the phagosome-lysosome fusion of other incoming liposomes. Indeed, an anionic sulphatide has been isolated from Mycobacteria tuberculosis, an organism which can evade lysosomal digestion, and medium from M.tuberculosis cultures will also inhibit the organelle fusion (Hart, 1979; 1981).

iv) Degredation.

The complete breakdown of complex biological structures (e.g.: bacteria and other parasites), which is a generally accepted function of macrophage lysosomes, probably holds true for most lysosomal systems. It is not surprising, therefore, to find that liposomes can be broken down in these organelles, indeed, the usual method of preparation of substrates for phospholipases involves the formation of liposomes.

Fowler and de Duve (1969) reported that phospholipids and sphingomyelin could be partially degraded in lysosomes although the type of degradation and the enzymes involved were not fully identified. Recently this subject has been reviewed by Waite et al.,(1976) and Van den Bosch (1980).

Most cellular and subcellular membrane preparations exhibit some phospholipase activity although how the enzymes remain inactive whilst surrounded by a sea of substrate remains uncertain. Interestingly, it has been suggested (Van den Bosh, 1980) that the plasma membrane of hepatocytes contains phospholipase A, orientated towards the exterior of the cell. Inclusion of this enzyme on the interior membrane of a phagocytic vacuole might allow the generation of monoacylglycerophophatide (e.g.: LPC) from liposomes and these molecules have been proposed as inducers of fusion between membranes (Lucy, 1978). This may, therefore, explain the mechanism of intracellular fusion proposed above.

Liposomes composed of dialkyl analogues of PC were reported to be three times more resistant to hepatic breakdown than the normal diester PC liposomes. The liver half-lives were of the order 8 hours (diester PC) and 24 hours (dialkyl PC) with the increase being ascribed to a resistance of the alkyl-PC to phospholipase hydrolysis (Deshmukh et al.,1978). The data on diester-PC agree quite well with the breakdown of labelled liposomal phospholipids reported by Glaumann and Marzella (1981) who found that the lysosomal half-lives were between 4 hours (^{32}P-labelled and ^{14}C-choline labelled PC) and 6 hours (^{14}C-glycerol labelled PC); cholesterol had a half life of 9 hours but removal of esters from E-CHOL occured with a half life of 3 hours. Using the same system unentrapped proteins and glycoproteins had half-lives of 0.8-2 hours.

Hwang et al., (1980) found a hepatic degredation (i.e.: release of entrapped drug) time of 3 1/2 hours for sphingomyelin: CHOL SUVs. However, this time probably does not reflect degredation of the liposomal lipids since Fowler and de Duve (1969) found that sphingomyelin was only hydrolysed to ceramide and phosphorylcholine. Hydrolysis of the ceramide either in lysosomes or in the whole liver was not found. Gotfredsen (1982), using larger SUVs containing PC, reported that the digestion products from entrapped ^{3}H-albumin were detectable in the plasma 15 minutes after I/V injection. The major portion of the ^{3}H radiolabel was excreted in the urine. Conversely, ^{14}C-PC was found to be excreted (as $^{14}C\text{-}CO_2$) in

the expired air of mice after a delay of two hours, thereafter excretion increased linearly up to 45 % of the injected dose at 12 hours; almost no $^{3}H\text{-}H_2O$ (from albumin) was found in the expired air. This data suggests that rapid hydrolysis of entrapped proteins preceeds liposome membrane digestion.

The hydrolase enzymes responsible for protein digestion are mainly present in the lysosomes (Gregoriadis, 1975; Dean and Barrett, 1976) and they are capable of reducing proteins and glycoproteins to dipeptide and amino acid units which may then permiate the lysosomal membrane (Goldman, 1976) to the outside. The short half-lives of intact proteins in the lysosomes may well account for the rapid fall in total hepatic ^{125}I-ASPase in this study.

Inhibition of lysosomal enzymes has been reported. The most relevant case is the effect of cationic amines on the breakdown of endogenous and exogenous polar phospholipids (Lüllman - Rauch, 1979). The mode of action of these drugs (e.g. chloroquine) remains uncertain but they may either bind to the phospholipid or the enzyme or act by altering the intra-lysosomal pH(Wibo and Poole, 1974; Ohkuma and Poole,1978) Larger complex amines (e.g. aminoglycoside antibiotics : Tulkens, 1979) probably have the same effect and one wonders if the presence of BLM might also inhibit phospholipid breakdown.

The activity of entrapped drugs against DNA and RNA synthesis suggests that liposome entrapment protects the drug from degredation until such time as it has acted. In the case of ACT-D, which contains two pentapeptide rings of amino acids (fig. 2:1a) this activity requires that the pentapeptide rings remain intact and allows only limited substitution of one amino acid for another without severe loss of its ability to bind to DNA (Goldberg, 1975; Sobell, 1974). For instance, when L-proline is replaced by L-hydroxyproline in only one of the pentapeptide chains biological activity is reduced to 5 % of the initial value. Goldman (1976) has suggested that the stereospecificity of the pentapeptide ring makes the peptide bonds resistant to enzymatic hydrolysis; probably because of the presence of the D-isomer of valine.

The carbohydrate moieties are common to all BLMs (Umezawa, 1973, 1974) and the glycosidic bond in 3-O-carbomyl-alpha-D-mannopyranosyl (1-2) alpha-D-gulopyranose should be hydrolysable by lysosomal enzymes. Perhaps the presence of gulose or its beta-1 linkage to hydroxyhistidine hinders breakdown. On the other hand, the action of cathepsins on the peptide portion of the BLM molecule should not be inhibited except perhaps by its unusual amino acid structure.

The stability of ASPase in the presence of lysosomal enzymes is unknown but there is no reason to suppose that free ASPase (i.e. not membrane bound) would be any more resistant to enzymatic attack than, for example, albumin. The presence of non TCA-pptable material in the whole liver suggests that this may be the case.

Finally, the presence of proteins on the liposome surface may have some effects upon liposome lysis and the breakdown of their contents in lysosomes; in this respect alpha-2-M is a unique protein. However, on teleological grounds it is difficult to see why a protein, which apparently acts like an opsonin in the plasma, should concurrently inhibit the digestion of the opsonized material.

The exact in vivo role of alpha-2-M is not understood. However, in vitro alpha-2-M will bind to many (perhaps all) endopeptidases; these include; lysosomal enzymes (cathepsins B, D and G, elastase, collagenases) and blood enzymes (plasmin, thrombin, kallikrein, plasminogen activator) (Barrett and Starkey, 1973; Werb et al., 1974; Harpel, 1976; James, 1980). This has led to the suggestion that alpha-2-M may operate as a scavenger of damaging enzymes released into the plasma as the result of cell death, phagocytosis, bacterial invasion or normal clotting mechanisms. (Harpel, 1976).

Other proposed actions of alpha-2-M have been reviewed by James (1980) and involve modulation of the immune system. Thus, alpha-2-M has been shown to bind to lymphokines, endotoxin, antigens and immune complexes as well as to colloidal material such as lipopolysaccharide, dextran sulphate, gold colloid (Dickson et al., 1981) and liposomes (Black and Gregoriadis, 1976).

The extent to which any or all of these effects are mediated intracellularly by the receptor-mediated endocytosis of alpha-2-M and its complexes is unknown but these data suggest the alpha-2-M may play a role in the control of the internal and external signalling which effects cell mediated immunity. The central question is whether alpha-2-M bound to liposomes might have similar effects. In particular, it would be important to know if alpha-2-M-liposome complexes can inhibit lysosomal digestion of lipid and proteins, at least at higher pHs, by "binding out" lysosomal enzymes : inhibition of, say, cathepsin B-alpha-2-M binding by SUVs would be easily testable and might reveal if the same portion of the alpha-2-M molecule is used for binding both structures.

b) Other mechanism of uptake: Fusion,lipid exchange and adsorbtion.

The forgoing extended discussion on endocytosis and its sequels reflects this authors belief that, in vivo at least, the major portion of liposome entrapped material enters cells by that method. Three other uptake processes have been suggested although other mechanisms or combinations of mechanisms may also occur.

Evidence that fusion between liposomes and cell membranes takes place in vitro has most recently been reviewed by Poste (1980). He has pointed out that certain criteria (e.g.: the demonstration that all endocytosis has been blocked and that entrapped material is present free in the cytoplasm in quantitatively similar amounts to the uptake of liposomal lipid) must be fulfilled before fusion can be evoked as the uptake process. The problems of interpreting these data have also been stressed (Poste and Papahadjopoulos, 1978; Poste, 1980) for, as has already been pointed out, intracellular fusion of liposomes with phagocytic vacuoles followed by membrane recycling would give similar results to those who claim that fusion has taken place extracellularly.

It is worth stressing again that in the presence of serum, or in vivo, unequivocable evidence that fusion takes place is lacking. Since we are concerned here with the possibility that the S fraction radioactivity is the result of liposome fusion in vivo, results which report the delivery of pharmacological molecules to cells or tissues, and which are subsequently found to be active, must be examined. Such a study presumes that : i) the unentrapped material will not normally enter cells;

ii) normally all endocytosed material is delivered to the lysosomes; iii) the material is sensitive to lysosomal digestion and cannot escape from these organelles and iv) the mode of action of the agent is not mediated by and does not act within the lysosomal compartment.

Data conforming to these criteria are not plentiful and often do not satisfy all the conditions. Examples in vivo come from the work of Gregoriadis and Ryman (1972b) who found an increased S fraction activity of entrapped invertase when compared with the same enzyme mixed in vitro with the whole liver prior to fractionation. Unfortunately, the distribution in vivo of unentrapped enzyme was not studied but these data suggest that it might bind to membranes. Similarly, Gregoriadis et al., (1974b) showed that although some neuraminidase activity could be found in the ML fraction this did not account for the total hepatic activity in mice injected with liposome-entrapped neuraminidase. Steger and Desnick (1977) found liposome entrapped enzyme in the S fraction for many days after injection.

These results (and those of this present study) are complicated by the fact that leakage of entrapped material from CHOL-poor liposomes may occur. Such may not be the case with macrophage activating agents entrapped in liposomes (Fogler et al., 1980; Hart et al., 1981; Fidler et al., 1981) which may have to bind to a surface receptor to be active (Poste et al., 1979). However, it is unclear if simple binding to this receptor is enough to activate the macrophage or if internalization (perhaps to the lysososmes?) is also required. Whatever the mechanism the liposome entrapped material released into the cytoplasm by either extracellular or intracellular fusion may be the active agent. Rahman (1979) has reported that EDTA is found almost exclusively within the S fraction when delivered to the liver by large MLVs despite the fact that normally EDTA does not enter this organ. It is possible that EDTA had entered and rapidly left the lysosomes or leaked from the liposomes intracellularly but its presence in S could also be due to fusion. However, it is difficult to equate this S fraction location with EDTA's action in removing heavy metals from the lysosomes unless the cytosolic drug is that which has already chelated its target.

Finally, liposome entrapment of polynucleotides results in interferon production in vivo and in vitro (reviewed Magee, 1980). It must be assumed that polynucleotides are sensitive to lysosomal digestion and, moreover, that their site of action is ultimately not in lysosomes because they must induce interferon production. Receptor mediated

endocytosis of these liposomes might deliver some smaller vesicles to the golgi-endoplasmic reticulum complex but it is possible that the finding of undegraded polynucleotides in the liver many hours after injection (especially when cationic liposomes were used) could also be due to fusion. Mayhew et al., (1977) have demonstrated that in vitro fusion is the mode of entry into the cytoplasm of polynucleotides entrapped in LUVs. As Magee (1980) has pointed out, the polynucleotides could induce interferon by acting at surface receptors which may mean that, like macrophage activators, they must either fuse with the extracellular membranes or else preferentially bind intracellularly to membranes which might then recycle to the cell surface.

Fusion in vitro has been demonstrated by experiments which have shown that large molecules sensitive to lysosomal digestion can be inserted into the cytoplasm; thus murine and human cell lines have been shown to translate rabbit globin m-RNA following interaction of liposome entrapped RNA with the cells (Dimitriadis, 1978; Ostro et al., 1978). Similarly Papahadjopoulos et al., (1980a) and Fraley and Papahadjopoulos (1981) have reviewed their work on the delivery of RNA, DNA and intact polio virions. These authors favour fusion as the method of entry of these large molecules into cells although they do not exclude other mechanisms. The amount of RNA delivered to the cells was estimated to be only 1 % of the entrapped nucleic acids and only 0.1 % of this was active; suggesting the fusion process is very inefficient. Addition of agents which promote fusion, e.g.:glycerol (Fraley et al., 1980) or lectins plus polyethelene glycol (Szoka et al., 1981), increase the uptake of molecules into the cytoplasm (but see Ralston et al., 1980 for contrary evidence).

Nevertheless, fusion has been suggested as the method of choice, because of its mild biologocal effects, for introducing plasmids (Fraley et al., 1979), DNA (Fraley et al., 1980) or whole chromosomes (Mukherjee et al., 1978) into cells of bacterial, plant or animal origin (Fraley and Papahadjopoulos, 1981). Szoka et al., (1979) have calculated that the increase in cell surface area would be up to 60 % if 1 % of SUVs fused with cells. (4×10^5 SUV per cell). This data was used to suggest that fusion did not take place because such extreme alterations in cell morphology have not been observed. In view of the recent recognition that fibroblastic L cells and macrophages (Steinman et al., 1976) can interiorize the equivalent (per hour) of 48% and 186%, respectively, of their membranes, it would seem that this may not be

a valid argument.

The incorporation of liposomal lipids or liposomal membrane-associated proteins (Poste et al., 1980; Poste, 1980; Gregoriadis, 1980a,b) has also been taken as evidence of fusion. As far as the latter are concerned, the recent work on the recycling of pinosome and receptosome membrane containing externally applied proteins (antibodies); (Tulkens et al., 1978; Tulkens, 1979; Schneider et al., 1979; 1981) would seem to indicate caution in this interpretation.

The known ability of lipids to exchange from liposomes to cell membranes (Pagano and Huang, 1975; Pagano and Weinstein, 1978; Sandra and Pagano, 1979) and thence to internal membranes suggests that, currently, these are not good indicies of fusion either. Using cultured fibroblasts, in the presence of serum, Mayhew et al., (1980) found most of the liposomal lipids and entrapped marker which were cell associated to be in the membrane fraction and no entrapped marker in the lysosomes; such a result is consistent with adsorbtion of the liposomes to the cell membrane.

Other workers have studied the uptake of liposomes, which have been in contact with cells, and then cultured with a second cell population. Papahadjopoulos et al., (1980b) reported that on second exposure the amount of membrane associated liposomal lipid was the same as in the primary exposure whilst the incorporation of entrapped marker (RNA) was reduced 10 fold. These authors take this to mean that less RNA leaked from the vesicle than before. On the other hand it is also consistent with the stabilization of the vesicle by prior contact with cells so that fusion no longer predominates (owing to the presence on the liposomes of some cell membrane derived lipids and proteins?) but that lipid exchange can still continue. Similar results had been previously reported by Dunnick et al., (1976b).

Finally, the adsorbtion of liposomes to cells in vivo and in vitro must be considered as a mode of cell/vesicle association. As has been previously mentioned, liposomes adsorbed in this manner may become detached during later proce dures (e.g.: homogenization) and give false intracellular distribution patterns. Mayhew et al., (1980) consider that, in the presence of serum, neither fusion nor endocytosis are of major importance in determining the total cell association of both LP and AP markers in cultured fibroblasts although they do not rule out some contribution from these mechanisms. These authors favor stable adsorbtion followed by phospholipid exchange and/or leakage of the

entrapped solute at the cell surface. In their initial study, Weinstein et al.,(1977) seemed to favour fusion as the method of liposome uptake by human lymphocytes but more recently (Blumenthal et al., 1979) they consider this less likely particularly in view of the lack of enhancement of solute uptake when known fusogens (LPC) were incorporated into the liposome (Ralston et al., 1980). Szoka et al., (1980) have also produced convincing evidence that in vitro SUVs adsorb to, but do not fuse with, various murine and human cells. Interestingly solid vesicles (e.g.: those with t_c > incubation temperature) adsorbed to a greater extent than fluid liposomes. The effects of charge on this adsorbtion were cationic >> neutral > anionic; fluid liposomes adsorbed to the same extent irrespective of charge. Similar results, i.e.: solid liposomes adsorb more avidly than fluid, have been reported by Mayhew et al., (1980) in the presence of serum.

Adsorbtion, in vivo, has been suggested as one mechanism which might account for the fact that only 50 % of injected liposomes were taken up by the liver (Souhami et al., 1981); Gotfredsen (1982) has shown that livers from liposome treated animals release liposomes when perfused. About 6 % of the injected dose of liposomes were loosely adsorbed to liver cells and were released by buffer and a further 2 % were released by collagenase perfusion.

In summary, the evidence in vivo points to the fact that much of uptake by the liver is due to endocytosis of intact liposomes and their contents. A smaller proportion of liposomes may become adsorbed to liver cell surfaces but it remains to be established if these liposomes will subsequently undergo fusion or lipid exchange or if they are adsorbed as a prelude to endocytosis.

CHAPTER 5.

Conclusions and future prospects.

Section 5:1. Conclusions

From the results presented in the previous chapters, the *in vivo* fate of liposome-entrapped agents following intravenous injection into rats may be summarised as follows :

i) Liposome preparations may contain a proportion of their entrapped agent outside (i.e.: leaked) of the bilayer structure depending upon the composition of the vesicle and this determines the rate of the release of the drug into the solution to be injected. In addition, some of the entrapped drug may reside on the outer surface of the liposome. Entrapment of the drugs used here does not appear to affect the overall anionic charge of liposomes but drugs sequestered in the lipid phase may, at high drug : lipid ratios, cause changes in the fluidity of the bilayers and aggregation of the preparation.

ii) Following injection (I/V), the entrapped drug is cleared from the circulation in a biphasic manner and this usually results in a longer plasma half-life of the liposomal agent when compared to the same dose of the free drug (but not in the case of ASPase).

During the first hour post-injection anionic MLVs, containing low amounts (20 mol %) of cholesterol, lose radiolabelled cholesterol to lipoproteins (LDL and HDL) whilst they are in the plasma but loss to erythrocytes does not appear to occur. The loss of CHOL is probably an exchange phenomenon rather than a net transfer.

Radiolabelled EPC in MLVs does not appear to transfer from MLVs to lipoproteins *in vivo* although some loss may occur from the SUV population.

iii) Other (non-lipo) proteins probably become adsorbed to MLVs during their sejour in the plasma. Experiments *in vitro* demonstrated that liposomes adsorb predominantly alpha-$_1$-macroglobulin from rat plasma and alpha-$_2$-macroglobulin from human plasma. Liposome/protein interactions in plasma results in the donation of a net negative charge to the vesicle irrespective of its initial charge.

iv) The tissue localization of drugs entrapped in anionic MLVs is, in general, different from that of the corresponding free drug. The liver and spleen capture most of the liposomal agents whilst other organs, e.g. : lung and small intestine, take up modest amounts. In tissues that do not contain substantial populations of phagocytosing cells, e.g. : kidney, muscle and brain, the uptake of liposome-entrapped drugs

is low or may be ascribed to blood contamination or leakage of entrapped drug. Except in phagocytosing cells or in tissues of particular architecture (e.g.: liver parenchymal cells), the ability of liposomes and their associated drug to escape from the bloodstream remains in doubt.

v) The mechanism of in vivo uptake of the liposomes used in this study is probably largely by endocytosis although other means cannot be ruled out. Intracellularly, liposomal drugs accumulate primarily in the lysosomal fraction of the liver from where some escape into other organelles (e.g.: nuclei) or compartments (e.g.: cytosol) is possible. Drugs which are either unable to escape from the lysosomes or are sensitive to lysosomal digestion may not be suitable as liposome-entrapped chemotherapeutic agents unless they can be released, e.g.: by intracellular fusion or other means, before reaching these organelles.

vi) Liposome-entrapped anti-tumour agents retain their ability to inhibit hepatic nucleic acid synthesis in vivo. However, only a proportion of the liposomal drug appears to be active, this may represent drug which has either leaked from liposomes or escaped lysosomal accumulation or entered the cell by a different mechanism. It seems possible that transfer of entrapped drug from one cell type to another may be limited and that the drug's activity against parenchymal cell nucleic acid synthesis is due either to capture of liposomes by these cells or to drug which has been released from the carrier at sometime.

Section 5:2 Future Prospects

i) In vivo uses of liposomes.

The initial reports of enzyme entrapment in liposomes (Sessa and Weissmann, 1970; Gregoriadis et al., 1971) and the subsequent demonstration that these liposomes could alleviate model storage diseases (Gregoriadis and Buckland, 1973; Colley and Ryman, 1974, 1976) raised the possibility that liposomes might be a universal carrier capable of delivering many types of therapeutic agents to diseased cells (Gregoriadis, 1973b, Gregoriadis et al., 1974a). These expectations have not been realised to any great extent mainly for the reasons discussed in this thesis, i.e. : i) leakage of entrapped agents from

liposomes, ii) instability of the carrier in the circulation with unwanted interactions with plasma components (e.g. : lipoproteins), and iii) a lack of tissue specificity or augmented tissue uptake into organs which do not have phagocytosing ability.

Nevertheless, early attempts at what might be termed "passive targetting", i.e. : allowing injected liposomes to find their own tissue distribution unaided, have produced much useful information on the characteristics of liposome disposition in vivo. This knowledge led to the design of a rational chemotherapy for Leishmania donovani infections in animals using liposome-entrapped antimonial drugs targetted to the liver and spleen lysosomes in which the parasites reside (Black et al., 1977). Similarly, the removal of toxic heavy metals, which also tend to accumulate in liver and spleen lysosomes, has proved to be possible using liposome-entrapped chelating agents whose distribution, in the free form, does not normally include these organs (Rahman, 1979, 1980). To-date neither of these useful applications have been applied to man. However, encouraging results have been reported by Gregoriadis (1979b, 1980a, b, 1981) on the use of a liposome-entrapped enzyme (human glucocerebroside : beta-glucosidase) in the therapy of a patient suffering from Gaucher's disease (a lysosomal storage disease) over a five year period. Clinically no untoward effects of this treatment have been reported and the liver size has not increased as would be expected in an untreated case. Similar clinical results have been reported by Tyrrell et al. 1976b) in another lysosomal storage disease. As yet it is uncertain if sites other than the liver have been affected by the liposome/enzyme therapy.

Other chemotherapeutic applications of liposome-entrapped drugs, e.g.: in the treatment of animal tumours, have not proved to be nearly so successful probably because of a lack of specificity for the tissues or subcellular sites. Although some augmented curative activity has been found in certain animal tumours using drugs entrapped in liposomes (reviewed : Gregoriadis, 1980a, 1980b; Ryman and Tyrrell, 1980) the 500-1000 fold increase in activity demonstrated against intracellular parasites has not been reproduced against tumours. If the antiparasitic activity of liposomal drugs is taken as a measure of the targetting capacity of liposomes then it is clear that the tumour results have hardly achieved any targetting at all. Indeed, current thinking now recognises that liposomes may be acting as circulating or stationary depots for the slow release of anti-tumour drugs (Mayhew

et al., 1979; Kaye et al., 1981) and that their anti-tumour effect will depend upon the co-incidence of this slow release with the phase in the tumour cell cycle sensitive to the drug's effect.

On the other hand, liposomes often reduce the acute toxicity of the entrapped drug (Black and Watson, 1980; Rahman et al., 1978) probably by preventing the interaction of the agent with sensitive tissues which would normally take up the unentrapped drug. Care must be taken, however, since if the site of drug toxicity is the liver or spleen entrapment of the drug in liposomes may increase its acute toxicity, for example : one site of toxicity for antimony-containing drugs is, like most heavy metals, the liver (Harvey, 1965); pentavalent antimony entrapped in liposomes has a 4-fold higher acute toxicity than the unentrapped drug and that of the entrapped trivalent drug is 10-fold higher : despite the fact that MLVs containing antimony are better than SUVs therapeutically against intracellular parasites, their acute toxicities are the same (Black; unpublished). Other workers have reported increased toxicity for antitumour drugs when in the liposome form (Rustum et al., 1979; Kaye et al., 1981). However, in some cases a reduced acute toxicity allows higher, curative, doses of the liposomal drug to be administered (Pirson et al., 1980; Pirson, 1982). Treatment of parasitic diseases in vivo with liposomal drugs is not always successful. Intracellular parasites in tissues other than the RES (e.g.: Trypanosoma cruzi infections of heart and skeletal muscle) are not amenable to treatment with liposome (MLV or SUV)- entrapped drugs even at doses which are several-fold higher than toxic dose of the unentrapped drug. In addition, parasites of the bloodstream (e.g.: protozoa, Tyrpanosoma brucei or metazoa, Schistosoma mansoni) which are treatable by liposomal agents do not appear to be more susceptible to the entrapped agent (whether in MLVs or SUVs) than the equivalent dose of free drug (Black and Watson, 1980 and Black and unpublished).

Passive targetting has proved useful in some other fields where advantage has been taken of the apparent inability of liposomes to escape from body cavities and so transport their contents to other tissues. Intra-articular therapy for treatment of experimental rheumatoid arthritis has been studied extensively using liposome-entrapped anti-inflammatory steroids (Knight and Shaw, 1979; Page-Thomas and Phillips, 1979). By administering liposomes directly into joint cavities these authors have overcome two problems simultaneously, i.e.: i) the carrier is administered directly at the site of required action and so does not

need to escape from the circulation, and ii) Uptake of liposomes by the RES is circumvented. Similar results have been obtained by Juliano and McCullough (1980) who administered liposomes as an aerosol into rabbit lungs and found no escape of entrapped drug into the circulation. Such data may be taken as good evidence that the liposome has very little capacity to directly traverse the cells lining a cavity. As discussed below and by Gotfredsen (1982), liposome escape from bodycavities or from local sites (Segal et al., 1975) may be by means of the lymphatic system. Other uses of phospholipids in the lungs of infants suffering from respiratory distress has been suggested by Morley et al., (1978) who proposed that the inhalation of dry phospholipids might relieve this condition.

An analogous situation to direct injection into body cavities is the local administration of liposomes by sub-cutaneous or intramuscular injection. Initial experiments on intra-testicular injection (Segal et al., 1975) demonstrated that small liposomes could localize in the lymph nodes draining the site of injection. These observations have been followed up by other groups of workers (Ryman et al., 1978; Osborne et al., 1979; Ryman and Tyrrell, 1980; Kaledin et al., 1981) who have shown that liposomes, containing an appropriate agent, can be used to outline the lymphatic system using imaging techniques. Of particular importance in this respect is the finding that liposomes can descriminate between normal and cancerous lymph nodes (Osborne et al., 1979). Again, local administration of liposomes circumvents RES uptake, at least initially, but still relies upon passive transport and uptake by the lymphatic system. Lymph node imaging may prove to be one area of potential for liposomes in clinical work. Without any specificity for tumour tissue, it seems unlikely that liposomes administered by other routes will be any better than other particle or colloidal systems for tumour imaging (Segal et al., 1976; Richardson et al., 1979).

Changes in the composition of liposomes have overcome some of the problems of passive targetting, e.g.: the incorporation of equimolar concentrations of CHOL into the phospholipid bilayers has reduced the leakage of entrapped solutes and the interactions of the carrier with lipoproteins (Gregoriadis and Davis, 1979; Kirby and Gregoriadis, 1980; Kirby et al., 1980a, 1980b). But in most cases these changes, whilst increasing the stability and prolonging the plasma half-life of the

vesicles, do not appear to result in marked changes to the passive targetting to the liver and spleen (Hwang and Mauk, 1977; Gotfredsen, 1982). However, recently Senior and Gregoriadis (1980,1982a,b) have made stable SUVs with long plasma half-lives. The authors state that the hepatic capture of these vesicles is reduced with increasing half-life. Blockade of the RES does not appear to substantially change the tissue distribution of MLVs or SUVs (Souhami et al, 1981).

Other alterations to the lipid composition of liposomes have been proposed by Yatvin et al., (1978; 1981) and Weinstein et al., (1979). In this case the vesicles were composed of a mixture of phospholipids such that the overall t_c of the carrier was a few degrees higher than the body temperature of the animals. Following injection, these liposomes circulate in the plasma but when they encounter an area of higher than normal temperature, supplied by the application of local hyperthermia, the lipid chains melt and release the liposome contents. This method does not prevent either unwanted protein interactions or the uptake of liposomes by the RES, indeed because they do not contain CHOL these liposomes may be more unstable in the plasma than some other preparations.

For the future, passive targetting may become restricted to certain areas of use:

i) Treatment of disease states with involve elements of the RES (e.g.: liver, spleen, lungs and perhaps bone marrow) where the disease can be shown to involve the stationary macrophages; although SUVs may have some applicability to liver parenchymal cells.

ii) local application into body cavities where RES uptake is minimised or where use is made of the lymphatic system to transport the vesicle.

iii) as protective carriers which reduce drug toxicity to sensitive tissues and/or which permit the slow general (or local) release of entrapped drug.

Consideration of many of the problems involved in "passive targetting" showed that a better recognition system was required than simply allowing the liposomes to find their own way. Obviously the recognition system should enable the carrier to interact specifically with a particular cell or tissue type and none other (i.e.: active targetting). Although theoretically molecules capable of specific recognition exist in large numbers (e.g.: certain glycolipids, glyco-

proteins, antibodies and some carbohydrates) it must be remembered that the uptake and clearance mechanisms of the RES appear to be mediated primarily by the physical, rather than chemical, nature of the material. For this reason, amongst others, convincing demonstrations of active targetting has been mostly restricted to in vitro work.

Liposomes containing antibody (Ab) raised against a specific cell type are able to select this type out of a mixed population of similar cells (Gregoriadis and Neerunjun, 1975b; Gregoriadis et al., 1977a; Magee et al., 1980). Similarly, specific antibodies either entrapped within, or covalently coupled to, liposomes have shown an increased avidity in various cell systems when compared to normal (non Ab-containing) liposomes. (Heath et al., 1980; Huang et al., 1980; Leserman et al., 1980a, 1980b; Gregoriadis and Meehan, 1981). However, some doubt remains as to whether increased attachment of liposomes to cells results in increased interiorization of the carrier or its contents (Weinstein et al., 1978; Leserman et al., 1979). Several factors appear to determine the interiorization of liposomes (discussed: chapter 4) but, in addition, in the case of active targetting such parameters as : i) the availability of the recognition molecule (Ab) on the liposome surface (Gregoriadis and Neerunjun, 1975b; Van Rooijen and Van Nieuwmegan, 1980; Huang and Kennel, 1979); ii) the density (Gregoriadis and Meehan, 1981) and/or the mobility (Humphries, 1980) of the Ab in the bilayer, and iii) the physiological capabilities of the target cell (chapter 4) will all have some effects on the uptake process. The type of protein used will also affect the results; Weissmann et al., (1975) showed that interiorization of IgG coated liposomes was efficient into phagocycles whilst albumin coated liposomes are not apparently captured by macrophages (Torchilin et al., 1980).

The success of these directional methods in vivo will ultimately depend upon three major factors : i) the reduction or blockade of uptake by the RES resulting in the subsequent prolongation of the plasma $T_{1/2}$ ii) the ability of liposomes to escape from the circulation whilst retaining the specificity due to the associated directional molecules and iii) the ability of immunologists to produce antibodies of high enough specificity which can recognise the diseased tissue or target site.

Application of active targetting in vivo has been attempted using Ab as the directional molecule. Gregoriadis et al., (1977a) attempted to follow up their initial in vitro work (Gregoriadis and Neerunjun,

1975b) by using anti-tumour Ab to increase tumour uptake of co-entrapped ^{111}In-BLM. These attempts were not successful apparently because of increased RES uptake. Using desialylated fetuin, however, an increased liver uptake was found for liposomes coated with this protein presumably because of its interaction with glycoprotein receptors known to exist on hepatocytes (Morell et al., 1971). Similar increased hepatic uptake has been reported using glycolipids in liposomes (Surolia and Bachhwat, 1980). Torchillin et al., (1979) administered antibody-bearing liposomes (Ab raised against cannine cardiac myosin) to dogs with myocardial infarctions. These liposomes appeared to concentrate to a higher extent in the damaged heart muscle than either normal (non-Ab) liposomes or liposomes bearing non-specific Abs. These results, therefore, suggest that some active targetting is possible in vivo. Much work will be needed to improve these prelimary results but the principle has been established.

Many of these same problems may also apply to other directional molecules (e.g.: carbohydrates) fixed to liposomes (Mauk et al., 1980a, 1980b). In this case, although the range of specificities may be more limited than that of Ab, the specificity also resides in the "receptor" on the cells. Many of these receptors remain unexplored so that this method relies upon further advances in membrane biology and differences between cell types may be less than those described by Ab.

Other directional systems have been used, e.g.: the use of lectin subunits in liposomes to introduce ricin into ricin-resistant cells (Dimitriadis and Butters, 1979; Gardas and McPherson, 1979) and viral spike proteins have been used to increase the fusion of liposomes with cells (Uchida et al., 1979).

In addition to their directional properties, antibody-bearing liposomes have also been used to remove proteins from the blood stream. Gregoriadis et al., (1981) have shown that liposomes carrying anti-IgM IgG can bind IgM in vivo. A similar system using anti-IgE IgG might prove very useful for the relief of acute anaphylactoid responses. Such a system has already proved useful in the case of digoxin overload (Tyrrell et al., 1978; Cambell et al., 1980) and to improve tumour detection (Barratt et al., 1981).

In the field of immunology it has long been recognised that many antigens require an adjuvant to exert their maximal effect as inducers of Ab. The original work of Gregoriadis and Allison (1974) and Heath et al., (1976) established that in some cases liposomes containing, or

mixed with, antigens could provide a safe adjuvant. More recently the adjuvant effect of liposomes has attracted more attention (Gregoriadis, 1980a, 1980b; Gregoriadis and Manesis, 1980; van Rooijen and van Nieuwmegan, 1980) and will no doubt continue to do so.

In one case liposomes containing a lipid soluble muramyldipeptide (MDP) derivative were shown to enhance the immunizing capacity of human malaria sporozoites in monkeys. (Siddiqui et al., 1978). However, care must be taken not to assume that liposomes are universal adjuvants. We subsequently showed (Mitchell et al., 1980), when a more severe test of adjuvancy was used (Mitchell et al., 1975), that the same liposome preparation mixed with a merozoite vaccine would not protect monkeys against a uniformly-fatal simian-malaria infection. Moreover, we also found that the best adjuvant results were found when the MDP was lipid soluble and entrapped in the LP of liposomes whose bilayers were above their t_c. From these results we were persuaded that the physical properties of an adjuvant may be as important as its chemical composition.

Other workers have used MDP and macrophage activating agents entrapped in liposomes in the chemotherapy of lung metastasis in mice as discussed previously (Fidler et al., 1980, 1981; Hart et al., 1981). Quite apart from being an exciting use of liposomes and an important area of research for the future, one wonders if the ability of MDP-liposomes to activate macrophages may not be intimately connected with the general property of these and other liposomes to act as immunological adjuvants (Tom, 1980). One further disquieting observation may nullify many of the attempts to use liposomes as adjuvants; Schuster et al., (1979) have reported that liposomes containing a lipid soluble antigen (lipid A) are able to induce Ab not only against the lipid A but also against the PC or sphingomyelin of the liposome carrier itself Empty liposomes or liposomes containing water soluble antigens do not apparently have this property. It seems possible that other lipid soluble agents entrapped in the LP (e.g.: drugs and some MDPs (?)) could have similar effects perhaps because membrane-bound agents can activate bone-marrow-derived lymphocytes (Kinsky, 1980) as well as macrophages. Nevertheless, the in vivo activation of macrophages by liposomal agents and/or the use of macrophages to transport liposomes and their contents across capillary and tissue barriers may prove to be an important use of liposomes which justifies the risk of antigenicity.

A final in vivo use of liposomes, which has been suggested but not developed as yet, is that of the circulating enzyme depot (Neerunjun

and Gregoriadis, 1976). Stable liposomes, with long $T_{1/2}$s, which contain an enzyme or other molecule, could be used to breakdown or chelate substrates in the circulation (and perhaps intracellularly too) so providing a circulating detoxifying system. Obviously a judicious balance must be stuck between the capacity of the entrapped molecule to leak and the ability of the substrate to enter the liposome but current knowledge allows this to be done (Kirby and Gregoriadis, 1981).

ii) In vitro uses of liposomes.

Because of their close similarity to cell membranes and their compositional versatility, liposomes have been, and will continue to be, used as model membranes but the major part of these studies are not directly concerned with the use of liposomes as carriers. However, interactions between proteins, especially lipoproteins, and liposomes will continue to add insight into the jigsaw which makes up the cell membrane as well as clarifying the structure and function of lipoprotein particles; all of which are relevant to the in vivo use of liposomes (chapter 3). In addition, our current knowledge of the mechanisms by which liposomes and/or their contents can enter cells (chapter 4) suggests that these uptake problems will have to be investigated in vitro because of the complexity of the in vivo situation. Studies on the mechanism of liposome uptake may prove to have implications beyond the use of carriers and will, in turn, perhaps illuminate some aspects of cell biology especially those concerned with membrane - membrane interactions in growth, differentiation and disease.

For the present, liposomes of various sizes and compositions have proved to be good vehicles for the introduction of molecules into cells, even if the mode of entry is not understood. The ability of liposomal agents to overcome cellular drug resistance (Papahadjopoulos et al., 1976; Mayhew et al., 1978; 1979; Dimitriadis and Butters, 1979) and to protect cells against virus infection (Magee and Miller, 1972; La Bonnardiere, 1978) suggests that these carriers may have important uses of their own in cell cultures.

An interesting current use of the liposome is to introduce "informational" molecules into cells of both animal and plant origin (Papahadjopoulos et al., 1980a, 1980b; Fraley and Papahadjopoulos, 1981). The most important fact is that molecules introduced in this manner can be "expressed" i.e.: they appear to be genetically active (Dimitriadis, 1978; Magee, 1978; Ostro et al., 1978; Papahadjopoulos et al., 1980a,

1980b). The use of liposomes to introduce nucleic acids into cells in vivo and so modify their functions, cannot be far away if these results can be translated into the whole animal.

The work of Kinsky and co-workers (Kinsky, 1972, 1977, 1978, 1980, Kinsky and Nicolotti 1977) has demonstrated that liposomal membranes can be rendered immunogenic by the incorporation of lipid haptens. These liposomes elicit humoral responses exclusively and can be lysed by the action of appropriate antibody and complement. With some advances in understanding of antigen presentation and mobility it should be possible to test for and quantify Ab raised against any liposome-immobilized antigen, in the presence of complement, by the release of an entrapped AP marker (radiolabel or other detectable molecule). Similarly, work on cell mediated immunity has demonstrated that liposomes bearing antigens can act as targets for cell-mediated immune attack (Hale et al., 1980; Henney, 1980). As well as advancing the knowledge of liposomes as immunological adjuvants, these data might lead to screening proceedures for diagnosis.

iii) General.

The initial enthusiasm of many workers for the concept of a universal drug carrier now appears to have abated to be replaced by a more sober assessment of the capabilities of both liposomes (Gregoriadis, 1980a, 1980b, 1981; Ryman and Tyrell, 1980) and other drug carriers (Gregoriadis ed., 1979a). There is no doubt that for diseases of the RES liposomes (and other particulate carriers) offer a very good vehicle for drug delivery : liposomes are probably the best primarily because they are normally non-toxic and biodegradable. (See Appendix). For other tissues, real advances in targetting await convincing demonstration that liposomes, bearing appropriate specificity, can evade the RES, escape from the circulation and be recognised by and taken up by the target tissue. Even should this be possible, modification of the subcellular distribution of the carrier, or its entrapped solute, may be necessary to ensure efficient delivery to its intracellular target.

Advances in liposomal stability, structure and directional molecules suggest that these problems could be overcome in the near future. If this does occur, the 75 year-old predictions of Paul Erhlich (1907) may truely come of age.

APPENDIX

TOXICITY OF LIPOSOMES.

TOXICITY OF LIPOSOMES.

Introduction.

The subject of liposome toxicity, i.e.: the toxicity of the liposome components in the absence of entrapped drug, is considered in this short appendix since it does not easily fit into the previous studies.

Methods.

Liposomes were prepared according to the standard method using EPC : CHOL : PA (7 : 2 : 1 molar ratio) with sterile 0.9 % NaCL entrapped in the aqueous phase. The quantities of lipid used depended upon the experiment but up to 50 times the standard quantities were used with one fifth or one tenth appropriate volume of saline (see methods). Where necessary rotary evaporation was performed in 1 litre flasks. Sonication was carried out for up to 30 minutes, depending upon the volume. In order not to overload the Sepharose 6 B columns, large quantities of liposomes were separated on a series of columns of equal height and volume. Finally, the quantity of lipid phosphorus was assayed in each preparation (Baginski et al., 1967) before use.

Animal experiments.

Male weanling rats (85-100 g) were injected I/V with either the empty liposomes or saline.

Two experiments were performed :

i) Rats received 50 mg/kg phospholipid (as judged by lipid phosphorus) daily for up to 3 weeks, the volume depending upon the body weight. Control rats received a similar volume of 0.9 % NaCl (short test).

ii) Some rats received weekly injections of 50 mg/kg phospholipid for periods up to 6 months (long test).

Animals participating in the short test were killed at weekly intervals; 6 animals (3 test, 3 control) were used for each time point. Animal body weights were measured daily and liver weights were taken at death.

In the longer test, animals were killed at monthly intervals (3 treated and 3 control animals at each point). Body weights were monitored weekly and liver weights were recorded at the time of death.

At death, tissues were removed for histological examination (liver, spleen, heart, lung, right kidney, whole ileum, pancreas, bone

marrow and brain). These tissues were fixed in 10 % formaldehyde for 24 hours. Paraffin embedded sections of each tissue were stained with haemotoxalin and eosin : kidney sections were stained with the PAS technique and bone marrow with Leishman's stain as used previously (Segal et al., 1974) for liposomes. Samples of each stained tissue section were examined by light microscopy.

Results.

Comparisons of means (Students 't' test) of body weights throughout the test between liposome and saline treated animals did not reveal any significant differences at any time. Similarly, there was no difference in liver weights between treated and untreated animals at death. No animal exhibited any untoward symptoms throughout the tests; no animals died.

Histological examination of the tissues (very kindly performed by Dr. Colin Green of the Animal Unit at this Centre) failed to find any tissues of abnormal appearance in any of the animals of either the long or the short term test. Of special interest was the lack of any increases in obvious fatty deposits of either the livers or the spleens of liposome treated animals when compared to the saline treated animals. Nor was there any apparent cellular infiltration of lung, spleen or liver tissues.

Discussion.

Until recently it has been assumed that liposomes, especially those composed of natural phospholipids and cholesterol, were not toxic. In general this is still the case (Gregoriadis, 1978; Kimelberg and Mayhew, 1978; Hart et al., 1981 and this work) although some exceptions have been reported.

Gregoriadis (1978) reported on the results obtained by Natterman Chemie who used, sonicated, pure soya-PC liposomes to test their toxicity in rats and dogs. Doses of up to 1g PC/kg were administered I/V to rats daily for 4 weeks. No acute toxicity was noted and no changes in health, behaviour or histology were found. Transient increases in plasma total fat, phospholipids and cholesterol were reported in rats receiving the highest dose.

Hart et al., (1981) treated (I/V) dogs and mice with empty MLVs containing PC, PS and EPC. Some increases above normal in serum enzyme levels (alkaline phosphatase, glutamine : oxaloacetic transaminase,

glutamic : pyruvic transaminase) and serum bilirubin levels were found in treated dogs but no evidence of liver damage was found during histological examinations. The authors attribute the changes to repeated venipucture of the animals. However, canine serum globulin levels were raised in one animal and one wonders if this could be in response to the removal of plasma alpha-2-macroglobulin by liposomes. Yet, Gregoriadis (1980a) reported that anionic liposome administration (100 mg lipid) to humans (I/V) did not change serum alpha-2-M levels for up to 3 days post-injection. In mice, Hart et al., (1981) failed to find any effects of liposome administration in the live animals and there were no inexplicable changes in the whole organs or in their histology.

Kimelberg and Mayhew (1978) reported that cationic liposomes (containing SA) were not toxic to mice when administered IP over a period of 3 months. Anionic liposomes (200 mg lipid/kg) have been injected (I/V) into rats with no ill effects (Colley and Ryman, 1976).

Exceptions to the general rule of non-toxicity have been found in the case of specific liposome constituents notably those used to impart charge to the vesicles.

Adams et al., (1977) reported that MLVs injected into the cerebral tissue of mice at high concentrations (250-500 mg lipid/kg) could cause epileptic seizures and tissue necrosis. This effect was most pronounced when SA or dicetylphosphate were used in the preparation. Other anionic phospholipids (e.g.: PA) or PC and CHOL alone were not toxic to rats when injected into the lateral cerebroventricals at 10-fold lower doses (Kimbelberg et al., 1978). The toxicity of cationic enzyme-containing liposomes (Steger and Desnick, 1977) has already been discussed (chapter 4).

The effect of liposomes of various compositions or clotting factors has also been discussed (chapter 3; Bangham, 1961; Papahadjopoulos et al., 1962; Juliano and Lin, 1980). Berdichevsky et al., (1979) have shown that liposomes composed of PE are more likely to decrease the aggregibity of platelets than EPC liposomes : platelet function was not altered. However, Gregoriadis (1980a) found no effects on clotting mechanisms in a patient injected with liposomes. Cationic liposomes have been reported to cause the agglutination and haemolysis of red blood cells (Martin and Mac Donald, 1976a).

Magee et al., (1974) found visible toxic effects when HeLa cells were exposed to cationic (SA) liposomes (2 mg lipid / 2 x 10^6 cells) but lower doses were not toxic. Using similar (SA) liposomes, Papaha-

djopoulos et al., (1974b) reported no effect upon the growth of 3T3 cells at doses of 0.2 mg lipid / 10^5 - 10^6 cells. The incorporation of SA or PS into ganglioside-containing DPPC : CHOL liposomes caused a reduction in the plating efficiency and growth of EMT-6 cells (Dunnick et al., 1976b). Finally, Chen and Keenan (1977) reported that liposomes containing EPC alone inhibited lymphocyte activation by attracting CHOL and reducing cellular CHOL levels.

In addition to SA, a large amount of work has been done on the toxicity of PS. This work followed initial reports (Bruni et al., 1976a, 1976b) that liposomes made from mixed brain phospholipids (mainly PS) markedly decreased brain energy metabolism in mice resulting in hyperglycaemia and accumulation of glucose in the brain : PS also caused the release of adrenaline from the adrenal medulla (Bruni et al. 1976b). Later, PS was implicated in increased catecholamine turnover in the hypothalamus (Toffano et al., 1978) of mice, a stimulation of acetylcholine release from mouse brain cortex (Mantovani et al., 1976), increases in both the dopamine-sensitive adenylate cyclase activity and the cyclic-AMP content of mouse brain (Leon et al., 1978) and changes in human pituatory function at the level of dopamine-mediated prolactin regulation (Masturzo et al., 1977). These effects could only be produced by brain PS (other sources of PS were not active; hence the composition of the acyl chains may be of crucial importance), other naturally occuring phospholipids (PC,PE,PI, DPPG) did not reproduce these effects. Furthermore, in order to be effective the liposomes had to be composed solely of PS; sonicated to produce SUVs and injected I/V(Bruni et al., 1976a, 1976b).

Since it is unlikely that liposomes cross the bloodbrain barrier the causes of these effects were assumed to be secondary to a metabolic transformation of the liposomes. Bigon et al., (1979a,b; 1980) have now shown that incubating PS with rat serum is followed by a strong increase in the toxic effects of PS and that these are due to lysophosphatidylserine (LPS). Identical effects can be produced by incubating PS with phospholipase A_2 from the pancreas. Other lysophospholipids (LPC, LPE) are not toxic. The action of LPS is apparently due to its ability to release intracellular catecholamines and histamine, an effect which is blockable by antihistamine and anti-adrenoceptor drugs; it is these released compounds that inhibit glycolysis. Isolated mast cells have also been reported to release histamine in the presence of LPS (Martin and Lagunoff, 1979). Chen et al., (1976) have reported that

liposomes composed to PC and PS can induce morphological differentiation in cultured neuroblastoma cells although it is not known if LPS is responsible.

Despite the fact that some liposome preparations can be toxic, there are some areas of physiology where the administration of empty liposomes might prove to be beneficial. Gregoriadis et al., (1977c) have reviewed this subject and have found several reports of the beneficial effects of liposomes. (e.g.: correction of liver phospholipid content to overcome dysfunction of the organ; reduction of plasma CHOL-levels, E-CHOL turnover and lipase activity in hypercholesterolaemic rabbits; and a reduction in aortic atherosclerosis in Japaneese quail).

The findings in this present work are in accord with other studies showing that in vivo EPC : CHOL : PA liposomes are not toxic. From the brief review it would seem that the liposome structure per se is not toxic but, as might be expected, individual components may cause untoward effects. In general, liposomes composed of PS, SA or dicetylphosphate should be avoided for in vivo and clinical work. Owing to the lack of alternatives to SA it is not known if cationic liposomes are toxic because of their charge or because of their composition.

The processes involved in liposome toxicity (apart from PS) are not understood. One might suspect that the exchange/transfer reactions between liposomes on the one hand and lipoproteins or cell membranes on the other hand are involved in the production of toxicity. Alterations in cell membrane composition and fluidity can be expected to have profound effects on cell or tissue viability.

Similarly relatively few studies have investigated the mechanisms of toxicity of liposomes containing entrapped agents apart from the measurement of the acute response. Liposomes containing drugs or proteins cannot be assumed to be non-toxic as shown by the adjuvanticity of some otherwise "non-toxic" liposome preparations.

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