ANALYTICAL APPLICATIONS OF LIPOSOMES

S. J. FROST
B.Sc., M.Sc., M.C.B., M.R.C.Path.,
C.Chem., M.R.S.C.

A thesis submitted in partial fulfilment of the degree of
Doctor of Philosophy, University of Surrey, 1994
Dedication

To my wife, Valerie, and my children, Kevin, Caroline and Rachel, for their encouragement and support, and for never complaining when they wanted to go somewhere and I was stuck in the loft.
Liposomes have established roles in drug delivery and cell membrane studies. Amongst other applications; they can also be used as analytical reagents, particularly in immunoassays. Liposomal immunoassays have potential advantages over alternatives; including sensitivity, speed, simplicity and relative reagent stability. The aim of these studies was to develop and evaluate novel examples of these assays.

When liposomes entrapped the dye, Sulphorhodamine B, a shift in its maximum absorption wavelength compared to free dye was observed. This was attributed to dimerization of the dye at high concentrations. If the liposomes were disrupted, the released dye was diluted into the external buffer, and the dye’s absorption spectrum reverted to that of free dye.

After optimization of dye entrapment, immunoassays were developed using these liposomes. Albumin-coated liposomes were used in a model assay to measure serum albumin. This assay employed complement-mediated immunolysis, commonly used in liposomal immunoassays. The liposomes were lysed by anti-albumin and complement, and this could be competitively inhibited by serum
albumin.

To improve sensitivity, Fab' anti-albumin liposomes were prepared. These enabled measurement of urinary albumin by a complement-mediated immunoassay, but using a sandwich technique.

Anti-albumin (intact) liposomes were shown to precipitate on gentle centrifugation after reaction with albumin. They were applied as a solid phase reagent in an heterogeneous immunoassay, using radioimmunoassay for urinary microalbumin as a model assay.

Liposomes containing Sulphorhodamine B were also used in a more novel assay; for serum anticardiolipin antibodies. Cardiolipin-containing liposomes were prepared. These were lysable using magnesium ions. Anticardiolipin antibodies (IgG) were found to augment this lysis, enabling their estimation. Similar imprecision and acceptable correlation with a commercial enzyme-linked immunosorbent assay (ELISA) were obtained.

The findings demonstrate Sulphorhodamine B release can be used as a marker in homogeneous colorimetric liposomal immunoassays; both in model assays and in potentially more useful clinical biochemistry applications.
## Contents

<table>
<thead>
<tr>
<th>section</th>
<th>page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>i</td>
</tr>
<tr>
<td>Contents</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>ix</td>
</tr>
<tr>
<td>Materials and Equipment</td>
<td>xi</td>
</tr>
</tbody>
</table>

### Chapter 1

**Introduction**

<table>
<thead>
<tr>
<th>section</th>
<th>page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Nature and Applications of Liposomes</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Immunoassays— the Search for Improved Labels</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Liposome Classification and Methods of Synthesis</td>
<td>5</td>
</tr>
<tr>
<td>1.4 Purification and Characterization of Liposomes</td>
<td>11</td>
</tr>
<tr>
<td>1.5 Varying Liposome Composition</td>
<td>15</td>
</tr>
<tr>
<td>1.5.1 Membrane Components and the Aqueous Phase</td>
<td>15</td>
</tr>
<tr>
<td>1.5.2 Protein Coupling Methods</td>
<td>21</td>
</tr>
<tr>
<td>1.5.3 Coupling Methods for Non-Proteins</td>
<td>30</td>
</tr>
<tr>
<td>1.6 Liposome Applications in Immunoassays</td>
<td>32</td>
</tr>
<tr>
<td>1.6.1 The Choice of Encapsulated Marker</td>
<td>32</td>
</tr>
<tr>
<td>1.6.2 Early Markers— Glucose and Electron Spin Labels</td>
<td>37</td>
</tr>
<tr>
<td>1.6.3 Enzymes, Chromophors and Fluorophors as Markers</td>
<td>39</td>
</tr>
<tr>
<td>1.6.4 Assays Using Cardiolipin Antibodies and Arsenazo III</td>
<td>40</td>
</tr>
<tr>
<td>1.6.5 Complement-Mediated Assays Using Enzymes</td>
<td>41</td>
</tr>
<tr>
<td>1.6.6 Complement-Mediated Assays Using Chromophors and Fluorophors</td>
<td>44</td>
</tr>
<tr>
<td>1.6.7 Melittin-Mediated Immunoassays</td>
<td>46</td>
</tr>
<tr>
<td>1.6.8 Assays Using Stabilized Liposomes</td>
<td>47</td>
</tr>
<tr>
<td>1.6.8 Solid Phase Liposomal Immunoassays</td>
<td>50</td>
</tr>
<tr>
<td>section</td>
<td>page number</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.6.9 Potentiometric Detection of Liposomal Markers</td>
<td>51</td>
</tr>
<tr>
<td>1.6.10 Other Immunological Applications</td>
<td>52</td>
</tr>
<tr>
<td>1.7 Non-Immunological Liposome Applications</td>
<td>54</td>
</tr>
<tr>
<td>1.8 Summary of Liposome Based Analyses</td>
<td>58</td>
</tr>
<tr>
<td>1.9 Aims of Project</td>
<td>61</td>
</tr>
</tbody>
</table>

**Chapter 2**

**Preparation of Liposomes, Optimization of Sulphorhodamine B Incorporation, and Examination of the Spectrophotometric and Fluorimetric Properties of the Free and Encapsulated Dye**

<table>
<thead>
<tr>
<th>section</th>
<th>page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction</td>
<td>63</td>
</tr>
<tr>
<td>2.2 Preparation of Liposomes Incorporating Iodine-Labelled Bleomycin</td>
<td>64</td>
</tr>
<tr>
<td>2.3 Examination of the Spectrophotometric and Fluorimetric Properties of Sulphorhodamine B in Aqueous Solution</td>
<td>68</td>
</tr>
<tr>
<td>2.4 Procedure for Preparing Liposomes Encapsulating Sulphorhodamine B by Reverse Evaporation Method</td>
<td>73</td>
</tr>
<tr>
<td>2.5 Dialysis as a Means to Assess Liposome Stability</td>
<td>77</td>
</tr>
<tr>
<td>2.6 Fluorescence Quenching of Liposome Preparations and its Use in Estimating Amount of Dye Entrapment</td>
<td>79</td>
</tr>
<tr>
<td>2.7 Comparison of Gel Filtration and Dialysis as Means of Purifying Liposome Preparations</td>
<td>83</td>
</tr>
<tr>
<td>2.8 Effect of Encapsulation on the Spectrophotometric Properties of Sulphorhodamine B</td>
<td>86</td>
</tr>
<tr>
<td>2.9 Optimization of Incorporation of Sulphorhodamine B</td>
<td>92</td>
</tr>
<tr>
<td>2.9.1 Selection of Procedure to Assess and Compare Incorporation and Stability</td>
<td>92</td>
</tr>
<tr>
<td>2.9.2 Effect of Various Lipid Compositions on Dye Incorporation and Stability</td>
<td>93</td>
</tr>
<tr>
<td>2.9.3 Effect of Dye Concentration on Incorporation and Stability</td>
<td>96</td>
</tr>
</tbody>
</table>

iv
### Chapter 2

**2.9.4 Effect of Buffer Composition on Incorporation and Stability**

**2.9.5 Effect of Cholesterol Content on Incorporation and Stability**

**2.9.6 Modifications to Original Reverse Phase Evaporation Procedure to Improve Incorporation**

**2.9.7 Effect of Phosphatidyl Ethanolamine on Liposome Incorporation and Stability**

**2.9.8 Effect of Cardiolipin on Liposome Incorporation and Stability**

**2.10 Comparison of Fluorimetric and Spectrophotometric Properties of Liposome Preparations**

**2.11 Comparison of Liposomal Encapsulations of Kodak and Sigma Sulphorhodamine B Dyes**

**2.12 Entrapment of Rhodamine B**

**2.13 Discussion**

### Chapter 3

**Liposomal Immunoassays for Serum and Urinary Albumin**

**3.1 Clinical Value of Serum and Urinary Albumin Assays**

**3.2 Aims of Liposomal Studies for the Measurement of Albumin**

**3.3 Preparation of Albumin-Coated Liposomes**

3.3.1 Formation of Dithiopyridyl-albumin (DTP-albumin)

3.3.2 Synthesis of Dipyridyl-Phosphatidylethanolamine (DTP-PE) and Liposome Formation

3.3.3 Coupling of DTP-albumin to Liposomes

**3.4 Effect of Complement and Antialbumin Antiserum on Liposomes**

3.4.1 Spectrofluorimetric Measurement

3.4.2 Spectrophotometric Monitoring of Complement-Mediated Dye Release
### Chapter 3

**Liposomal Assays for Anticardiolipin Antibodies**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.3 Monitoring Entrapped Dye Release Using a Cobas Bio Centrifugal Spectrophotometric Analyser</td>
<td>146</td>
</tr>
<tr>
<td>3.4.4 Inhibition of Complement-Mediated Immunolysis by Albumin</td>
<td>157</td>
</tr>
<tr>
<td>3.5 Preparation of Antialbumin Liposomes and Effects of Complement</td>
<td>163</td>
</tr>
<tr>
<td>3.6 Complement-Mediated Immunolysis of Liposomes Coated with Antialbumin Fab' Fragments</td>
<td>166</td>
</tr>
<tr>
<td>3.6.1 Preparation of Fab' Antialbumin Liposomes and Complement-Mediated Immunolysis</td>
<td>166</td>
</tr>
<tr>
<td>3.6.2 Assessment of Potential Interferences in Microalbumin Estimation</td>
<td>176</td>
</tr>
<tr>
<td>3.6.3 Urinary Microalbumin Assay Performance</td>
<td>190</td>
</tr>
<tr>
<td>3.7 Immunoprecipitation by Antialbumin-Coated Liposomes</td>
<td>195</td>
</tr>
<tr>
<td>3.7.1 Evidence of Competitive Label Binding</td>
<td>195</td>
</tr>
<tr>
<td>3.7.2 Use of Liposomes as a Solid Phase</td>
<td>199</td>
</tr>
<tr>
<td>3.8 Discussion</td>
<td>205</td>
</tr>
</tbody>
</table>

### Chapter 4

**Liposomal Assays for Anticardiolipin Antibodies**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>237</td>
</tr>
<tr>
<td>4.1.1 Clinical Applications of Anticardiolipin Antibodies</td>
<td>237</td>
</tr>
<tr>
<td>4.1.2 Existing Non-Liposomal Assays for Antiphospholipid Antibodies and Anticardiolipin Antibodies</td>
<td>243</td>
</tr>
<tr>
<td>4.1.3 Liposomal Immunoassays for Anticardiolipin Antibodies</td>
<td>247</td>
</tr>
<tr>
<td>4.1.4 Objectives of Anticardiolipin Studies</td>
<td>249</td>
</tr>
<tr>
<td>4.2 Complement-Mediated Immunolysis of Cardiolipin-Containing Liposomes</td>
<td>250</td>
</tr>
<tr>
<td>4.3 MgCl₂-Dependent Lysis of Cardiolipin-Containing Liposomes</td>
<td>259</td>
</tr>
<tr>
<td>4.3.1 MgCl₂-Induced Lysis Using Manual Spectrophotometer and Fluorimeter</td>
<td>259</td>
</tr>
<tr>
<td>4.3.2 Application of MgCl₂-Dependent Lysis to the Cobas Bio Analyser</td>
<td>260</td>
</tr>
</tbody>
</table>
I would like to express my extreme gratitude to Dr. G. B. Firth, Consultant Clinical Biochemist at the Princess Royal Hospital, Haywards Heath, for his support, encouragement and advice throughout this project, particularly in the techniques of liposome preparation and immunoassays.

I also wish to thank Dr. J. Chakraborty, Senior Lecturer, School of Biological Sciences, University of Surrey, for his invaluable assistance in the preparation, planning and content of this thesis.

I am grateful to Miss Dawn Chescoe, School of Materials Sciences, University of Surrey, for the electron microscopy of my preparations.

In addition, I thank Mr. N. Loring, Department of Histology, The Princess Royal Hospital, for his assistance in the production of light microscope photographs.

I am also grateful to Dr. J. Faux, Churchill Hospital, Oxford, for the supply of anticardiolipin antibody reference sera; and to the staff in those other laboratories who kindly supplied patients samples containing anticardiolipin antibodies.

Finally, I am pleased to acknowledge the financial assistance of the South West Thames Locally Organised Research Scheme, The British Heart Foundation and The Television South Trust, whose grants enabled these studies to be carried out.
Abbreviations

a area of lipid molecule in a bilayer
A absorbance
ACA anticardiolipin antibody
APA antiphospholipid antibody
APS antiphospholipid antibody syndrome
B₉-GPI B₉ Glycoprotein I
BCG bromocresol green
C centi (prefix) or concentration
Celsius (centigrade)
C clostridium
Ca calcium
CH50 unit of complement activity
cholesterol
Ci curie
Cl chloride
conc concentration
CRP C-reactive protein
CSF cerebrospinal fluid
CV coefficient of variation
D dalton
D₁ the number of dye molecules per liposome
DNA deoxyribonucleic acid
DOPE dioleoyl phosphatidyl ethanolamine
DPPC dipamitoyl phosphatidyl choline
DTP dithiopyridyl (prefix)
duplicate
Dm molar absorbance coefficient
EDCI 1-ethyl-3-(3-dimethylamino-propylcarbodiimide)
EDTA ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
em emission wavelength
EMIT enzyme multiplied immunoassay technique
ESR electron spin resonance
ex excitation wavelength
f fluorescence
FIA fluorescence immunoassay
ft fluorescence after lysis
G gram
GPL unit of IgG ACAs
h bilayer thickness
H hydrogen or hexagonal
HSV Herpes simplex virus
I iodine
Ig immunoglobulin
IRMA immunoradiometric assay
k kilo (prefix)
K potassium
1 litre or optical path length
LA lupus anti-coagulant
LUV large unilamellar vesicle
m milli (prefix) or metre
M molar (moles per litre)
MBS m-maleimidobenzoyl-N-hydroxysuccimide ester
Mg magnesium
MLV multilamellar large vesicle
mol mole
MPL unit of IgM ACAs
n nano (prefix)
N number of lipid molecules per liposome
Na sodium
NETRIA North East Thames Radioimmunoassay Unit
NHSP N-hydroxysuccinimide ester of palmitic acid
NMR nuclear magnetic resonance
p probability
PAPS primary antiphospholipid antibody syndrome
PC phosphatidyl choline (dipamitoyl)
PE phosphatidyl ethanolamine (dipalmitoyl)
PG phosphatidyl glycerol (dipalmitoyl)
P number of protein molecules per liposome
PTA phosphatidic acid (dipalmitoyl)
Qv Quenching Coefficient
r correlation coefficient
R reagent or radius (of liposome)
REV reverse phase evaporation vesicle
RIA radioimmunoassay
s second
sd standard deviation
sing singleton
SLE systemic lupus erythematosus
SMPB N-succinimidyl 4-((p-maleimidophenyl)butyrate
SPDP N-succinimidyl 3-(2-pyridyldithio) propionate
SUV small unilamellar vesicle
T4 thyroxine
TAMSMB 2-(2-thiazolylazo)-4-methyl-5-(sulphomethylamino) benzoic acid
TM trade mark
u micro (prefix)
U unit
x times (e.g. magnification)
Materials and Equipment

Materials

Lipids (of the highest purity available unless otherwise stated), purified albumin (essentially fatty acid and globulin free) and the dye, Sulphorhodamine B were supplied by Sigma Chemical Company Ltd., Poole, Dorset. This dye was used throughout except when stated a Kodak dye supplied by Phase Separations Sales, Deeside, Clwyd, Wales was used.

Cholesterol was measured using a colorimetric procedure (‘CHOD-PAP method, BDH Ltd, Poole, Dorset) using an EPOS analyser (BDH Ltd.)

Protein was measured using a manual Coomassie blue assay (Biorad Laboratories Ltd, Watford, Hertfordshire).

BM-Test 3 or BM-Test 5 were used in the initial assessment of urine samples for protein and pH (Boehringer Mannheim Ltd, Lewes, East Sussex).

Other chemicals and AnalaR grade reagents were supplied
Gel filtration materials and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were supplied by Pharmacia Ltd, Milton Keynes, Buckinghamshire.

Phosphate buffer, 0.05M, pH7.4 was used throughout except when varied experimentally as stated.

"L Buffer" contained 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanosulphonic acid) (HEPES) and 145 mM sodium chloride, pH 8.0.

"Buffer A" contained 50 mM tris titrated to pH 7.5 with HCl and containing 0.15 M NaCl, 0.5 mM MgCl₂ and 0.15 mM CaCl₂.

A radioactive marker, I-125 labelled bleomycin, was kindly donated by Dr G B Firth, Princess Royal Hospital, Haywards Heath.

Radioactive I-125 labelled human albumin (specific activity 16.4 uCi/ug) was supplied by North East Thames Regional Immunoassay, London (NETRIA).

Polyclonal anti-human albumin antiserum (batch 608, xii
purified immunoglobulin G fraction), normal animal sera and donkey anti-sheep antiserum were obtained from Guildhay Antisera Ltd., Guildford, Surrey.

Fab' fragments of their sheep anti-human albumin antiserum were prepared by Immunogen International Ltd, Llandysul, Dyfed; by papein digest, dialysed into phosphate buffer.

Animal complement was obtained from Sera-lab Ltd., Crawley Down, Sussex. Human complement was supplied by Sigma Chemical Company.

Anticardiolipin Reference Sera (1100 GPL units) was kindly donated by Dr J Faux, Churchill Hospital, Oxford. Other anticardiolipin positive samples were kindly supplied by the following Immunology Departments; Kings College Hospital, London; Royal Hallamshire Hospital, Sheffield; Royal Sussex County Hospital, Brighton and St. Helier Hospital, Carshalton, Surrey.

Anticardiolipin antibody enzyme-linked immunosorbent assay (ELISA) kits were supplied by Cambridge Life Science Ltd, Ely, Cambridgeshire
**Equipment**

Sonication was achieved by a Soniprep 150 probe sonicator (MSE Scientific Instruments Ltd, Crawley, West Sussex).

An LKB-Wallac Clinigamma counter (Pharmacia Ltd) was used to measure radioactivity.

Gel filtration was performed using 0.7 mm x 20 cm 'Econo Columns' (Bio-Rad Laboratories Ltd).

Dialysis employed dialysis tubing supplied by Medicell International Ltd, London. This was 18/32 inch size unless otherwise stated.

Liposomes were filtered using 200 nm 'Minisart NML' filters (Sartorius Ltd, Epsom, Surrey).

Spectrophotometric scans and absorbance measurements were made using a Pye Unicam SP1800 spectrophotometer (Philips Scientific, Cambridge, Cambridgeshire) and an Hitachi U-2000 spectrophotometer (Hitachi Scientific Instruments, Wokingham, Berkshire) or using a Cobas Bio automated analyser (Roche Products, Welwyn Garden City, Herts).
Fluorescence measurements were made using a Perkin Elmer scanning fluorescence spectrophotometer (Perkin Elmer Ltd, Beakonsfield, Buckinghamshire). A solution of $2 \times 10^{-6}$M Sulphorhodamine B was used to set the scale reading to 100% (scale 4) which equals 900 arbitrary fluorescence units (excitation wavelength 400 nm, emission wavelength 580 nm).

ELISA plates were read using a Multiscan MCC/340 plate reader, supplied by Labsystems Ltd, Basingstoke, Hampshire.

Centrifugation employed a Burkhard Koolspin centrifuge (Uxbridge, Middlesex) and a MSE Mistral 61 (Crawley, West Sussex).

The rotary evaporator used was type 349/2 from Corning Ltd, Stone, Staffordshire.

The following methods were used to measure urinary analytes as described by the manufacturer, (Bayer P.L.C., Basingstoke, Hampshire) using a Bayer Axon analyser: phosphate, ammonium molybdate; magnesium, xylidyl blue; and calcium, cresolthalein.
Urinary sodium and potassium were analysed using a flame photometer (Instrumentation Laboratory 943, Warrington, Cheshire).

A Corning-Eel model 10 pH meter was used to measure pH (Corning Ltd).

Light microscopy photographs were obtained using a Leitz Diaplan microscope with a 35 mm film magazine (Leica, Milton Keynes).

Electron micrographs were kindly produced by Ms Dawn Chescoe, School of Materials Sciences, University of Surrey, Guildford.

Statistical calculations were performed using an Amstat1 parametric statistics package on an Amstrad PCW 9512 computer (Amstrad plc, Brentwood, Essex); except that regression equations were calculated using Deming's method as described by Cornbleat and Gochman (1).

LP3 and LP4 plastic tubes were supplied by Luckhams Ltd, Burgess Hill, Sussex.
The following hand pipettes were used; Eppendorf, Oxford Scientific Instruments; SMI hand pipettes, Alpha Laboratories Ltd, Eastleigh, Hampshire.
1.1 Nature and Applications of Liposomes

Liposomes are synthetic spheroidal vesicles consisting of a lipid bilayer membrane, usually composed mainly of phospholipids, enclosing an aqueous interior. A variety of materials including lipids or proteins can be incorporated into or attached to the membrane, while water soluble substances such as drugs, enzymes or dyes can be entrapped in the aqueous interior.

Thermodynamically unstable structures, liposomes do not form spontaneously when amphiphilic molecules (e.g. phospholipids) are brought into contact with an aqueous phase. In theory, they eventually will always tend to convert to more stable phases such as lamellar or hexagonal structures (2). However in practical terms; liposomes can be very stable, readily formed by a variety of techniques and often lose their integrity only slowly. These considerations, together with the ease and subtlety with which their composition can be altered, has
led to their application in a number of fields. These have ranged from their use as models of cell membranes and as a means of studying the physical chemistry of lipid-lipid and lipid-protein interactions, to their role as carriers of drugs or other therapeutic agents. These applications have been extensively reviewed (2-6). Liposomes have recently become familiar as ingredients of cosmetics (7). The potential of liposomes as analytical tools, the subject of this thesis, is another comparatively recent area of study.

1.2 Immunoassays - the Search for Improved Labels

Since the development of radioimmunoassay (RIA) by Yalow and Bersen (8) in the early 1960s; the technique has held a central importance in the measurement of analytes at low concentration, despite there being inherent disadvantages of the use of a radionucleotide as the label in the immunoassay. These include the limited shelf life, the need for specialised counting equipment, the need to incorporate a separation stage of the antibody-bound label from the free label and the health and safety risks of the use and disposal of radioactive materials. While radioimmunoassay is still popular there has been a gradual move to safer, stabler and more
convenient labels. The various alternatives have been reviewed and compared (9), and the impetus to improve labels shows no sign of abating (10).

An ideal label would retain the advantages of radioimmunoassay while avoiding its disadvantages. No single label to date has fulfilled this aim completely, perhaps explaining why there are now so many alternative immunoassay techniques available.

Radiolabels can be counted with a high degree of sensitivity leading to low limits of analyte detection. Some of the alternatives have approached this sensitivity, for example enzyme linked immunosorbent assay (ELISA) (11). This technique achieves sensitivity, however, by an enzymatic reaction step which can be time consuming and involves reagents with limited stability.

Under certain circumstances assays can be constructed that avoid the separation stage of radioimmunoassay (e.g. enzyme multiplied immunoassay (EMIT) (11) or plane polarised fluorescence immunoassay (12)). However these tend to lack sensitivity, cannot usually be developed by the user (unlike radioimmunoassay), generally apply only to small analytes such as drugs or non-protein hormones and are expensive.
Some assay techniques such as fluorescence (12) or chemiluminescence (13) assays require equipment that is not readily available in all laboratories.

These considerations, together with improvements in radiolabelled assay performance such as the development of immunoradiometric assays (IRMAs) (14) and the use of affinity purified or monoclonal antibodies (15) have ensured that radiolabels are still widely used. New developments however, particularly in the commercial field, tend to utilize alternative non-isotopic labels (10).

The possibility of using liposomes as carriers of labels for immunoassays has received a limited, though growing, consideration. Liposomes are potentially very versatile as label-carriers since they can be made to entrap a wide range of measurable substances. These can include enzymes, fluorophors, colorimetric dyes, chemiluminescent molecules and potentiometrically measurable ions (16). Antigen or antibody can be incorporated within the lipid membrane or attached to the liposome surface. It may be possible to measure the signal in intact liposomes although more usually the liposome is lysed, for example by detergent, enabling the encapsulated substance to be measured.
1.3 Liposome Classification and Methods of Synthesis

Liposomes fall into three main categories depending on their size. These were defined by the New York Academy of Science (17) as multilamellar large vesicles (MLVs), of sizes ranging between 0.1-5 μm; small unilamellar vesicles (SUVs), size range 20-50 nm and large unilamellar vesicles (LUVs), size range above 60 nm (typically 200 nm - 1 μm (18)). Giant and "cell size" liposomes have also been synthesized (19,20). Each synthetic method tends to produce a certain type of liposome, though often with a small proportion of other types.

When a mixture of lipids including phospholipid is deposited on the wall of a vessel by evaporation of the organic solvent and then shaken with an aqueous solution liposomes are formed. They consist of large concentric layers of lipid enclosing aqueous solution centrally and between each layer. These are multilamellar large vesicles and are of the type first described by Bangham and Horne in 1964 (21). While very useful, for example in membrane studies, these liposomes are less suited to immunoassay applications because of their heterogeneity and low aqueous capture volume.
Small unilamellar vesicles (22) are formed if multilamellar large vesicles are sonicated, usually for a few minutes. Although relatively homogeneous these also have a low capture volume.

A better capture has been achieved using large unilamellar vesicles. A convenient method for their synthesis was described by Szoka and Papahadjopoulos (18). This involves first sonication for a few minutes of a mixture of an organic phase, containing lipid, and an aqueous phase which usually contains the material to be entrapped. Sonication produces a water-in-oil emulsion. This is followed by rotary evaporation under reduced pressure. As the organic phase evaporates the mixture becomes viscous and usually briefly forms a gel. The gel suddenly collapses, often violently, and disperses, resulting in the creation of bilayered large unilamellar liposomes (LUVs). These liposomes are also known as reverse phase evaporation vesicles (REVs).

A more homogeneous preparation of the reverse evaporation vesicles is obtainable by filtration through suitable sized, usually polycarbonate, filters (e.g. 200 nm pore size) (23).
Consideration of surface area/volume ratios would suggest a greater entrapment of aqueous phase by large unilamellar vesicles compared with small unilamellar vesicles or multilamellar large vesicles. This seems generally to be the case. Szoka and Papahadjopoulos (18) reported between approximately 24 and 60% of the aqueous volume was entrapped within large unilamellar vesicles (8.1-15.6 l/mg of lipid) compared with approximately 16.5% (4.1 l/mg of lipid) for multilamellar large vesicles and 1.8% (0.5 l/mg of lipid) for small unilamellar vesicles.

Large unilamellar vesicles are likely, therefore, to be the liposomes of choice for high encapsulation. This may be desirable for liposomes used as immunoassay labels; as it will increase the signal (e.g. release of entrapped dye or enzyme) produced by the lysis of a given number of antigen or antibody-coupled liposomes. However it is possible that the signal strength of an assay might not be a significant consideration. Alternatively, the amount of lysis may be dependent on high amounts of coupled antibody or antigen. In those circumstances small unilamellar vesicles, with their increased surface area to volume ratio, may be preferable.
Small unilamellar vesicle (and multilamellar large vesicle) preparations have the advantage that there is virtually no risk of contact between organic solvent and labile antigens or antibodies which could cause denaturation. However this is not a major disadvantage of large unilamellar liposomes, since a variety of coupling procedures enable attachment of proteins to large unilamellar vesicles subsequent to their synthesis and organic solvent removal (see Section 1.5.2).

There are a number of other techniques to prepare liposomes (2-4,24). For large unilamellar vesicles, reverse evaporation is probably the method of choice (24), particularly for small experimental batches. A detergent dialysis technique has been claimed to have the advantage of producing liposomes of greater homogeneity and more easily controlled size (25-300nm, i.e. intermediate between small unilamellar vesicles and large unilamellar vesicles) (25). Lipid is dissolved using a detergent such as sodium cholate to form mixed lipid/detergent micelles. The detergent is then reduced in concentration by dialysis, either using a dialysis bag or commercially available equipment (25). When the detergent concentration falls below a critical level liposomes are formed. The technique has the advantage of avoiding potentially damaging organic solvents. However
it is not easily applicable to retaining dialysable material (e.g. dyes) within the liposomes.

Solvent injection methods have also been used. In these techniques vesicles are formed by the injection of lipid in organic solvent into a large aqueous volume. The ethanol injection technique of Batzri and Korn (26) is a simple method but produces small unilamellar vesicles of low encapsulation. The ether infusion method of Deamer and Bangham (27), involving the injection of organic mixture into aqueous solution at 55-65°C or under reduced pressure at 30°C, does produce large unilamellar vesicles. However it suffers from the potential disadvantage of denaturation of organic or heat sensitive molecules and of producing liposomes of heterogeneous size (28).

Papahadjopoulos et. al. (29) have used a calcium infusion method to prepare liposomes from acidic phospholipids. By the addition of calcium to certain types of small unilamellar vesicles multilamellar structures termed cochleate cylinders are formed. Addition of EDTA coverts these to large unilamellar vesicles. Despite the restriction to certain types of lipid this method has the advantage of using gentle reaction conditions.
Another method which produces liposomes of LUV dimensions which are oligo and multilamellar has been described by Kirby and Gregoriardis (30). This involves dehydration, typically by freeze drying, followed by controlled rehydration and is claimed to be a simple method giving high entrapment yields. Ligands can be coupled to liposomal surfaces prior to entrapment of material within the liposome using this method. This might have advantages if the entrapped material is sensitive to coupling reagents (31).

Liposomes of desired characteristics, e.g. homogeneity, can be formed by reducing the size of large liposomes by passing through a small orifice. Hamilton et. al. (32) used a French press to convert multilamellar large vesicles to small unilamellar vesicles. It is also a common practice to use filtration through polycarbonate membranes to improve the homogeneity of large unilamellar vesicles (23). A similar technique can be used for multilamellar large vesicles to produce smaller more homogeneous preparations (33).
1.4 Methods of Purification and Characterization

Once liposomes are formed, physical methods are usually used to separate the liposomes from unencapsulated aqueous phase as well as un-incorporated lipid. The choices of methods used vary depending on the size and nature of the liposomes and the type of entrapped material.

Gel filtration, for example using Sepharose 4B or Sepharose 6B, is widely used. Large unilamellar vesicles normally elute in the void volume in these gels, while small unilamellar vesicles often are separated into two peaks, one in the void volume and one that is filtered more slowly. A potential problem with gel filtration techniques is absorption of lipid onto the gel column (34).

Recently an alternative physical separation method, high pressure liquid chromatography, has also been used. This was shown to have advantages over gel filtration, including small sample size and rapid run times (35).

Dialysis is another technique which is useful if the entrapped material is dialysable and can give an indication of liposome leakage rate.
Centrifugation techniques are also available. Ultracentrifugation can be used to separate liposomes, both from impurities and according to size. In addition, centrifugation can be used to precipitate out large liposomes and sonication probe fragments from small unilamellar vesicle preparations (36).

A useful alternative which can avoid high centrifugation forces is to use a density gradient medium such as Ficoll-Paque (T.M. Pharmacia) (37). Since liposomes are less dense than this medium they float above it and can be separated from other components which remain in the aqueous phase or are precipitated. Alternatively the density gradient medium could be added to the encapsulated solution to increase the liposome's density and facilitate their centrifugation.

An assessment of the physical properties of the prepared liposomes is usually desirable. If liposomes are to be used analytically this will include assessing their instability. While many liposome preparations do not show appreciable loss of their contents over several months this is not always the case (see Section 1.5.1). Loss of internal contents can be by leakage or rupture (38). The term "lysis" strictly speaking implies liposomal rupture. However it is often used to indicate release of contents.
from liposomes without implying knowledge of the specific mechanisms involved. "Lysis" will be used as a term in this general sense throughout this report.

A convenient method to assess instability is by dialysis, provided an encapsulated component is dialysable. A sophisticated mathematical treatment has been applied to obtain accurate measurements of liposome permeability (24). Dialysis can also be used, however, to simply estimate the stability of a liposomal preparation.

Another approach to the assessment of liposome instability; which, like dialysis, can also be used to assess the amount of encapsulation of aqueous phase material, is the measurement of the degree of quenching of an encapsulated fluorophor. Carboxyfluorescein (38,39) and calcein (40) have been used for this technique. It is based on the observation that when a fluorophor is incorporated at a concentration sufficient to cause self-quenching (by an inner filter effect) the fluorophor within the liposome remains quenched and the liposomes do not fluoresce. However if the liposomes are lysed, for example by detergent, the fluorophor is diluted and fluoresces. The increase in fluorescence upon lysis of a sample of a preparation (for example by detergent) can be
used to calculate the percentage incorporation of fluorophor, while the gradual increase in fluorescence of a liposome preparation (in absence of detergent) can be used to assess the stability. Since the kinetics of quenching loss is different if liposomes leak than if they rupture, this technique can be used to differentiate between the two types of loss of contents (39).

The size of a liposome preparation and its homogeneity can be assessed by several techniques. Electron microscopy has been widely used, negative staining being more commonly used to assess size (23) while freeze fracture techniques are more useful to assess morphology (41). While electron microscopy is applicable to large unilamellar vesicles or small unilamellar vesicles; multilamellar large vesicles are large enough to be assessed by light microscopy, for example by the inclusion of a hydrophobic fluorescent probe into the bilayer (42). Light scattering studies have been used to assess liposome sizes and can be used to estimate the degree of contamination of a preparation by large liposomes (43-45). Thin layer chromatography has also been used to provide an approximate estimate of liposome size (46). Kirkland et. al. (47) used the centrifugal technique of sedimentation field flow fractionation to
size liposomes. This is based on the differential flow rates of different sized particles through a narrow orifice under centrifugal force. Another approach is the use of a Coulter™ particle analyser (48).

The size of the liposome, together with an estimate of the encapsulation ratio (litres aqueous phase entrapped / mol lipid), can be used to calculate the number of liposomes in a preparation and their surface area (49). The lipid content of a liposome preparation can usually be assessed by measurement of phosphate or cholesterol. Alternatively a radiolabelled tracer (e.g. 14Carbon labelled phospholipid) could be used to assess lipid recovery.

Finally, free-flow electrophoresis has been used to study the surface charge density of liposomes (50).

1.5 Varying Liposome Composition

1.5.1 Lipids and Internal Aqueous Phase

Although liposomes formed from single chain amphiphiles such as dodecanol/sodium dodecylsulphate, octadecyl sulphate or octadecylphosphate have been reported(51),
the large majority of liposome studies have involved phospholipid liposomes. Their lipid membranes contain one or a mixture of phospholipids, often including dipalmitoyl phosphatidyl choline (DPPC), sometimes with other lipids, proteins and perhaps various other substances. There is clearly a wide choice of compositions which can be used and will affect the liposomal properties such as size, encapsulation volumes and stability.

Liposome membranes can exist in two states, a gel state at lower temperatures and a more fluid liquid crystalline state at higher temperatures. Above the phase transition temperature liposomes become very leaky. Hence liposomes are generally maintained below their phase transition temperature, although higher temperatures may be used during their formation, as is the case in the reverse evaporation method (18). The phase transition temperature is 20-25°C for most lipids but 45°C for DPPC. Mixed lipid liposomes may not have a single phase change but may have a more gradual transition, consisting of mixed phase localized domains.

Various physical techniques can be used to determine fluidity, including nuclear magnetic resonance (NMR), electron spin resonance (ESR), fluorescence spectroscopy
or Raman spectroscopy (52). It is generally desirable to reduce fluidity in order to increase stability (i.e. to reduce leakage). Physical studies (52) indicate a potentially complex effect of the fatty acyl groups on fluidity; in terms of their length, number of double bonds and the presence or absence of cis-double bonds, which pack less compactly than trans-double bonds. The phospholipid head groups also affect the fluidity; as does cholesterol, which reduces membrane fluidity by perpendicular "wedge like" packing at a level just below the head groups. Cholesterol tends to reduce the permeability of the membrane up to a molar ratio of 1-1, above which it may have the reverse effect.

The in-vivo fluidity of membranes is thought to have important biological effects; most cellular membranes existing predominantly in the gel state with fluid domains around membrane proteins, many of which function better with greater mobility.

The addition of lipids of net positive or negative charge can alter the rate of incorporation or subsequent loss of charged molecules. Stearylamine is often added to produce positively charged liposomes and phosphatidic acid to produce negatively charged liposomes. Positively charged liposomes tend to be impermeable to cations whilst
negatively charged liposomes are generally freely permeable to cations. Anions, however, tend to diffuse through liposomes irrespective of their charge. The permeability of all charges of liposome membranes to protons is generally low. This may be helpful if a pH gradient must be maintained.

The incorporation of specific non-lipids can have effects on structure and permeability. For example, the permeability to specific ions can be selectively increased by the incorporation of valinomycin, certain antibiotics or cyclic peptides (53). Various substances, including glycophorin, a transmembrane protein (54) and acylated antibody (55), can act to stabilize unsaturated phosphatidylethanolamine liposomes. These are otherwise unstable at physiological conditions, rapidly transforming to non-bilayer, hexagonal II phase, structures with the release of their aqueous contents (56).

The lipid type can influence the liposome size and hence affect the encapsulation rate. For example, cholesterol at a molar ratio of 1-1 with phospholipid has been shown to increase incorporation of sucrose and cytosine arabinoside, an effect which was related to the size of the liposomes (18).
The degree of encapsulation of an aqueous substance and the subsequent stability of the liposome depends not only on the choice of membrane components and the synthetic technique but also to a degree on the aqueous phase. In addition to the relationship between the permeability to ionic species and the lipid charge, the ionic strength of the solution has an effect. In general encapsulation is increased at low ionic strengths (18).

An example of the importance of the aqueous phase is seen in the specific effects produced by calcium ions. These can induce fusion of small unilamellar vesicles containing acidic phospholipids to form multilamellar cochleate cylinders (28). Studies at the molecular level indicate that calcium ions can draw together negatively charged phospholipid groups and can also produce a localised phase change from fluid to gel state with close packing of fatty acyl chains. This may be a mechanism by which calcium ions exert an influence on cell fusion in vivo (52,57).

In the case of cardiolipin, the neutralization of negative charges on the head groups by divalent cations seem to draw them together and induce a phase change from lamellar to hexagonal (II) phase. Hexagonal (II) phases are rod like structures based on reverse micelles (i.e
monolayers with the fatty acyl chains protruding outwards) (2). Their formation can have the effect of destabilizing liposomes, releasing their contents. Cardiolipin is concentrated in mitochondria, and hexagonal (II) structures may have a biological function in providing pores in mitochondrial membranes creating channels for the passage of proteins (58).

Liposomes can be destabilized by a number of other aqueous phase substances which could be internal or external to the liposomes. The addition of general anaesthetics such as ether or chloroform have been shown to induce cation leakage (59). The addition of certain proteins to liposome preparations induces anion and glucose leakage. These include lysozyme (60), myelin basic protein (61) and immunoglobulins (62).

Complement will cause the release of the contents of liposomes which have first undergone an antibody reaction against membrane components such as cardiolipin (63). Leakage seems to be the mechanism, shown by analysis of the fluorescence quenching effects as marker is released (64). Leakage of calcein from small unilamellar vesicles was induced in vitro by calf serum (65), and both the complement system and lipoproteins have been implicated in lysis of liposomes in vivo (66). While these

20
observations have implications to the success of liposomes as delivery systems in vivo they should not generally cause excessive problems at the dilutions used for in vitro analytical techniques.

Liposomes can readily be lysed by detergents such as Triton X100 or by high concentrations of organic solvents such as ethanol or methanol. Care must be taken to avoid contamination of glassware, particularly by detergents as this may lyse liposomes and lead to erratic experimental results.

Because of the potential complex interaction of aqueous phase and lipid phase it is often difficult to predict on theoretical grounds the optimum conditions for entrapment of a particular aqueous material. It is generally necessary to combine predictions from previous experimental results with a degree of trial and error.

1.5.2 Protein Coupling Methods

The earliest attempts to incorporate proteins into liposome membranes involved non-covalent bonding which occurs due to electrostatic and hydrophobic interactions. This was demonstrated in multilamellar large vesicles
(67) and examined in small unilamellar vesicles (68), in which it was greater in liposomes containing anionic phospholipids. However, little study has been reported on the reproducibility of this method, the irreversibility of the coupling or the stability of the coupled liposomes. The majority of more recent studies have centred on the use of covalent coupling methods.

Methods to chemically couple proteins to liposomes have been extensively reviewed (34,37). This can be attributed to the importance of coupling macromolecules such as antibodies or protein antigens to liposomes. An advantage of these techniques is their transferability, i.e. the applicability of individual methods to a large number of different proteins.

Protein coupling methods fall into two main categories. The first involves attachment of protein directly to preformed liposomes by a chemical reaction with a lipid species, commonly the primary amine group of phosphatidylethanolamine. The second approach involves the prior modification of the protein with a hydrophobic "anchor" such as a fatty acid which can then be intercalated into liposomes, either at their preparation or subsequently.
The major advantage of the first approach is that liposomes can be preformed under conditions that have been optimized for entrapment, stability and other variables. Another advantage is the avoidance of preparative conditions which might denature the protein, such as the presence of organic solvents or high temperatures. The methods may involve comparatively few analytical steps and it may be easier to define and control the protein/lipid ratio.

The second approach, i.e. coupling the protein to lipid prior to liposome formation, has an advantage of avoiding the likelihood of liposome-liposome or protein-protein coupling which are certainly a potential problem in some of the earlier direct methods. It may also be desirable in some applications to be able to isolate and characterize the protein-conjugate prior to inclusion in the liposomes. The approach suffers however from the restrictions it imposes on the method of formation of the liposomes which must include the fatty acyl-protein derivative and which must avoid denaturing the protein. There is also the possibility of reduced exposure of incorporated protein if it is positioned on the inner surface of the membrane.
The main application of this approach has been by Huang et. al. (69). Their method involves the coupling of protein (e.g. antibody) to the N-hydroxysuccinimide ester of palmitic acid(NHSP) (which is first formed by a condensation reaction of palmitic acid and N-hydroxy succinimide). This method has been successfully used for small unilamellar vesicle coupling (70).

The method's use for coupling to large unilamellar vesicles commonly utilizes the detergent dialysis technique. Although this is a valuable technique for encapsulating macromolecules it is less suited to entrapping small dialysable materials such as dyes. A reverse-phase evaporation method has been reported (69) This involves addition of the modified protein at a stage after the evaporation but in the presence of a small amount of organic solvent. After mixing and standing for two hours in the presence of detergent, the mixture is dialysed. This technique is, therefore, in effect to some extent a hybrid of the reverse evaporation and detergent dialysis methods and may suffer the same limitation regarding the entrapment of small molecules.

The earliest methods which covalently coupled protein directly to preformed liposomes utilized bifunctional crosslinking reagents. These include glutaraldehyde (71),
carbodiimide (normally the water soluble 1-ethyl-3-(3-dimethylamino)-propylcarbodiimide (EDCI) (62) and dimethyl superimidate (72). These couple to amino groups on the protein and on the liposome, usually introduced through the incorporation of phosphatidylethanolamine. The methods are particularly susceptible to cross linkage, producing protein-protein bonding which may form large aggregates that can be difficult to separate from liposomes using gel chromatography or centrifugation. These reagents are also limited in the extent of their reaction.

Other techniques have been developed which circumvent the problem of protein-protein crosslinkage. If the protein contains carbohydrate residues, for example horse radish peroxidase, these can be oxidized to provide reactive aldehyde groups that can then be coupled to phosphatidylethanolamine or stearylamine containing liposomes (73).

The requirement of this approach for carbohydrate residues in the protein was removed by an alternative method of oxidizing glycosphingolipids incorporated into the liposome membrane (74) which could then react with amino groups on the lysyl residues of the protein.
Coupling through thiol groups has given rise to a number of useful methods based on the development of heterobifunctional reagents that can react both with amino and thiol groups. An example of these reagents is m-maleimidobenzoyl-N-hydroxysuccimide ester (MBS) (75). Thiol groups on the protein can be reacted with phosphatidylethanolamine incorporated into liposome membrane using this reagent (37). This technique gives high levels of coupling but requires the formation of unstable free thiol groups on the protein which must be conjugated immediately.

Another heterobifunctional reagent, N-succinimidy1 3-(2-pyridyldithio) propioniate (SPDP) (76) has been used in a similar way to MBS to couple, for example, Fab’ fragments of immunoglobulin to liposomes (77). The particular strength of SPDP is its ability to form a linkage between amino groups on the lipid membrane (e.g. phosphatidyl ethanolamine) and on the protein.

SPDP can be reacted with phosphatidyl ethanolamine prior to liposome synthesis to give a molecule containing a protected, relatively stable, thiol group; N-(3-(2-pyridyldithio)propionyl)phosphatidyl-ethanolamine (DTP-PE). The DTP-PE can then be incorporated into liposomes. SPDP is then reacted with the protein to give another
protected thiol group. Conjugation is achieved by first cleaving the disulphide group in the derivatized protein using dithiothreitol and mixing the protein and liposomes. The 2-pyridyl disulphide group on the phospholipid undergoes a thiol disulphide exchange producing a covalent dipropionyl-disulphide linkage between the phospholipid and the protein.

This technique is relatively straightforward to perform, uses moderate reaction conditions, produces a high degree of coupling and has the advantage that the reagent is commercially available (78). A potential disadvantage, however, of dipyridyldithiol reagents is the susceptibility of the disulphide bond to reduction.

Martin and Papadjopoulos (79) have used N-succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) to activate phosphatidyl-ethanolamine which can then be incorporated within liposomes and reacted with either the endogenous thiols of rabbit Fab' (69) or with thiols introduced into monoclonal antibodies by SPDP (80). The latter approach introduces a more stable bond than that produced by SPDP alone while avoiding the difficulties associated with the instability of free endogenous sulphide groups. A similar approach has been used with the reagent, N-succinimidylidoacetic acid (81).
Finally the versatility of recent indirect conjugation techniques, utilising either protein A or the avidin-biotin reaction, has been extended to liposome-protein conjugation. Liposomes can be coupled to protein A which can then be used to bind to the Fc portion of antibodies (82).

The avidin-biotin system can be used in several ways (83-86). Avidin can be conjugated to liposomes which will then bind to biotinylated antibody. Alternatively biotinylated antibody can be coupled to biotinylated phosphatidyl ethanolamine through an avidin bridge. The potential of these techniques is that a "universal" liposome preparation could be made which could react with a range of antibodies or other proteins. However careful optimization of reaction conditions is necessary to prevent unwanted cross-linkage reactions.

Once coupled it is necessary to separate the liposomes from the unreacted reagents and assess their characteristics. Many of the techniques used to purify liposomes and assess their stability can equally be applied to this stage. It is important to ensure that protein-protein aggregates do not separate with the liposome fraction during purification. This can best be assessed by the inclusion of appropriate control
experiments. The use of density gradient medium centrifugation techniques can be useful in the separation of protein aggregates from liposomes (37).

In order to assess the efficiency of coupling, the protein/lipid ratio can be measured. A convenient method to assess bound protein is to use a labelled protein as a tracer (37). Heath et. al. (74) studied the effects of varying protein/lipid concentration ratios and found that, using the technique of periodate oxidation of membrane bound glycosphingolipid, the bound protein/lipid ratio was dependent on the protein concentration in the reaction mixture much more than the lipid concentration.

It will also be necessary to investigate whether the bound protein is still active. The immune reactivity of a protein can be assessed by its binding to a radiolabelled antibody or antigen, as assessed by association of the radioactivity with the liposomes on physical separation (e.g. gel filtration or density gradient centrifugation). This may be adequate for application in an in vitro assay but may not indicate biological activity, which can be affected by alterations in, for example, antibody valency. It has been reported that enhanced antibody affinity can occur derived from an
increase in effective antibody valency when it is conjugated to liposomes (85).

1.5.3 Coupling Methods for Non-Protein Molecules

While hydrophobic substances can be incorporated into membranes as part of the liposome preparation by incorporation with the lipid in the organic phase, it may be necessary to couple small molecules such as haptens or other non-protein to lipid either before or after liposome formation.

Liposomes can bind by electrostatic or hydrophobic forces to a variety of polymers, such as dextrans (87) or polyamino acids (88). Both types of molecule can induce aggregation (88,89) and certain polyamino acids alter liposome permeability (72).

Electron spin resonance is a technique that has been applied to the study of membrane components at the molecular level (90). Spin labels can be introduced into liposome membranes. The probe TEMPO, for example, associates non-covalently with lipid (91). It is also possible to introduce probes by chemical coupling to phospholipid fatty acyl chains (92).
A number of haptens have been coupled to liposomes for in vitro or in vivo immunological uses. Thyroxine (T4) can be coupled to dipalmitoyl phosphatidylethanolamine prior to large unilamellar vesicle formation. This involves the reaction of the phospholipid with succinic anhydride and triethylamine to form a hemisuccinimide derivative which is then able to react with the thyroxine (93). Tan et al. (94) used an alternative procedure to synthesise 5-N-thyroxine-2,4-dinitrophenyl phosphatidylethanolamine.

Since, due to their sugar moiety, digoxin conjugates often are unstable, O'Connell et al. (48) used the aglycone of digoxin, digoxigenin, to form liposomes with the same antigenic determinants. They made use of the reactive amino group of phosphatidylethanolamine to couple it to 3-ketodigoxigenin prior to large unilamellar vesicle formation.

Canova-Davis et al. (95) synthesized a theophylline derivative of phosphatidylethanolamine, again prior to large unilamellar vesicle formation. This involved the reaction of glutaric anhydride and 4,5-diamino-1,3-dimethylpyrimidine-2,6-dione, followed by thionyl chloride to form the hydrochloride of 8-(3-carboxypropyl)-theophylline which then was coupled with
dipalmitoyl phophatidylethanolamine through a peptide linkage.

Six et al. (96) and Urema et al. (97) have reported methods for synthesizing dinitrophenylated phosphatidylethanolamine for model studies of antibody and complement reactions with liposomes.

As a final example, Ligler et al. (98) reported a method for coupling mycotoxin T-2 (hydroxydiacetoxyisovaler-oxyepoxytrichothecene) to phosphatidylethanolamine prior to liposome formation. T-2 was first succinylated, converted to its acid chloride and then coupled to the amino group of phosphatidylethanolamine.

1.6 Liposome Applications in Immunoassay

1.6.1 The Use of Liposomes and Choice of Encapsulated Marker

In most conventional immunoassays, it is usual for a single molecule of label (e.g. fluorophor) to be linked to a single molecule of either antigen or antibody. The
immunoassay is based on the ability to measure the label with adequate sensitivity.

Liposomal immunoassays can be designed, in contrast, in which many molecules of a label are entrapped within a liposome, and antibody or antigen can be coupled to the membrane surface. If the labels conventionally produce sensitive assays (e.g. fluorophors), the liposomes can be used to amplify the signal and result in even greater sensitivity.

Coupling a single chromogenic molecule to an antibody or antigen would be unlikely to produce a measurable signal in an immunoassay. In the case of a liposomal chromogenic assay, however, the amplification can be used to provide sufficient sensitivity to produce a measurable spectrophotometric signal. The advantage in this case might be assay speed and simplicity, rather than extreme sensitivity.

In clinical biochemistry laboratories, chemical analyses of common analytes (e.g. enzymes, glucose, urea etc.) are generally performed by spectrophotometric analysers. These almost always use liquid reagents which are optimized to avoid separation stages, and enable rapid analysis of large numbers of samples. To be performed on
these analysers, immunoassays require colorimetric markers and generally must be homogeneous (i.e. without a separation step).

Immunoassays are at present usually performed on separate dedicated instruments (e.g. by radioimmunoassay (RIA) (5), fluoroimmunoassay (8), chemiluminescence immunoassay (9) or enzyme-linked immunosorbent assay (ELISA) (7)). They can be homogeneous (e.g. using fluorescence polarization immunoassays (8)) or heterogeneous, requiring a separation step (e.g. ELISA (7)).

The development of homogeneous immunoassays with spectrophotometric detection might enable both chemical assays and immunoassays to be performed on a single spectrophotometric analyser. This would be likely to confer considerable organizational benefits.

There are a limited number of alternative homogeneous immunoassays which allow colorimetric detection. Immunoturbidimetry (99) is one example. It is based on light scatter resulting from antibody-antigen reactions and is primarily restricted to large antigens (usually proteins). The performance of this technique may be compromised because of interference due to light
scattering by biological fluids and dust particles in reagents.

Immunoturbidimetry is generally less sensitive than alternative immunoassay techniques (e.g. RIA). It is usually used to measure proteins of relatively high serum concentration, e.g. immunoglobulins, (approximately 0.5 - 30 g/l). Urinary microalbumin (molecular weight 69000 D), probably represents close to the limits of the basic technique, with respect both to molecular size and also to limit of detection (approximately 5 - 20 mg/l).

Although the basic immunoturidimetric technique is limited, particle enhancement (i.e. coupling of antibody or antigen to particles) can improve sensitivity (100). Latex is commonly used, although gold sols and erythrocytes have also been applied (100). Modifications to the technique also allow the measurement of smaller antigens, including drugs such as gentamycin and small hormones such as thyroxine. One such approach (manufactured by Bayer plc) (101) involves linkage of the antigen to Ficoll (TM Pharmacia). Ficoll has a high density, (it is also used as a density gradient medium), and agglutinates with microparticle-bound antibody in the absence of free antigen. The agglutination is inhibited
by the presence of free antigen, which competes for the antibody binding sites.

There are a few other homogeneous immunoassay techniques which can be performed using spectrophotometric analysers, particularly for small molecules such as drugs (e.g. enzyme-multiplied immunoassay technique (EMIT) (TMSyva) (7) and CEDIA (TMMicrogenics)(102)).

Although these alternative spectrophotometric assays are successful, none has become the established method of choice. They employ expensive reagents, and, because of the complexity of reagent composition, (and sometimes patent restrictions), it is not normally feasible for users to themselves develop new assays by these techniques.

EMIT is generally based on inhibition of an enzyme’s binding site by antibody in the absence of antigen. CEDIA involves the binding of two inactive portions of an enzyme; one of which is covalently bound to an analyte. Antibody binding to this conjugate inhibits activation of the enzyme, except in the presence of free antigen.

In both assays, therefore, enzymes are used as the marker. The enzymes produce products which can be
measured spectrophotometrically. The enzymatic reaction is a further step which usually adds to the assay time and adds more components to the reaction mixture (e.g. substrates and co-factors). Some of these components, including the enzymes, may limit reagent shelf-life.

A liposomal chromogenic marker, on the other hand, can produce a rapid signal (usually only depending on the rate of chromogen release), and the reagents will usually be simpler and more stable.

The choice of liposomal marker may depend on several considerations and may not be the same for all applications. Aspects which may be important include technical feasibility and convenience, both in the assay and the availability or synthesis of reagents; safety, sensitivity, speed of analysis, matrix affects of the medium to be analysed, transferability of methods and the cost, both of reagents and equipment.

1.6.2 Early Markers - Glucose and Electron Spin Labels

The ability of complement to lyse antibody-coated liposomes was an early observation (103). IgM antibodies
were found to be more effective than IgG in initiating complement damage. These studies, mainly in the 1970s, used glucose as a marker \(96,103\) of complement-mediated lysis. The increase in absorbance at 340nm after reaction with hexokinase, glucose-6-phosphate dehydrogenase and NADP was measured. This only occurred if the glucose was released from the liposomes. It was thus unnecessary to separate the free glucose from the intact liposomes. This formed the basis of an approach later developed for homogeneous liposome immunoassays. Glucose is not an ideal marker because of its presence in a range of biological fluids. Nevertheless complement-mediated release of glucose has been used to test for specific immune reactions against lipids \(104\) and lipopolysaccarides \(105\) incorporated into liposome membranes.

Another early approach \(92\) involved the use of electron paramagnetic resonance spin labels to study liposomal membrane properties \(90\). Humphries and McConnell, \(106,107\) incorporated the water soluble electron resonance spin label, tempocholine, into liposomes. The encapsulated label produced a broad paramagnetic resonance signal of low amplitude. This was due to the close proximity of the spin molecules which could exchange signals. On release by complement-
mediated damage, the tempocholine was diluted and the signal alteration measured.

A similar spin label technique was used to measure thyroxine chemically coupled to liposomal membrane (94). Complement-mediated lysis was competitively inhibited by thyroxine in the sample.

The use of spin labels has some advantages. Since they are not measured optically, they are less susceptible to some matrix effects of biological fluids, such as colour, turbidity or fluorescence. Spin resonance markers are also likely to have good sensitivity. However a limiting factor in its application is the need for sophisticated and expensive equipment which is not widely available.

1.6.3 Enzymes, Chromophors and Fluorophors as Markers

It was not until the mid-1980s that a series of publications began to appear using liposomes containing markers that might be more suited to routine analytical use, for example in clinical biochemistry laboratories. The main liposomal markers have been enzymes, fluorophors or chromophors (generally dyes). Each type of marker has its own particular potential advantages.
Enzymes have a capacity for sensitivity due to the amplification afforded by the enzyme reaction. However the need for the additional stages of the enzymic reaction may be disadvantageous compared to chromophors or fluorophors which can be read instantly. Fluorophors are probably capable of greater sensitivity than chromophors; however chromophors have the advantage of being measurable using the ubiquitous spectrophotometer. Assays using these various markers have been developed concurrently in recent years. Usually large unilamellar liposomes have been used. Some assays have been homogeneous (e.g. complement-mediated lysis) and some have been heterogeneous (e.g. coated tube) type assays.

1.6.4 Assay Using Cardiolipin Antibodies and Arsenazo III

In 1983, Janoff et al. reported an assay for the autoantibodies which are found in the sera of patients with systemic lupus erythematosus (108). These antibodies react with cardiolipin. When cardiolipin is incorporated into liposomal membranes, it can be induced to re-arrange from a lamellar to hexagonal (II) phase by divalent ions. In the technique, magnesium (II) ions were used to destabilize and lyse the liposomal membranes. The presence of systemic lupus erythematosus serum prevented this re-
arrangement. The marker used was the dye, Arsenazo III. This dye undergoes a colour change from red to blue on release from the liposomes due to sensitivity to the divalent cations in the extra-liposomal solution (109). The method required only a one minute incubation at room temperature. Although developed as a method for qualitative screening of sera, the colour change was claimed to be quantitatively measurable.

1.6.5 Complement-Mediated Assays Using Enzymes

In 1984 applications using alkaline phosphatase as the enzyme marker and based on guinea-pig complement-mediated damage were reported for thyroxine and immunoglobulin G (IgG) (93). These were both homogeneous, "one stage" immunoassays (although sodium hydroxide was added to terminate the enzyme reaction).

The assay for thyroxine involved a competition of thyroxine in the patients sample and thyroxine coupled to the liposomal membrane for free antithyroxine antibody. The antibody which became bound to the liposomes was inversely related to the concentration of the thyroxine in the sample. Complement in the solution invoked lysis and the released alkaline phosphatase was measured by its
action on the substrate, p-nitrophenylphosphate. The reaction was terminated by sodium hydroxide after thirty minutes and the absorbance at 410nm used to calculate the thyroxine in the sample.

The assay for immunoglobulin G was also competitive and made use of the competition for complement of IgG in patients' sera and liposomal membrane coupled human IgG F(ab')2 fragments. The reaction conditions and timing were similar to those for the thyroxine assay. Both methods correlated well with existing methodologies. It should be pointed out, however, that the IgG assay made use of the special property of immunoglobulin for binding to complement. The assay is not universally applicable in its described form to other macromolecules which do not bind to complement. Since 1984, several assays using complement have been reported but most have measured small molecules.

In 1986 Canova-Davis et al. (95) measured theophylline by a similar approach to that for thyroxine (93); a theophylline derivative of diphosphatidylethanolamine being incorporated into the liposomal membrane. This was used to induce lysis by antitheophylline antibody and complement. Lysis was inhibited by free theophylline from the patient's sample. The technique used glucose-6-
phosphate dehydrogenase as a marker. This had an advantage over alkaline phosphatase that it is not present in large amounts in serum. The enzymatic reaction was coupled with a dye, 2,6-dichlorophenolindophenol. The absorption of the dye at 610nm was reduced by the presence of NADH, avoiding problems of absorption of reaction components (e.g. complement) in the ultra-violet range. The reagents, some in lyophilized form, were shown to be stable for at least four months at 5°C.

In the same year (1986), Bowden et al. (64) used the complement reaction against antibody-sensitized liposomes to measure complement activity itself. Liposomes were produced incorporating a dinitrophenol derivative and reacted with antidinitrophenol antiserum and complement. The results obtained correlated with the haemolytic complement test (correlation coefficient(r) = 0.80) and also with complement components C3 (r=0.62) and C4 (r=0.74). Alkaline phosphatase was used as the liposomal marker. The test was claimed to have the advantages of stability of reagents, simplicity and speed compared to other techniques to measure complement.

Alkaline phosphatase was also used subsequently as a marker to demonstrate that albumin-coated liposomes
could be lysed by antialbumin antiserum and complement 110,111). Competitive complement-mediated assays for phenytoin, phenobarbital and carbemazepine have also been described, using entrapped glucose-6-phosphate dehydrogenase (112).

1.6.6 Complement-Mediated Assays Using Chromophors and Fluorophors

Fluorescent markers become quenched if incorporated into liposomes at high enough concentration. The fluorescence increases as the fluorophor is diluted on lysis of the liposomes (38-40,113,114).

Umeda et al., in 1986, (115) reported a sandwich liposomal immunolysis assay for C-reactive protein using complement and carboxyfluorescein as marker. The assay is of interest as one of the few examples of a complement-mediated assay for a protein. Antibody to C-reactive protein was coupled to the surface of multilamellar liposomes. Incubation with C-reactive protein and unbound antibody caused complement-mediated lysis.

In the following year Ligler et al.(98) produced a
competitive assay for the mycotoxin T-2 (hydroxydiacetoxyisovaleroxyepoxytrichothecene) from Fusarium using a complement-mediated assay. This also used the fluorescent marker, carboxyfluorescein. Liposomes incorporating membrane-coupled T-2 were employed. These activated complement if incubated with a monoclonal anti T-2 antibody, but only in the presence of polyclonal anti-mouse immunoglobulin as a second antibody. In the presence of free T-2 toxin the lysis was reduced. This assay, which required only a thirty minute incubation, was claimed to be approximately ten times more sensitive than a conventional enzyme immunoassay which used the same antibodies.

The same group subsequently described the coupling of Fab' fragments to liposomes using the method of Martin and Papahadjopoulos (with SMBP reagent) (79). They suggested these liposomes could be used in sandwich-type assays for large molecules but did not present supportive data (116).

Nomura et. al. (117) showed that thyroxine can be measured by the inhibition of complement-mediated lysis using a colourimetric label. (Thyroxine had been measured earlier with an enzyme marker(83)). The label which was used was 2-(2-thiazolylazo)-4-methyl-5-(sulpho(-)
methylamino) benzoic acid (TAMSMB), which was encapsulated within small unilamellar liposomes. This reagent became coloured in the presence of cobalt(III) ions which were added to the reaction mixture at levels high enough to prevent interference from metal ions in the patient's sample. A good sensitivity (limit of detection almost $10^{-10}$ mol/l) was claimed using this approach.

More recently, fluorescence release has been used to measure levels of antibodies to cytochrome C and myoglobin. Complement-mediated lysis was induced in the presence of the respective antibodies. Carboxy-fluorescein-containing liposomes coated in antigen were employed (118). Gelonin, a polypeptide plant toxin, has also been estimated by a competitive complement-mediated assay, using gelonin-coated liposomes containing calcein (119).

1.6.7 Melittin-Mediated Immunoassays

Freytag et al. (113,114), in 1984, reported an assay for digoxin which was not dependent on complement. In this assay antidigoxin antibody competitively reacts with digoxin from the patient's sample and an ouabain-
melittin conjugate. (Ouabain was substituted for digoxin because of greater solubility).

Melittin is a biological cytolysin which can lyse liposomes. If the ouabain-melittin conjugate is bound to its antiserum it loses lytic activity. This binding is competitively reduced by free digoxin (from the patient). The release of encapsulated alkaline phosphatase was monitored by the absorbance after reaction with p-nitrophenylphosphate. Although no assay performance data was presented this approach seems to offer the basis of a simple and rapid homogeneous assay for small molecules. It has the advantage that the liposomes used did not have to be modified by conjugation.

A similar melittin-mediated assay for digoxin has since been reported, based on a chemiluminescence detection of released glucose oxidase, by measuring the enzymatically produced hydrogen peroxide using isoluminol (120).

1.6.7 Assays Using Stabilized Liposomes

Ho and Huang (54), in 1985, reported an approach that was innovative in two major respects. Firstly, it was the
first practical application of the reduction in quenching of a fluorophor, in their case calcein, as it is diluted as a marker in an immunoassay. Fluorescence quenching loss had been used five years earlier to assess the serum induced leakage of liposomes (65).

The second innovation was the use of stabilized dioleoyl phosphatidylethanolamine (DOPE) liposomes. DOPE does not form stable liposomes at neutral pH and room temperature. However if certain materials such as the derivatives N- (dinitrophenylaminocaproyl)- phosphatidylethanolamine (DNP-cap-PE) or glycophorin A, a glycoprotein, are incorporated into the membranes the liposomes can be stabilized. Ho and Huang (54) showed that if stabilized liposomes are incubated with immobilized antibody to either dinitrophenol or glycophorin A, the liposomes are destabilized and lysis occurs. This can be explained by the rapid random diffusion of antigen to the immobilized antibody where it is bound. Thus a lateral phase separation of membrane components leads to a localized relative depletion of the stabilizing antigen in a portion of the liposome. When this happens, the DOPE converts to an hexagonal (HII) phase and the liposomes lyse.
In the presence of free antigen or antibody the solid phase immunoreaction was inhibited and lysis was reduced. This formed the basis of an immunoassay for either antigen or antibody. The assays described had some practical limitations. The antigens (dinitrophenol and glycophorin A) are of restricted practical application. Also small unilamellar vesicles were used, (facilitating the incorporating of the stabilizing substances at the time of liposome synthesis). Finally the assay design was inconvenient (coated glass slides being used as a solid phase). Nevertheless the report was a novel and innovative immunoassay approach.

The same group subsequently reported a technique to detect cells infected with Herpes Simplex virus (HSV) (121). Again unsaturated phosphatidylethanolamine liposomes were used, but in this case stabilized by acylated-antibody against the virus. The antibody stabilized the liposomes until it bound to the virus membrane, when destabilization occurred, probably by the mechanism described in the earlier report. The assay avoided the inconvenient solid phase of the previous study by, in effect, using the virus as a solid phase. Calcein and alkaline phosphatase were both used as markers.
1.6.8 Solid Phase Liposomal Immunoassay

O'Connell et al. (48), in 1985, used antibody-coated tubes in an immunoassay for digoxin. Polypropylene tubes were coated with digoxin antiserum in glycine buffer (pH 9.6). These were used in a competitive solid phase immunoassay, free digoxin from patients' samples competing with digoxinogen-coupled liposomes for binding to the tubes. (Digoxinogen is the aglycone of digoxin which is immunologically similar and is used because of greater stability). After a thirty minute incubation; the tubes were drained, rinsed and their contents released by lysis with detergent. The marker in this assay was the dye Sulphorhodamine B which was encapsulated in large unilamellar liposomes and which could be measured by its absorbance at 565nm. The liposomes showed good stability over six months; and the assay correlated well with radioimmunoassay and had acceptable precision.

In a brief poster presentation (122), Vonk et al. entrapped a europium complex within liposomes in-order to detect the signal by time-resolved fluorescence. They claimed good sensitivity in micro-titre plate solid phase assays for human chorionic gonadotrophin and for thyrotropin.
One of the few commercially available liposomal immunoassays is a qualitative test based on a solid phased assay. In the Q Test for Group A Streptococci (TM Beckton Dickinson), throat swabs are inoculated onto a membrane impregnated with antibodies specific to the bacterial antigens. Specific antibody-coated liposomes are then added, together with a wash liquid. Liposomes remain bound to the centre of the membrane only in the presence of bacterial antigen (123).

1.6.9 Potentiometric Detection of Liposomal Markers

Monroe (16), in 1988, described the use of potentiometric detection of the components released from lysed liposomes. Ions which were impermeable to liposome membranes (e.g. trimethylphenylammonium or tetraphenyl-ammonium ions (124)) were able to be detected directly by ion selective electrodes. In an alternative approach, released markers such as enzymes (125) or glucose (126) could participate in reactions producing detectable species such as oxygen. This could confer an amplification to the signal. While requiring a degree of technical complexity, this approach has some advantages, such as the lack of interferences by turbidity of serum samples.
A procedure to measure theophylline has been reported using complement lysis of theophylline-coated liposomes and antiserum; in which horseradish peroxidase was used as the enzyme marker. The lysis was inhibited competitively by free theophylline from patients' samples. Once released the enzyme produced oxygen from hydrogen peroxide, which was detected by an oxygen microelectrode (125). A thirty minute incubation was sufficient to allow complement-mediated lysis and electrode equilibration. An alternative approach (16) used melittin conjugates to detect theophylline, (as described earlier (113,114)), and also used horseradish peroxidase to produce an oxygen electrode signal.

1.6.10 Other Immunological Applications

Durst et. al. (127) reported a liposome-enhanced flow injection immunoanalysis system. This was designed to allow competition between immobilised Fab' antibody fractions, antigen-coated liposomes encapsulating carboxyfluorescein, and free antigen from samples. The liposomes and sample were fed into an immunoreactor containing the F(ab)′ fragments coupled to glass beads. The unbound liposomes then flowed into a chamber where they were lysed by surfactant injection and the released
contents passed to a fluorescence detector for measurement. The strength of signal was claimed to be related directly to the amount of free antigen from the sample. Critical aspects of this technique, such as the hydrodynamics of liposome flow, have been studied by the same group, (128) who have developed a competitive assay for theophylline using this approach.

Antibody-coated liposomes have been widely applied as means to target cells containing antigenic determinants of interest; either to enhance histological staining, for cell-biological studies or to assess the therapeutic potential of targeted liposomes prior to in-vivo use (36,37,129). Truneh et al. (36) applied mixed populations of two different antibody-coated liposomes, one type containing sulphorhodamine and one carboxyfluorescein, as multicolour immunofluorescence markers for both flow cytometry and fluorescence microscopy. A flow-cytometric assay for anticardiolipin antibodies has also been described (130), based on altered light scattering when the antibodies reacted with cardiolipin-containing liposomes.

Antibody-bearing liposomes can also be used to enhance latex agglutination assays. An application for the detection of human rheumatoid factor has been
reported (131). Human rheumatoid factors are a class of immunoglobulin, predominantly immunoglobulin M, which binds heat-denatured immunoglobulin G. The conventional latex agglutination technique involves the agglutination of latex beads coated with heat-denatured immunoglobulin G by samples containing rheumatoid factor. The addition of liposomes coupled at their surface with anti-human immunoglobulin M, which bind to the rheumatoid factor sensitized latex, increases the speed and sensitivity of the latex agglutination. This enhancement seems to rely on the membrane characteristics of the liposomes since anti-human immunoglobulin M-coated latex particles do not themselves enhance agglutination.

1.7 Non-Immunological Liposome Applications

Not all the analytical applications of liposomes involve immunological techniques. One of the earliest applications, based on the loss of quenching of carboxyfluorescin following the lysis of liposomes, measured the lytic enzymatic activity of phospholipase C, an enzyme produced by C. welchii (132). The method involved a thirty minute incubation. It was claimed to be more sensitive, rapid and convenient than the conventional method which involves the digestion of a
lecithin dispersion followed by titrimetric estimation of the released fatty acids. A similar release of fluorescence was seen with phospholipases from other sources.

Depending on the site of cleavage of the phospholipid molecule, other phospholipases may give similar release of liposomal contents. Phospholipase A is of clinical interest, since its concentration is raised in acute pancreatitis. Since a product of phospholipase A is lysophosphatidyl choline which has a detergent action, a degree of liposomal lysis seems likely. The phospholipases have been conventionally measured by titrimetry. Although simpler enzymic methods are now available (133), these still involve additions of several reagents. Liposomal assays might be considerably simpler and quicker.

Another application is interesting since it made use of chemiluminescence (13), which has also been used as label in liposomal immunoassays. The application employed unusual small unilamellar vesicles made from the heterophilic compound, didodecyldimethylammonium bromide, which in combination with uranine and sodium hydroxide forms a chemiluminescent system (134). This system is sensitive to free cyanide ions, which could be measured
at levels much lower than can be obtained by conventional spectrophotometric or ion selective electrode methods. The sensitivity is thought to be due to an enhanced quantum efficiency and/or energy transfer efficiency in the ordered environment of the liposomal membrane.

The effects of free radicals are another possible non-immunological area for liposomal assay development. Liposomes can be used to study or measure the effects of lipid peroxidation. This is a topical area; in light of the interest in free radical reactions, particularly relating to ageing processes, and in the therapeutic effects of anti-oxidants. Lipid peroxidation has been studied by analysis of the effects of factors such as ultra-violet light on the lipid composition of liposome membranes (135). An additional effect is the release of liposomal contents by membrane perturbation. This has been shown for liposomes containing glucose in response to chemical peroxidation by ferrous ions and ascorbate (136) and to ultra-violet light (137). It has also been demonstrated using chromium ion-containing liposomes in response to superoxide anions (138). A difficulty in this approach is the lack of stability of unsaturated phospholipids on storage. If this could be overcome; liposomes might provide a straightforward test for free radical reactions and anti-oxidant activity, perhaps by
dye release, as well as shedding light on the molecular and cellular processes involved.

Two final applications suggest a role for liposomes in enhancing the performance of reagents used in other analytical techniques. In the first, Ullman et al. (139) used liposomes to compartment mutually reactive reagents within a stable single mixture. They applied this approach to an homogeneous EMIT (enzyme multiplied immunoassay technique) for theophylline. This normally requires separate addition of antitheophylline antibody and theophylline-glucose-6-phosphatase conjugate to the sample; premature mixing rapidly causing inhibition of the conjugate by the antibody. The authors encapsulated the conjugate within liposomes and found that more than 92% of the conjugate activity was still present after one year in the presence of the antibody. At the time of assay, the liposomes were lysed by detergent in the buffer used to dilute the samples. The approach of combining reactive reagents using liposomes may have practical benefits for other techniques that use mutually reactive reagents.

Another application of liposomes was an homogeneous signal amplification system (140). Dioleoylphosphatidyl-ethanolamine liposomes were stabilized by incorporation
of ganglioside GM1 (an alternative to glycophorin or dinitrophenol as used by Ho and Huang (54)). Ganglioside GM1 is a substrate for β-galactosidase, an enzyme used in some immunoassays. On exposure to β-galactosidase the liposomes were destabilized and lysis occurred. This released entrapped glucose-6-phosphate dehydrogenase, which could be measured with a degree of amplification over the signal produced by β-galactosidase.

The β-galactosidase produced by the CEDIA (TM Microgenic) assay for digoxin was used to lyse the stabilized liposomes. This resulted in enhanced sensitivity due to signal amplification. Although the increase in sensitivity in this particular application was probably of limited clinical use, liposomes were demonstrated to have a role as secondary reagents to enhance the sensitivity of other immunoassays.

1.8 Summary of the Analytical Applications of Liposomes

Although the use of liposomes as analytical tools is quite a recent development there are already a number of reported applications.
A range of small molecules or "haptens" have been analysed including thyroxine (93, 94, 117), theophylline (16, 95, 128), digoxin (48, 113, 114) and mycotoxin T2 (98). Cyanide has also been measured by a non-immunoassay technique (134).

Assays for antibodies against membrane components have also been relatively numerous, including assays for antibodies against cardiolipin (108), dinitrophenol and glycophorin A derivatives (54) and other membrane lipids or lipopolysaccharides (105-107). These assays either utilize complement-mediated lysis (105-107) or rely on the delocalization of stabilizing liposomal components (54). The latter technique was also applied to the measurement of Herpes Simplex Virus (121).

There have been surprisingly few methods to measure protein antigens, despite the relative ease of protein conjugation methods and the theoretical simplicity of certain methods (e.g. coated tube techniques).

No applications of either the membrane destabilization techniques (54, 105-107, 121) or melittin-induced lysis (113, 114) have been reported for the measurement of proteins. Perhaps surprisingly, the solid-phase coated-tube technique (48) has not been widely used for
macromolecular antigens; although the only commercial application has been a qualitative solid phase assay for Group A Streptococcal antigens (123).

Complement-mediated lysis has also been used to measure complement itself (64) and to assay immunoglobulin G, where the immunoglobulin competed for complement with membrane-bound immunoglobulin fragments. The enzymatic estimation of phospholipase C (132) and the augmentation of the latex agglutination method for rheumatoid factor(131) have also been reported.

Assays involving complement-mediated lysis of liposomes have tended to measure either small molecules incorporated in the membrane or other lipid components. They have been shown to be a means of measuring the concentrations of antibodies specific to protein antigens on the liposomal surface (110,111,118). The plant polypeptide, gelonin, has been measured by a competitive, complement-mediated assay (119).

There have been very few reports that antibody-coated liposomes can react with antigen and free antibody to induce complement-mediated lysis. This type of sandwich technique would be a simple and attractive means to measure protein antigens. One report has claimed success
in this approach, using liposomes coated with intact antibodies to CRP in a sandwich-type assay for C-reactive protein (115).

Despite the limited number of published examples of its use to measure protein antigens, complement-mediated lysis is the most frequently adopted approach to liposomal immunoassays.

1.9 Aims of the Project

The primary aim of this project is to design liposomal assays and to examine their role in measuring analytes of clinical biochemical interest. Their advantages and disadvantages, relative to the wide range of alternative techniques available, will be examined.

The choice of the encapsulated marker is necessarily a compromise between various advantages and disadvantages. The use of Sulphorhodamine B as a liposomal marker will be investigated. This dye can be measured spectrophotometrically, giving the possibility of assays easily applicable on readily available equipment; and also fluorimetrically, with the possibility of greater
sensitivity. It has the advantages of a high absorbance coefficient and is also highly soluble in aqueous solutions.

An attempt to develop homogeneous liposomal immunoassays using Sulphorhodamine B will be undertaken. If Sulphorhodamine B is a useful marker it will be applied to model complement-mediated assays, since these are the commonest homogeneous type of liposomal assay and have been shown to be applicable to a wide range of analytes.

The possibility of developing homogeneous complement-mediated assays for protein antigens will also be examined. Despite the potential usefulness of this approach, there have been few reports of successful assays of this type.
Chapter 2

Preparation of Liposomes; Optimization of Sulphorhodamine B Incorporation and Spectrophotometric and Fluorimetric Properties of the Free and Encapsulated Dye

2.1 Introduction

A wide range of substances have been encapsulated within liposomes. Maximum incorporation is dependent on physical considerations such as size and charge of the encapsulated "marker". Despite this, it is usually difficult to make an accurate prediction of the conditions likely to optimize incorporation of a given marker because of the number of variables involved. It is necessary to assess factors such as the types and combinations of lipids used, buffer pH and ionic strength in order to optimize incorporation. Consideration is also required as to whether these factors are most appropriate to produce liposomes with the desired characteristics for a given application; such as their stability under conditions both of storage and of use.

The relative merits of differing types of liposomes in immunoassays was discussed in chapter 1 (section 1.6.1).
The reverse evaporation method is a convenient technique which produces large unilamellar vesicles (LUVs). These tend to have a higher capacity to incorporate aqueous material than other types of liposomes (section 1.3).

The dye Sulphorhodamine B was considered as a potential aqueous "marker" to be incorporated into the liposomes. It has both a high absorbance coefficient and also a high aqueous solubility. These properties are predictable from the dye's structure (figure 2.1). The amino group is characteristic of the group of rhodamine dyes, which tend to be basic in character. However the presence of sulphite groups render Sulphorhodamine weakly acidic, with a net negative charge in near neutral pH solutions. The heterocyclic aromatic structure stabilizes the otherwise unstable pyrylium cation (-O\(^{+}\)-). These ring structures are characteristically highly coloured.

2.2 Preparation of Liposomes Incorporating \(^{125}\)Iodine-Labelled Bleomycin

Radiolabelled bleomycin was incorporated into liposomes. This was to obtain information of entrapment using the reverse evaporation method; with a view to its use as a
Figure 2.1

Chemical Structure of Sulphorhodamine B
reference with which to compare subsequent incorporation of Sulphorhodamine B.

A total of 66 umol of purified lipid consisting of PC/Chol/PTA (molar ratio 10:2:1) was dissolved in 2ml chloroform and 2ml petroleum ether. 1 ml of phosphate buffer containing I-125 bleomycin was added and the mixture sonicated for 2 minutes in a cold water bath at an amplitude of 10um. A further 1ml of phosphate buffer was then added and the suspension transferred to a long neck evaporating flask warmed in a water bath to 45°C. The organic solvent was removed by evaporation under reduced pressure. The suspension rapidly formed a gel which then collapsed to produce an opalescent aqueous suspension containing liposomes. The liposomes were immediately purified by separation using Sepharose 4B gel filtration.

Liposome preparation (0.5 ml) was applied to a 0.7 mm x 20 cm gel filtration column containing Sepharose 4B, which had been pre-equilibrated with phosphate buffer. Fractions (1 ml) were collected using an automated fraction collector.

The radioactivity of each fraction was counted over 100s in a gamma counter (figure 2.2). The liposomes eluted in
Radioactive bleomycin-containing liposomes were formed by the reverse evaporation procedure and then separated using a Sepharose 4B gel filtration column. Fractions (1ml) were collected and their radioactivity measured (as described in section 2.2).
the void volume, as clearly seen by opalescence in these fractions, while the free bleomycin was retarded by the gel and formed a second peak of radioactivity. The radioactive counts in the liposomal fractions were divided by the counts in both peaks. This indicated a 30% incorporation of the radioactive bleomycin into the liposome fraction, which was similar to the incorporation of bleomycin obtained previously using a similar procedure (141).

2.3 Examination of the Spectrophotometric and Fluorimetric Properties of Sulphorhodamine B in Aqueous Solution

Solutions (0.1 mol/l) of Sulphorhodamine B supplied by Sigma was prepared both in distilled water and phosphate buffer. The impurities of the Sigma dye (30% by weight) were stated by the manufacturer to be non-chromogenic material, although the impurities could not be identified. They were not included in the molarity calculation. A confirmation that the impurities were non-chromogenic was obtained by comparing absorbances and scans with solutions of dye from Kodak which was more than 99% pure. Both solutions were diluted as appropriate, scanned spectrophotometrically and the
absorbance coefficient of the dye and their absorbance maxima determined. The spectrophotometric scans (figure 2.3) and the molar absorptivity for both dyes appeared to be the same, discounting the Sigma impurities. This was calculated to be $1.13 \times 10^5 \text{ mol}^{-1} \text{ cm}^{-1}$ at a wavelength maximum of 565 nm. At this wavelength Beer's law was found to be obeyed up to a concentration of approximately $1.5 \times 10^{-5} \text{ mol/l}$. Use of either distilled water or phosphate buffer did not alter the optical properties significantly.

The dye solutions were found to fluoresce. The fluorimetric characteristics of the dye were examined and are described in appendix 2.

There were no detectable fluorimetric differences between the two sources of dye, whether distilled water or phosphate buffer was used as a solvent, using an excitation maximum of 400 nm with an emission peak at 580 nm. These wavelengths were selected as a means of measuring dye concentrations in subsequent studies. Using these wavelengths; the fluorescence of the dye was linearly related to dye concentration up to approximately $6 \times 10^{-6} \text{ mol/l}$, but above this concentration showed a quenching effect characteristic of fluorophors (figure 2.4).
The fluorescence of Sulphorhodamine B was measured at various concentrations in distilled water. The excitation wavelength was 400 nm and the emission wavelength was 580 nm. A definition of fluorescence arbitrary units has been given in the materials and methods section.
As the properties of the two manufactures' dyes were similar the Sigma dye was used subsequently (except in later experiments to confirm similar liposomal entrapment of the two sources of dye (section 2.11)).

As the units of fluorescence were arbitrary, the fluorimeter was calibrated so that a $2 \times 10^{-6}$ M dilution of Sulphorhodamine B produced 900 arbitrary units; using 10 nm excitation and emission band widths. Under these conditions (ex. 400nm; em. 580nm), 4 arbitrary fluorescence units was approximately equivalent to an absorbance of 1 mA at 565 nm. However the fluorescence sensitivity could be increased by widening the band widths (as described in appendix 2).

To examine the effect of pH on the absorption and fluorescence of the dye, 0.05M phosphate buffers were made up between pH2 and pH12 at pH2 intervals (using a phosphoric acid, phosphate salts and sodium hydroxide as appropriate). The spectra of the dye solutions were examined by both spectrophotometry and spectrofluorimetry. The effect of varying buffer strength was also examined by preparing a range of buffers of between 0 and 0.5M at 0.1M intervals. Neither alteration
of pH or buffer concentration between these limits had any detectable effect on the spectrophotometric or fluorimetric properties of the dye solutions.

The effect of adding either ethanol or Triton X-100, both of which can be used to release the contents of liposomes, to solutions of dye was examined. Neither ethanol or Triton X-100 had any detectable effect on the spectrophotometric or fluorimetric properties of the dye at the maximum concentrations used in subsequent experiments (dilutions of 1:50 for Triton X-100, and 1:10 for ethanol).

2.4 Procedure for Preparing Liposomes Encapsulating Sulphorhodamine B by Reverse Evaporation Method

Liposomes were prepared as described above (section 2.2) incorporating 1ml of 0.1 mol/l Sulphorhodamine B in 0.05M phosphate buffer as the aqueous phase (rather than bleomycin). The incorporation was assessed using Sepharose 4B. A small volume (20 ul) of eluted 0.5ml aliquots was mixed with 100ul ethanol (to release entrapped dye from liposomes), then diluted in phosphate
buffer to give a measurable absorbance (total dilution usually 1/1000). Good separation of the liposomal and free dye peaks was obtained by Sepharose 4B chromatography (figure 2.5), the liposomes eluting in the void volume. The percentages of dye in the liposomal fractions were calculated to be 8.9% and 9.3% in consecutive preparations under these conditions.

When, as a control, diluted free dye alone was applied to the column no detectable dye eluted in the void volume. As a further control, the reverse evaporation technique was performed with free dye but without lipid. The position of the free dye peak was unaltered, indicating that the liposomal peak was not an artifact due to an effect of the procedure on the dye. The fractions containing liposomes were collected and stored at 4°C for further investigation.

Ion exchange chromatography was carried out for comparison to gel filtration. The liposome preparation was eluted through a column containing Sepharose 4B-DEAE-CL. As in the case of Sepharose 4B the liposomes were eluted in the void volume and the percentage of the total dye in this fraction (elution of two preparations, 7.8% and 9.5% respectively) was similar to Sepharose 4B. However the free dye could only be eluted using 6M HCl.
Figure 2.5

Separation of Sulphorhodamine B-Containing Liposomes from Free Sulphorhodamine B Using Gel Filtration

Sulphorhodamine B dye-containing liposomes were prepared by the reverse evaporation method and then separated from free dye using a sepharose 4B gel filtration column. Fractions (0.5ml) were collected and measured spectrophotometrically (as described in section 2.4).
which was less practical and this technique was not pursued further.

The presence of liposomes was confirmed by several means. Firstly, on microscopic examination of the liposomal fraction under high magnification (x400), liposomes were clearly visible as minute red coloured spheres (appendix 1). The liposomal fraction, although very red, was noticeably more turbid than the free dye fraction. The turbidity cleared on addition of ethanol or Triton X-100, which was presumed to be due to liposome lysis.

It was found difficult to directly measure the amount of lipid eluted to establish that most was present in the void volume, in order to confirm this to be the liposomal fraction, because of the high absorbance of dye present. To provide evidence that this was the case, a separate identical liposome preparation was prepared in which the dye was replaced by phosphate buffer. The cholesterol was measured using a cholesterol oxidase procedure and compared to that included in the liposome preparation. 90% of the added cholesterol was calculated to be eluted in the liposomal fraction (i.e. in the void volume).
2.5 Dialysis as a Means to Assess Liposome Stability

The stability of liposomes can be defined as their resistance to loss of encapsulated material. As referred to in section 1.4, the term "lysis" is used to describe the process leading to release of this material, although the loss could be either by leakage or by rupture.

To assess liposome stability the rate of dye loss from the liposome fraction obtained from Sepharose 4B filtration was measured. The fraction was dialysed against 40 times its volume in phosphate buffer at room temperature with rotary mixing. The absorbance of the dialysed dye was measured at frequent intervals.

Under these conditions there was an initial rapid rate of dye dialysis. This decreased in an exponential manner towards equilibrium in 2-4 hours using 9/16 inch diameter dialysis tubing; or approximately 12 hours using 1/4 inch diameter dialysis tubing. This was mainly attributable to dialysis of free dye present in the liposomal fraction. To show this free dye alone was dialysed and the dye completely equilibrated with the dialysis buffer within these time periods. Typically approximately 10% (range 5% to 20%) of the dye present in
the liposome fraction was found to be released at this stage.

There followed a slower release of dye which was usually at a rate of less than 1% per 24 hours. This was attributed to the release of entrapped dye from the liposomes (Figure 2.6). This secondary slow rate gave an indication of the length of time a preparation would be usable as a reagent. To assess this, the dialysate absorbance was measured at least once a day over at least two weeks. The rate of release was found to be approximately linear over this period. Stabilities of liposomal preparations could be compared by calculating the percentage of the dye within the dialysis tubing released per 24 hours. The first 24 hours was excluded to reduce the effect of dialysis of free dye from the preparation.

Similar experiments were conducted at 4°C to assess the stability of the liposomes under refrigerated storage conditions. For practical reasons, daily mixing was used rather than continuous mixing. At 4°C, the secondary rate of dye release was either very low (typically <0.1% per day) or immeasurable over a period of up to six months.
The absorbance of the dialysate was measured periodically, when Sulphorhodamine B liposomes (fraction from sepharose 4B) were dialysed against phosphate buffer (x40 volume) at room temperature (as described in section 2.5). The symbols on the graph refer to the following: (a) = period of release of free dye from within the dialysis tubing; (b) = period of release of liposome-encapsulated dye; (x) = extrapolated absorbance deduced to be due to free dye present at the initiation of dialysis.
2.6 Fluorescence Quenching of Liposome Preparations and its Use in Estimating Amount of Dye Entrapment

The spectrofluorimetric properties of the free dye have been described in section 2.3. At the dye concentration incorporated into the liposomes the fluorescence was quenched (figure 2.4). The fluorescence remained quenched even when the intact liposomes were diluted since the concentration within the liposomes remained high. On lysis of the liposomes, the dye became sufficiently dilute that the solution fluoresced. This phenomenon, referred to as quenched fluorescence release, has been applied as a marker in immunoassays, as described in sections 1.4 and 1.6.6.

Provided the entrapped dye is completely quenched and the liposomes are diluted sufficiently that on lysis there is no quenching, an estimate of the percentage of dye that is encapsulated in a preparation can be calculated. The following formulae, derived from the work of Weistein and Ralston (38) on the fluorophor, carboxyfluorescein, were applied to the prepared liposomes.
Fraction of dye in the liposomes = 
\[
\frac{(1 - f/ft)}{Qv} \tag{1}
\]

\(f\) = fluorescence before lysis

\(ft\) = fluorescence after lysis

\(Qv\) = Quenching Coefficient

\[Qv = 1 - av \tag{2}\]

\(av\) = fluorescence of dye in liposomes (fluorescence of same concentration without quenching)

If the concentration is sufficiently high, \(av\) will be 0, in which case;

\[
\%\text{encapsulation} = (1 - f/ft) \times 100\% \tag{4}
\]

To estimate encapsulation in practice, a small amount of a liposome preparation (e.g. 20\textmu l) was diluted in five times its volume of ethanol (e.g. 100\textmu l), then vortexed briefly. A second aliquot was diluted to the same degree
in phosphate buffer. The mixtures were then both diluted in phosphate buffer to give fluorescences in the linear range (usually at least 1000 fold). The ratio of the two fluorescences were applied to formula (4) to calculate the percentage entrapment. (Duplicates were measured to minimise pipetting errors.)

It follows from equation (4) that the amount of fluorophor-release from a liposome preparation can also be estimated from the change in fluorescence. This can be calculated using equation (5), as long as the liposomes contained quenched fluorophor and the released fluorophor is sufficiently dilute not to be quenched.

$\text{fluorophor-release (\%)} = \frac{(\text{final fluorescence} - \text{initial fluorescence})}{(\text{maximum fluorescence (ft)} - \text{initial fluorescence})} \times 100\%$  

(5)

The entrapments of dye in two liposome preparations (prepared as in section 2.5, but prior to gel filtration) were calculated from equation (4). This indicated slightly higher entrapments of 12.5% and 11.2% respectively, compared to 9.6% and 8.9% respectively for entrapments calculated by gel filtration.

The validity of the technique depends on the absence of light scatter effects. This was assessed by extensive
dialysis of the preparations obtained by gel filtration (dialysed three times, each for 24 hours at 4°C, against fresh phosphate buffer) to remove free dye. The amount of dye release in the final dialysis was less than 0.1% of the total. The fluorescences of a dilution of 1/1000 of the dialysed preparations were 2 and 4 arbitrary units respectively, while after lysis the fluorescences were 450 and 459 respectively. This indicated an entrapment of over 99% in the dialysed preparation. The low fluorescence of the liposomes in the near absence of free dye suggested a lack of interference by light scatter from the liposomes.

2.7 Comparison of Gel Filtration and Dialysis as Means of Purifying Liposome Preparations

For several preparations, the entrapment calculated by gel filtration and dialysis were compared. In order to ensure the complete removal of the large amount of free dye from the liposome preparations (not subjected to gel filtration), they were each dialysed for two hours at 4°C against 40 times their volume of phosphate buffer, then dialysed three times more, each for 24 hours at 4°C against fresh phosphate buffer.
A higher percentage entrapment using the dialysis data was found (12.5% and 11.5%) compared to gel filtration data (9.6% and 8.9% respectively). Lack of complete dialysis seemed unlikely in view of the low release of dye in the final dialysis. Furthermore; fluorescence quenching release calculations of the level of entrapment (section 2.6) agreed quite closely with the dialysis data, and indicated greater than 99% encapsulation of dye in the final dialysed preparations.

The apparent relative underestimation by gel filtration of entrapped dye in the liposome fractions, compared to dialysis or fluorescence quenching data, might appear to contradict the observation in section 2.5 that the post- gel filtration liposome fractions contain free dye. However, the two observations can be reconciled, by disruption of liposomes in passage through the column resulting in loss of entrapped dye from the liposome fraction. This would explain the lower calculated dye entrapment using gel filtration compared to the other techniques. The free dye in the liposome fraction suggests further destabilization of the liposome fraction resulting in further dye release at or soon after its elution from the column.
To investigate whether this apparent release of dye on gel filtration was simply related to the gel pore size, a liposome preparation was fractionated through Sepharose 6B gel. This has a larger pore size than Sepharose 4B. A very similar separation and calculated entrapment was found for both types of gel (9.2 and 9.0% for Sepharose 6B compared to 9.6% and 8.9% respectively for Sepharose 4B).

Thiomersal (0.01%) was used as a gel preservative and washed out with buffer before use. Loss of incorporation could be due to thiomersal; a residual amount of which may have been retained on the columns. To test this, liposomes were purified by repeat dialyses (as above), mixed with 0.01% thiomersal for 30 minutes, then redialysed. The release of dye was very small; being calculated as 0.2% dialysed. Furthermore, the thiomersal-treated liposomes had entrapment of greater than 99% of the dye using fluorescence quenching measurements.

No marked difference in calculated entrapment by gel filtration was observed whether or not either 1 g/l bovine albumin (9.1%) or 1% normal sheep serum (9.5%) was added to the pre-equilibration and elution buffers; nor if distilled water was used in place of phosphate buffer.
to elute the liposomes (8.8%). (Figures in brackets refer to the means of calculated entrapments from duplicate experiments).

2.8 Effect of Encapsulation on the Spectrophotometric Properties of Sulphorhodamine B

The optical properties of the dye in solution have been described (section 2.3). On encapsulation there was an unexpected change to the spectrophotometric scan of the dye. Figure 2.7 shows the scan between 500 nm and 600 nm of liposomes containing dye. The liposomes were prepared as in section 2.5, purified by Sepharose 4B, then dialysed three times against x40 volumes of fresh buffer at 4°C, each for 24 hours, to remove free dye as completely as possible. The free dye's absorbance peak of 565 nm was greatly reduced when the dye was entrapped in liposomes. There was a large second absorbance peak at 530 nm for the entrapped dye whereas there was only a slight shoulder on the free dye scan.

The liposomes were subsequently lysed by adding ethanol in the ratio of one volume to five volumes of the liposomes. When the liposomes were lysed the dye's
Wavelength scans of solutions of liposomes or free Sulphorhodamine B. The scans represent: (a) liposomes containing dye; (b) liposomes containing dye after lysis using ethanol; (c) free dye.
absorbance pattern changed to that of free dye (figure 2.7). There was an increase in absorbance of approximately 2.2 times at 565 nm when liposomes containing entrapped dye were lysed.

The effect was shown not to be an artifact of adding ethanol, by the alternative use of a detergent. Addition of a small volume of Triton X-100 to the liposomes (volume ratio 1 - 50) produced the same effect. Sonication of the liposomes for two minutes caused a similar reversion to a free dye type scan by physical rather than chemical disruption of the liposomes.

When a very low volume of Triton X-100 was added to liposomes diluted in phosphate buffer (one volume of Triton X-100 to 500 volumes of liposomes) the rate of decrease in absorbance at 530 nm and the increase at 565 nm could be monitored spectrophotometrically (figure 2.8). The rate of absorbance change at either wavelength appeared to be constant over several minutes but eventually plateaued. This was concluded to be due to complete release of dye from the liposomes, since further addition of detergent did not cause any further absorbance change. The rate of absorbance change was dependent on the volume of Triton X-100 added, becoming
Figure 2.8

Absorbance Changes on Lysis of Sulphorhodamine B-Containing Liposomes by Triton X-100

Absorbance changes with time are shown. Triton X-100 was added to liposomes which contained Sulphorhodamine B (left). With the same solution in place in the spectrophotometer, the wavelength was rapidly changed from 565nm to 530nm, and later to 565nm again. This was to demonstrate the reverse direction of absorbance changes at the two wavelengths.
almost instantaneous at higher detergent concentrations (e.g. 1 - 50 ratio of detergent to liposomes).

The absolute absorbances of a sample of dilute liposomes at either wavelength was found to be a combination of both entrapped and free dye. This meant that the absorbance at one or other wavelengths could not easily be used to calculate the absolute percentage entrapment of dye. In an assay, however, the total entrapment would be of less importance than whether the rate or extent of absorbance change reflected the rate or extent of dye release from the liposomes.

The relationship between absorbance at either 530 nm or 565 nm and the relative amounts of entrapped and free dye was investigated by combining free dye and a liposome preparation purified by dialysis (as above) in which more than 99% of the dye was estimated to be entrapped. These were both diluted to give approximately equal absorbances at 530 nm (figure 2-9a), then in a separate experiment to give approximately equal absorbances at 565 nm (figure 2-9b). The absorbances when various proportions of free dye and liposome dilutions were combined are shown in figures 2-9c and 2-9d respectively. There was an approximately linear inverse relationship between percentage of liposomes in the mixture and
Absorbance Changes on Combining Diluted Liposomes Containing Sulphorhodamine B with Diluted Free Dye

Sulphorhodamine B solution was mixed, in various proportions, with liposomes containing the same dye. The liposome preparation had first been extensively dialysed to remove free dye. In (a) solutions of liposomes and free dye of equal absorbances at 530 nm were mixed in various proportions. Figure (c) shows the resulting absorbances at 565 nm. In (b) dye and liposomes of equal absorbances at 565 nm were mixed. Figure (d) shows the resulting absorbance changes at 530 nm. The percentages shown in each figure are the percentages of liposomes in the liposome-free dye mixture (inferred to be the percentages of liposome-entrapped dye).
absorbance at 565 nm in the first case, and a near linear positive relationship at 530 nm in the second case.

These results are consistent with the view that the change in absorbance at 530 nm or 565 nm could be used to monitor release of Sulphorhodamine B dye from liposomes.

This can be summarised by the following relationships:

\[
\Delta \% \text{ dye entrapped} = -k_1 \Delta A_{565 \text{nm}} \quad (6)
\]

\[
\Delta \% \text{ dye entrapped} = k_2 \Delta A_{530 \text{nm}} \quad (7)
\]

\[
\Delta \% \text{ dye entrapped} = k_3 \Delta (A_{530 \text{nm}} - A_{565 \text{nm}}) \quad (8)
\]

2.9 Optimization of Incorporation of Sulphorhodamine B

2.9.1 Procedure to Assess and Compare Incorporation and Stability

Liposome preparations were submitted to Sepharose 4B gel chromatography and the percentage of the total dye in the
liposome fraction calculated as described in section 2.4). The liposomes were then subjected to dialysis, (as described in section 2.5) with mixing, against x40 volume of phosphate buffer. This allowed the stability of the liposomes at room temperature to be assessed. Stability was also assessed at 4°C by dialysis. While for practical reasons this did not employ constant mixing, dialysis experiments were mixed by hand daily.

2.9.2 Effect of Various Lipid Compositions on Dye incorporation and Stability

The liposomes prepared using the conditions described in section 2.5 had given disappointing incorporations of dye of approximately 9% in the liposomal fraction after gel filtration. They were very stable however with very little dye release on dialysis over several weeks.

Several other combinations of lipids were used to prepare liposomes using the same reverse phase evaporation technique. The results are shown in Table 2.1. Although it appeared that in general a slightly higher incorporation was obtained using lipids which were neutral, these liposomes showed poorer long term stability. It did not seem possible to improve
incorporation greatly by changing the type of phospholipid used.

Since the dye is negatively charged at near neutral pH, the use of stearylamine containing liposomes, which are positively charged, might have been expected to increase incorporation by electrostatic attraction. However it proved impossible to form these liposomes. The dye and stearylamine appeared to interact to prevent liposome synthesis. No gel-phase formed during the rotary evaporation stage. After prolonged rotary evaporation, the lipid/dye/sterylamine mixture, which was milky in appearance, stayed at the top of the gel filtration column. Under the microscope, aggregates were observed. In a control experiment when dye was omitted, stearylamine liposomes formed readily. 94% of the turbidity at 250 nm of the sample applied to gel filtration eluted in the liposomal fraction.
<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>%Dye Encapsulated</th>
<th>Lipid Charge</th>
<th>Instab.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-PTA-Chol (10-1-2)</td>
<td>9.3</td>
<td>negative</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>PC-PTA (10-1)</td>
<td>8.6</td>
<td>negative</td>
<td>0.75%</td>
</tr>
<tr>
<td>PC-Chol (10-2)</td>
<td>4.2</td>
<td>neutral</td>
<td>0.75%</td>
</tr>
<tr>
<td>PC only</td>
<td>10.6</td>
<td>neutral</td>
<td>1.5%</td>
</tr>
<tr>
<td>PC-Chol-PG-PE (30-10-3-1)</td>
<td>12.4</td>
<td>neutral</td>
<td>0.9%</td>
</tr>
<tr>
<td>PG only</td>
<td>13.5</td>
<td>neutral</td>
<td>2.5%</td>
</tr>
<tr>
<td>PC-Chol-Stearylamine</td>
<td>not formed</td>
<td>positive</td>
<td>not applicable</td>
</tr>
</tbody>
</table>

* Instability calculated as % dye loss / 24 hrs on dialysis at room temperature excluding first 24 hours. Values are means of duplicate preparations.

2.9.3 Effect of Dye Concentration on Incorporation and Stability

Liposomes incorporating Sulphorhodamine B at varying concentrations were prepared to assess the effect of the dye concentration on encapsulation and stability. The dye
was found to be soluble at 0.1 mol/l but at higher concentrations became saturated. To avoid this 0.1 mol/l was the maximum concentration used.

The effect of dye concentration is shown in Figure 2.10. A greater percentage entrapment was found at lower concentrations such that, for a 0.02 mol/l dye solution there was incorporation of approximately 25% which is close to that found for bleomycin (section 2.3). However the higher incorporation was achieved by the undesirable means of initially diluting the dye, the intensity of which might be a major advantage in immunological applications.

A measure of the total amount of dye entrapped can be calculated from the initial dye concentration, the initial volume of dye and the percentage incorporation. It was found that, although the percentage incorporation was increased by diluting the dye, the total amount of dye incorporated in each preparation was lower. This removed any advantage of using a more dilute dye (figure 2.10).
The concentration of dye used in the liposome synthesis was varied as indicated. Dye was dissolved in phosphate buffer (0.05M). The symbols used were as follows: dye incorporated within liposomes, expressed as a percentage ●; incorporated dye expressed as an amount (calculated from the percentage entrapment and the amount of dye used in the liposome synthesis) □; and rate of dye release from liposomes (percent per 24 hours) O.
2.9.4 Effect of Buffer Composition on Incorporation and Stability

Another variable which was examined was the effect of the strength of the phosphate buffer used to make up the dye solution. The incorporation and stability effects of varying buffer concentration are shown in figure 2.11. There was an increase in incorporation from approximately 9.5% to approximately 13% if distilled water was substituted for 0.05M phosphate buffer.

If the poor incorporation were due to an electrostatic effect, varying the pH of the buffer might alter the net charge on the dye or on components of the lipids and increase the incorporation. To test this, dye was prepared in 0.05 mol/l phosphate buffers between pH 2 and pH 12. A dye concentration of 0.1 mol/l was used initially, but the experiment was repeated using 0.02 mol/l dye. At the lower dye concentration, the higher percentage entrapment gave a clearer indication of any incorporation changes due to pH. The results are shown in figures 2.12 and 2.13. At the lower dye concentration the highest incorporation was obtained at near neutral pH but incorporation was not very sensitive to pH over a range of approximately 5 to 9. The effect of pH on entrapment
The concentration of phosphate buffer used to make up the Sulphorhodamine B solution was varied, and following liposome preparation, the liposome-incorporated dye (%) • and rate of dye release from the liposomes (% in 24 hours) ○ were measured.
Figure 2.12

Effect of pH of Buffer Used to Prepare Dye Solution on Liposomal Dye Entrapment and Leakage of Dye from Liposomes

The pH of the phosphate buffer (0.05 mol/l) used to prepare the solution of Sulphorhodamine B (0.1 mol/l) was varied and following liposome preparation, the liposome-incorporated dye (%) • and rate of dye release from the liposomes (% in 24 hours) ○ were measured.
The pH of the phosphate buffer (0.05 mol/l) used to prepare the solution of Sulphorhodamine B (0.02 mol/l) was varied and following liposome preparation, the liposome-incorporated dye (%) and rate of dye release from the liposomes (% in 24 hours) were measured.
using the higher dye concentration was too small to be clearly defined.

2.9.5 Effect of Cholesterol Content on Incorporation and Stability

Cholesterol has been reported to have effects on liposome formation and stability (51). Various amounts of cholesterol were added to liposomes, without altering the molar ratio of PTA and PC. Figure 2.14 illustrates that cholesterol increased the incorporation although the liposomes became less stable. A cholesterol content of 40% seemed a reasonable compromise between improved incorporation and loss of stability.

2.9.6 Modifications to Original Reverse Phase Evaporation Procedure to Improve Incorporation

The indications were that incorporation of dye could be increased both by reducing the buffer concentration and by increasing the cholesterol content of the liposomes. Liposomes were prepared containing 40% cholesterol and encapsulating Sulphorhodamine B (0.1mol/l) in distilled water. This procedure gave incorporations which were
Figure 2.14

Effect of Cholesterol Content of Lipid Used in Liposome-Synthesis on Liposomal Dye Entrapment and Leakage of Dye from Liposomes

The cholesterol content of the lipid used in liposome preparation was varied and following liposome preparation, the liposome-incorporated dye (%) • and rate of dye release from the liposomes (% in 24 hours) ○ were measured. All overlaid points represent duplicate measurements.
considered satisfactory and approached that obtained for bleomycin encapsulation (section 2.3). There was a moderately increased instability at room temperature, although this was still probably acceptable for most applications. Table 2.2 compares the encapsulation and stability of these liposomes with the "original procedure" described in section 2.5.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>%Dye Encapsulated</th>
<th>Instability</th>
</tr>
</thead>
<tbody>
<tr>
<td>original</td>
<td>8.9</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>modified</td>
<td>20.0</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>1.0%</td>
</tr>
</tbody>
</table>
2.9.7 Effect of Phosphatidyl Ethanolamine on Liposome Incorporation and Stability

Phosphatidyl ethanolamine (PE) is commonly incorporated into liposome membranes to introduce amino groups which can be subsequently used in coupling reactions. The effect of adding various amounts of PE to liposomes was examined. The remaining lipid was in the same molar ratio as described in section 2.9.6.

Figure 2.15 shows that in low amounts PE has little effect on either the amount of dye incorporation or the stability of the liposomes.

2.9.8 Effect of Cardiolipin on Liposome Incorporation and Stability

The introduction of cardiolipin into liposomes can enable their use as reagents in the detection of anticardiolipin antibodies (108). Cardiolipin was supplied in ethanolic solution, which had to be removed to prevent possible disruption of the liposomes. An appropriate volume (usually approximately 6 ml) of cardiolipin solution was dried in a rotary evaporator, redissolved in 6ml chloroform, again evaporated to
The phosphatidyl ethanolamine content of the lipid used in liposome preparation (66 umol) was varied. Following liposome preparation, the liposome-incorporated dye (%) • and rate of dye release from the liposomes (% in 24 hours) ○ were measured. All overlaid points represent duplicate measurements.
dryness and finally redissolved in 2ml of chloroform. To this solution the other lipids, 2ml of petroleum ether and 1ml of dye were added and the reverse evaporation procedure carried out as described in section 2.5.

The effect of incorporating cardiolipin into liposomes in various proportions of the total lipid is shown in figure 2.16. As PTA and cardiolipin are both negatively charged lipids, it was suspected that PTA might not have a significant effect on entrapment in the presence of larger amounts of cardiolipin. Furthermore, decreasing the percentage incorporation of PTA in proportion to the other lipids at high cardiolipin contents reduced the PTA content to a very small, and not very accurately weighable, amount. Figure 2.16 shows that the presence or absence of PTA at two cardiolipin concentrations had little apparent effect and PTA was omitted from the third. Apart from PTA; the other lipids, cholesterol and PC, were incorporated in the same molar ratio as in section 2.9.6.

The incorporation of dye was not found to vary greatly at molar compositions of cardiolipin up to 40% of total lipid. Incorporation of cardiolipin seemed to improve liposome stability at room temperature. Stability at 4°C
Figure 2.16

Effect of Cardiolipin Content of Lipid Used in Liposome-Synthesis on Liposomal Dye Entrapment and Leakage of Dye from Liposomes

The cardiolipin content of the lipid used in liposome preparation (66 umol) was varied. Following liposome preparation, the liposome-incorporated dye (%) (closed symbols) and rate of dye release from the liposomes (% in 24 hours) (open symbols) were measured. All overlaid points represent duplicate measurements. Cardiolipin was incorporated either in the presence (circles) or absence (squares) of PTA (otherwise using the modified procedure, as described in section 2.9.6).
was measured over several months and indicated negligible release of dye.

To compare experimental findings with those of Tashiki et al (142) who used liposomes of a lower cardiolipin content, liposomes were also prepared according to their directions. These contained 2.5% cardiolipin, 48.75% cholesterol and 48.75% PC. The liposomes were found to give an entrapment of 19.8% which was similar to that obtained for other cardiolipin compositions.

2.10 Comparison of Fluorescence and Spectrophotometric Properties of Liposome Preparations

A large number of the liposome preparations described in Section 2.9 were found to exhibit similar spectrophotometric scans to those shown in figure 2.7. This indicated that the shift in absorbance wavelengths compared to free dye was not dependent on the lipid types used to form the liposomes. Neither was there detectable variation in fluorescence properties when liposome preparations of various lipid compositions were compared.
2.11 Comparison of Liposomal Encapsulations of Kodak and Sigma Sulphorhodamine B Dyes

Despite the presence of non-chromogenic impurities (30% by weight), the Sigma dye was demonstrated to show similar optical characteristics to a purer source of dye (Kodak) (section 2.3). Sigma dye was used subsequently. However, the dependence of dye entrapment on sodium phosphate buffer strength was subsequently demonstrated (section 2.9.4). To assess whether the impurities in the dye might have affected percentage dye entrapment; liposomes containing the Kodak dye were prepared, using two different liposomal lipid contents and dye concentrations. The results, compared to those obtained using the Sigma dye, are shown in table 2.3.

As additional evidence that the impurities did not seem to affect entrapment, a 0.5ml aliquot of Sigma dye (0.1M in distilled water) was added to a Sepharose 4B gel filtration column, and eluted using phosphate buffer (pH 7.4, 0.05M). The eluted dye fractions close to the dye peak (2 x 0.5 ml) were collected. The eluted dye was adjusted to 0.02M by addition of phosphate buffer (pH 7.4, 0.05M), and its entrapment was compared to that of
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dye Concentration</th>
<th>% Entrapment</th>
<th>Kodak</th>
<th>Sigma</th>
</tr>
</thead>
<tbody>
<tr>
<td>original</td>
<td>0.05M</td>
<td>16.5</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>(section 2.5) (in phosphate buffer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>modified</td>
<td>0.1M</td>
<td>22.5</td>
<td>21.7</td>
<td></td>
</tr>
<tr>
<td>(section 2.9.6) (in distilled water)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are means of duplicate preparations.

dye diluted to the same concentration. The mean entrapment of two preparations of the eluted dye was 23.0%, while compared to the mean entrapment of the diluted dye of 24.3%. This again suggested that the impurities did not reduce entrapment, as might be predicted from figure 2.11 if the impurities were simple salts. In this experiment, however, there remained a possibility that impurities eluted with the dye.
2.12 Entrapment of Rhodamine B

Although the studies centred on the use of Sulphorhodamine B, attempts were made to prepare liposomes containing Rhodamine B. Rhodamine B is a dye in the same class as Sulphorhodamine B, although it lacks the sulphite group, rendering it more basic.

The dye was dissolved in water at a concentration of 0.02M, and an absorbance coefficient of $6.45 \times 10^4$ l mole$^{-1}$ cm$^{-1}$ was found (maximum absorbance at 554 nm) (compared to $1.13 \times 10^5$ l mol$^{-1}$ cm$^{-1}$ for Sulphorhodamine B at 565 nm). The dye was found to be insoluble in water at concentrations above 0.02M, and precipitated out rapidly on standing at 0.1M (the concentration used for Sulphorhodamine B). Liposomes were prepared using Rhodamine B (0.02M in water) (under the conditions of the original procedure, section 2.5), but no separation between free and entrapped dye was evident on gel filtration. None of the eluted fractions showed detectable shifts in absorption maximum. The failure to demonstrate significant entrapment, together with the lower absorbance coefficient and solubility, suggested the dye was a less suitable candidate for a chromogenic marker than Sulphorhodamine B.
2.13 Discussion

Sulphorhodamine B was investigated as a potential marker because of its high molar absorptivity and its good solubility in aqueous solutions. Fluorescence added to its potential, as well as increasing the range of analytical approaches available for assessing entrapment and dye release.

A variety of techniques were available to assess entrapment and stability; including gel filtration, dialysis, fluorescence quenching release and centrifugation. The choice of techniques depended on their convenience in individual experiments. Gel filtration is a well established, simple, rapid and convenient method. It can also be subject to less sources of imprecision than dialysis which involves careful estimation of small volumes of preparations. For these reasons; gel filtration was used to obtain comparative data on the entrapment of dye in different liposome preparations, even though it was found to underestimate the entrapment approximately 10% compared to dialysis and fluorescence data. Another concern with gel filtration was the presence of a small proportion of free dye in the liposome preparation. These observations suggest the disruption of a small proportion of the liposomes during
their passage through the column. It was not possible to influence this underestimation by altering the pore size of the gel or factors which might affect electrostatic binding (e.g. buffer concentration).

Problems with the use of gel filtration in fractionation of liposomes have been reported previously, due to non-specific adsorption of liposomes onto gels (34). It seems likely that similar mechanisms could have accounted for the observed loss of liposome contents in this report.

While gel filtration was considered acceptable to obtain comparative data, the potential losses of entrapped dye involved suggest that dialysis might be a preferable means to purify liposomes as part of liposomal preparations. However, in some instances dialysis would not be an option; for example if macromolecules such as proteins are being entrapped. It was not thought necessary in these studies to employ another alternative, centrifugation, as a purification technique.

Dialysis was found more easily applicable to calculations of liposome stability.
The calculation of entrapment using the fluorescence quenching release technique was a very rapid method to assess new preparations. By taking sequential readings, it could also be used to assess liposome stability. The entrapments calculated in this way were as a rule within a few percent of dialysis data while somewhat higher than the data from gel filtration. Nevertheless, potential errors in this approach have not been extensively studied. Data obtained by this technique were generally used only as a check on data obtained by either gel filtration and dialysis, and were not included in this report.

The use of Sigma dye, which contains non-chromogenic impurities, rather than a more pure Kodak dye, was considered justified because of its very similar optical properties. The presence of non-chromogenic impurities was not found to reduce entrapment; as might be predicted from the effects of buffer concentration (section 2.9.4), if the impurities were simple salts. If they were salts, it is possible that the entrapment was not dependent on their concentration below that found in the Sigma dye. Alternatively the impurities may be another material such as sucrose, which might not affect entrapment to measurable degree.
The differences in spectrophotometric absorption spectra of the entrapped dye, compared to free dye, were unexpected and do not appear to have been previously reported in liposomes. They seemed to be independent of the lipid composition of the liposomes.

It is possible to deduce a mechanism for these differences based on earlier studies (143,144). Sulphorhodamine B is a member of the group of rhodamine dyes. Using optical cuvettes with very short path lengths; a similar type of shift in absorption spectra has been observed for other members of this group of dyes, using absorbance measurements at concentrations not normally measurable by conventional spectrophotometry. The altered absorption spectra at high concentrations have been attributed to dimerization of zwitterionic forms of the dyes, caused by hydrophobic interactions of the alkyl substituents. The blue shift in absorption maximum is characteristic of these interactions for the other rhodamine dyes, including Rhodamine B (143). The failure to show this for Rhodamine B in the present study (section 2.12), probably indicates that the entrapment conditions were unsuitable for Rhodamine B, which is less negatively charged at neutral pH than Sulphorhodamine B. The blue shift in absorption maximum is presumably accounted for by increased electron delocalization.
In these studies, the dye is held at high concentration within the liposomes. On release of the dye from the liposomes; it is diluted into the surrounding aqueous medium, resulting in a shift in absorption spectrum.

It appeared possible in principle that the change in absorbances at 565 nm and at 530 nm, either alone or in combination, could be used as a novel means to monitor liposome lysis. This would enable colorimetric assays to be developed which might be simpler or more convenient than alternatives such as fluorescence or enzyme release (as described in section 1.6.2).

Because of the marked absorption shift due to Sulphorhodamine B dimer formation, another possible reason for an absorption change on encapsulation of a chromophor was not examined. It might be predicted that, at high enough concentrations, entrapped chromogen would have a lower absorbance than the equivalent amount of chromogen in free solution. This is because it is hard to see how the very high concentration of liposome-entrapped chromophor could obey the Beer-Lambert law (equation 3.1, section 3.4.3).

This proposition is illustrated by the following hypothetical example. Suppose the concentration of dye
were such that virtually all the light waves falling on a liposome were absorbed in passing half way through it. The remaining 50% of dye in the liposome would not contribute to the absorption. At the same time, some light may pass through the solution without encountering a liposome. The absorption due to this portion of the light would be zero (or low, dependent on any non-liposomal absorbance). The net effect should be a reduction in absorbance compared to the equivalent amount of free dye, which would have a much lower concentration. This situation would seem analogous to the quenching effect of fluorescence at very high concentrations (described in section 2.6).

Ethanol and detergent at high concentrations caused rapid release of liposome contents, apparently by complete disruption of the liposomes. At low detergent (Triton X-100) concentrations a slower release of dye was observed. The mechanism for the effect of Triton X-100 on liposomes is believed to be the formation of mixed micellar structures, after incorporation of the detergent into the lipid bilayer (145).

The poor incorporation of dye could have been due to the choice of lipids, which would have formed negatively charged liposomes because of the inclusion of PTA.
degree of exclusion of the dye may have occurred, since
the dye is also negatively charged at near neutral pH.
Although, in general, neutral liposomes showed a slightly
higher entrapment than negatively charged liposomes they
were also less stable. Positively charged liposomes
containing stearylamine failed to form, apparently
because of an interaction between stearylamine and the
dye. It could be speculated that stearylamine-dye
complexes may have formed by electrostatic interaction.
These complexes may have aggregated by adopting micellar
structures (2).

Diluting the dye resulted in greater percentage
entrainment, but less dye was entrapped in absolute
amounts. One mechanism to explain the lower percentage
incorporation at higher dye concentrations would be
exclusion of dye by electrostatic repulsion. However,
over quite wide pH ranges (pH 5 - 9), entrainment seemed
insensitive to changes in pH.

Although initial dye entrapment was poor, extensive
optimization of preparation conditions led to entrapments
of over 20%, which was considered acceptable for the use
of the liposomes in subsequent studies. This was achieved
in part by increasing the cholesterol content. This was
not unexpected, since cholesterol has been reported to
reduce the fluidity of liposome membranes by perpendicular packing at a level below the phospholipid head groups (52). An unpredicted increase in dye leakage occurred at the highest cholesterol content, of approximately 2% per day compared to 1% per day for 40% cholesterol content. This was despite a higher percentage entrapment at the higher cholesterol content. (figure 2.14).

If the stabilizing effect of cholesterol is due to a decrease in membrane-fluidity, a parallel increase in liposome entrapment and stability might have been expected. It could be speculated that another mechanism may have contributed to the higher amount of leakage at 60% cholesterol content. While liposomes composed of 50% cholesterol have been found to be stable; small unilamellar liposomes with 66% cholesterol content have been shown to be metastable, forming larger structures on standing (146). One possibility is that the liposomes containing 60% cholesterol in this study showed a small degree of structural rearrangement by a similar mechanism, which was sufficient to release dye at a higher rate. However, no microscopic evidence of such an effect was found.
Decreasing the buffer molarity also increased entrapment, perhaps by reducing electrostatic effects.

The finally adopted preparative conditions showed acceptable stability of less than 1% leakage per day at room temperature and very low leakage rates at 4°C. Small amounts of phosphatidyl ethanolamine, necessary for coupling of proteins to liposomes, had only a small effect on entrapment and stability. The inclusion of cardiolipin, used as an antigen in subsequent studies, marginally improved entrapment and liposomes were more stable at room temperature.
Chapter 3

Liposomal Immunoassays for Serum and Urinary Albumin

3.1 Clinical Value of Serum and Urinary Albumin Assays

Albumin is normally the most abundant protein in the human plasma and it is a commonly measured analyte in clinical biochemistry laboratories. Its estimation in serum or plasma may be useful in assessing liver disease, poor nutritional status or prognosis in myeloma, in patients undergoing long term albumin replacement (e.g. parenteral nutrition) and in elucidating the cause of oedema. Albumin concentrations are also useful in assessing the free levels of constituents which are partially albumin-bound in the plasma, such as calcium or unconjugated bilirubin (147).

Serum albumin is usually measured by chemical dye-binding methods which have been shown to lack specificity (148). In particular, bromocresol green (BCG) has been shown to overestimate results, particularly at low serum albumin concentrations. This is largely due to reaction with globulins, which can cause falsely elevated results in
the presence of acute phase proteins. The bias can be reduced by using short reaction times. An alternative dye-binding method, using bromocresol purple, has much greater specificity. Despite this, bromocresol purple has been slow to be adopted, mainly because animal-based sera react differently to human serum and cannot be used as calibrators. Participation in animal sera-based quality assurance schemes is also difficult, particularly if most other participants use a BCG method.

Immunological methods are considered to be more specific than BCG. However, they are more cumbersome to automate and are less popular. Assay methods have included radial immunodiffusion, electroimmunoassay, and more recently immunoturbidimetry and immunonephelometry. Both homogeneous and heterogeneous "labelled" immunoassays have also been described, for example using fluorescent labels (149). In addition, it is also possible to measure albumin by scanning densitometry following electrophoresis; though this is relatively imprecise and is rarely used as a routine method.

Urinary protein above 200 μg/minute (or approximately 200 mg/l) is found in frank proteinuria. Albumin may be a major component of this proteinuria, for example in renal glomerular damage. Alternatively, there may be an "over-
flow" proteinuria caused by increased plasma proteins; for example immunoglobulin light chains in myeloma, or myoglobin in muscle trauma. The clinical significance of proteinuria has recently been reviewed (150).

Urinary protein is usually measured by a chemical method, either as a semi-quantitative strip test or by a quantitative laboratory method. Both approaches lack specificity and may have different sensitivity to various types of protein (151,152). For these reasons urinary albumin, measured by an immunological method, may be preferable to total protein as an index of glomerular disease.

Urinary albumin in the range between normal concentrations and those detected by the chemical methods normally used in strip tests (approximately 20 - 200 mg/l), can be associated with subsequent nephropathy and other vascular disease. These levels are a significant risk factor in Diabetes mellitus. They are usually termed "microalbuminuria"; implying low (but increased) concentrations of albumin, rather than suggesting the presence of abnormally small albumin molecules. The term "pauci-albuminuria" has been suggested as a more appropriate description of microalbuminuria but not widely adopted.
In both insulin-dependent and non-insulin dependent diabetics, microalbuminuria is associated with poorer glucose control, raised arterial blood pressure and lipid abnormalities. As well as nephropathy, increased levels are associated with cardiovascular mortality and retinopathy (153, 154). Microalbuminuria has also been shown to be a predictor of vascular disease in non-diabetics (155), and of increased mortality in the elderly population in general (156).

Microalbuminuria clearly reflects generalized vascular disease and not only renal disease. A mechanism to explain this was put forward by Deckert et al. (157), who postulated a genetic polymorphism of enzymes involved in the metabolism of heparin sulphate proteoglycan. According to the theory, some diabetics have iso-enzymes that are susceptible to poor diabetic control. This results in a reduction in heparin sulphate proteoglycan, a component of the glomerular basement membranes, aortic myomedial cells, mesangium and endothelial plasma membranes. The consequential loss of glomerular membrane anionic charge results in albuminuria.

Loss of heparin sulphate has a number of other consequences; including disruption of the microstructure of the glomerular basement membrane and increased pore
size, leading to a more generalised proteinuria. Heparin sulphate proteoglycan also has anti-thrombogenic properties. Its loss could result in microthrombi and/or platelet plug formation, which might ultimately lead, for example, to retinopathy. Heparin sulphate proteoglycan also binds to lipoprotein lipase, stimulating its activity, and inhibits smooth muscle cell proliferation in arteries. Its loss could be a promotor of atherosclerosis. The proposed genetic polymorphism would explain why only certain diabetic individuals develop microalbuminuria.

The value of measuring microalbuminuria rests on the belief that careful treatment can reverse both microalbuminuria and its associated clinical consequences. This has been bourne out in trials; for example, one large study showed that intensive treatment of diabetes could reduce the development of microalbuminuria and its progression to frank proteinuria. The progression of retinopathy and neuropathy were also slowed. (158). Treatment relies mainly on control of glycaemia, blood pressure and protein intake.

Accurate measurement of the low albumin concentrations found in microalbuminuria requires a specific
immunoassay. Gel-based techniques, such as radio-immunodiffusion (159) have been used to measure microalbumin. Current methods, however, mainly employ radioimmunoassay (160), assays using alternative labels such as fluorescence (161), and immunonephelometry or immunoturbidimetry (99).

There have also been near-patient tests developed, which allow an estimate of microalbuminuria to be obtained quickly, perhaps during a patient's consultation. Like the laboratory methods, these are based on immune reactions, including latex agglutination methods (162) and ELISA methods (163). These methods often lack sufficient sensitivity to completely replace laboratory methods, at least for the present.

There has been considerable debate as to the type, timing and storage conditions of microalbumin specimens which are optimal. Microalbuminuria has been defined as an albumin excretion rate of 20-200 ug/minute, which is approximately 30-300 mg/24 hours. Because of the inconvenience and difficulties in accurate timing of 24 hour or overnight collections, untimed collections are usually collected. The first morning sample is often favoured, as this has been found to correlate most closely to a timed collection.
Some centres advocate simple concentration measurements, with a positive result being above approximately 20 mg/l. Errors in rates of water excretion are reduced by taking at least three specimens over several months. Two out of three increased levels is often taken as indicating incipient diabetic nephropathy. Others measure the albumin:creatinine ratio (164). This reduces the effect of changes in water excretion, but creatinine measurements in diabetics can themselves be problematical (165) and analytical errors may be compounded.

There is little consensus as to whether samples should be stored refrigerated or frozen. Silva et al. (166) showed no significant difference between results of samples stored at -20°C for one month, or 4°C for either 18 hours or one week. Centrifugation of samples after freezing, to remove precipitate, did not appear to significantly alter the results.

While albumin and microalbumin are frequently measured as indicators of renal disease and incipient renal disease respectively, it is not yet clear whether specific measurements of other urinary proteins might have greater prognostic value. For example, transferrin excretion is more often raised than microalbumin, both in
diabetic nephropathy (167) and in diabetic retinopathy (168).

3.2 Aims of Liposomal Studies for the Measurement of Albumin

Complement-mediated liposomal immunoassays have been described using a number of markers, such as enzymes, fluorophors and chromophors, as describes in section 1.6. Complement is used to lyse liposomes which have undergone an immune reaction at their surfaces. After optimization of entrapment of the dye, Sulphorhodamine B (section 2.9) it was shown to have potential uses as either a fluorogenic marker or as a novel chromogenic marker in immunoassays. The possible use of these liposomes containing Sulphorhodamine B in a complement-mediated immunoassay was examined, using serum albumin as a model assay to demonstrate the assay principle. The aim was to extend this assay to urinary albumin, should the model assay be viable. There do not appear to have been any previous reports of a liposomal immunoassay of human albumin in serum or urine.
3.3 Preparation of Albumin-Coated Liposomes

Liposomes coated with albumin were prepared using the heterobifunctional reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (described in section 1.5.2) (78). The coupling method is summarized in figure 3.1.

3.3.1 Formation of Dithiopyridyl-albumin (DTP-albumin)

DTP-albumin was prepared by a standard method essentially as described by Truneh et al. (36). An aqueous solution of purified human albumin (4 ml of 10g/l) was mixed gently with SPDP reagent in 140 ul methanol to give a molar ratio of SPDP to albumin of 10:1. The mixture was incubated at approximately 23°C for 30 minutes with occasional stirring. The pH was then reduced to approximately pH 4.5 by the addition of 250ul of acetic acid, pH 3.0.
1. **Introduction of 2-pyridyl disulphide structures onto albumin (A) giving DTP-albumin.**

2. **Introduction of 2-pyridyl disulphide structures onto phosphatidyl ethanolamine (B) to give DTP-PE.**

3. **Formation of liposomes incorporating DTP-PE.**

4. **Thiolation of DTP-albumin using dithiothreitol (DTT).**

5. **Conjugation of liposomes and thiolated albumin.**
3.3.2 Synthesis of Dipyridyl Phosphatidyl Ethanolamine (DTP-PE) and Liposome Formation

DTP-PE was prepared using a standard method (36) adapted to allow its synthesis and its incorporation in the formation of liposomes to be completed within a working day. PE (6.9 mg in 300 ul chloroform), SPDP (3.75 mg in 300 ul methanol) and triethanolamine (2.7 ul) were mixed, solubilized by brief heating to 30°C and then incubated at room temperature for two hours. The organic phase was then washed once in phosphate buffer and twice in distilled water, centrifuging at 2000 x g for five minutes and discarding the aqueous phase at each stage. Chloroform (1 ml) was added prior to the last centrifugation step to facilitate complete removal of the final aqueous phase without excessive loss of the organic phase. A white precipitate which formed at the solvent interface could be reduced but not eliminated by the addition of a small amount of methanol. This precipitate was discarded after the final centrifugation, leaving a clear organic phase containing DTP-PE.

The concentration of DTP-PE formed was estimated by the release of dithiopyridine as described by Truneh et al. (36). An aliquot (25 ul) of the organic phase was gently evaporated to dryness then immediately redissolved in "L
Buffer" (1 ml) with vigorous vortexing. The absorbance before and after adding 50μl dithiothreitol (100 mM) was measured and the molar concentration of DTP-PE calculated as:

\[
\text{(change in absorbance at 343 nm x (dilution factor)) / molar absorbance coefficient of dithiopyridine at 343 nm (8.08 x 10^3 M^{-1}cm^{-1})}.
\]

The DTP-PE was immediately incorporated into liposomes by the reverse phase evaporation method, described in section 2.9.6, including 2 μmol of DTP-PE (typically approximately 0.5 ml). Aliquots of two preparations of liposomes showed entrapments of Sulphorhodamine B of 19.0% and 18.9% respectively on gel filtration which is similar to that which had been achieved when PE was incorporated in place of DTP-PE (figure 2.15).

The liposomes were immediately filtered by passage through a 200 nm polycarbonate filter to increase homogeneity, then dialysed overnight (as section 2.8) to remove unencapsulated dye. The DTP-liposomes were then coupled to DTP-albumin within two days.
3.3.3 Coupling of DTP-Albumin to Liposomes

Immediately prior to coupling with the liposomes 50 μl of dithiothreitol (500 mM) was added to the DTP-albumin preparation which was then incubated for 20 minutes at room temperature. The DTP-albumin was separated from the other reagents by gel filtration using Sephadex G50 equilibrated in phosphate buffer. The protein content of the protein fraction, eluting in the void volume, was measured using the Coomassie blue method.

Equal volumes of the liposomes and DTP-albumin reagent were mixed and incubated overnight at room temperature. The mixture was then applied to a Sepharose 4B column, the antigen-coated liposomes eluting in the void volume while the residual free dye and uncoupled protein was retarded by the gel (the latter demonstrated by the Coomassie blue method).

The protein content of the preparation was determined by the Bio-Rad Coomassie blue method, modified for this purpose by solubilizing the lipid by the addition of 50 μl ethanol to 20 μl of liposome preparation. The standard colorimetric procedure was then followed by adding 1 ml saline and 200 μl of Coomassie blue reagent, incubating at room temperature for 15 minutes and then measuring the
absorbance at 580 nm. It was also necessary to subtract a blank for the absorbance of Sulphorhodamine B at the same wavelength. In this case 9 g/l ("normal") saline replaced the Coomassie blue reagent. Ethanol (50 ul) was also added to the standards to compensate for the small additional effect of ethanol on the reagent.

As mentioned previously (section 2.4), measurement of the lipid content of the liposomes was complicated by the interfering intense absorbance of Sulphorhodamine B. This was avoided by overnight dialysis of the preparations in the presence of detergent (Triton X-100) against a 1000 fold excess of distilled water. Detergent lysis of the liposomes allowed the dye to be dialysed while the lipid was retained. Cholesterol was assayed by the cholesterol oxidase procedure after dialysis (allowing for dilution during dialysis) and the total lipid calculated as (moles cholesterol x 2.5).

The retention of lipid on dialysis in the presence of detergent was verified using liposomes which did not contain dye (prepared as in section 2.4). In two experiments, the mean cholesterol concentration of the dialysed liposomes was 5.2 mmol/l. This agreed acceptably with the cholesterol content without
dialysis (but diluted slightly to allow for a small volume increase during dialysis) of 5.0 mmol/l.

Using these calculations a binding of 54 ug albumin / umol lipid was calculated to have been obtained. The protein content of the final preparation was 0.8 g/l. The absorbance was inferred to be 120 A at 565 nm (by measurement, following dilution to an absorbance below 2). The liposomes formed were stored at 4°C in a glass tube until used, showing negligible loss of dye over several months. In an assessment of instability (as described in section 2.6), the liposomes showed a leakage of 0.2% per 24 hours at room temperature.

3.4 Effect of Complement and Anti-Albumin Antiserum on Liposomes

The reaction of complement and antiserum when combined with albumin-coated liposomes was examined by fluorimetric monitoring, using a spectrophotometer (SP1800), and also using an automated Cobas Bio centrifugal analyser.
3.4.1 Spectrofluorimetric Measurement

To assess whether the release of dye by the addition of complement and antiserum could be monitored fluorimetrically, fresh guinea-pig complement (20µl) and liposomes (5µl) (free dye having been removed by dialysis) were combined with "buffer A" (2ml) in a fluorimeter cell and mixed. The cell contents were monitored (ex. 405nm, em. 580nm) for five minutes without detectable fluorescence change. Antialbumin antiserum (20µl) was then added and the contents rapidly mixed and monitored. There was a rapid increase in fluorescence indicating liposomal lysis with dye release (figure 3.2). The complement-mediated lysis was found to be initially rapid but slowed so that only a small rate of fluorescence change occurred later than four minutes after mixing.

As the lysis slowed but did not cease completely after a few minutes, the effect of complement over longer incubation times was investigated. Test tubes were incubated using the same components as above (figure 3.2) and the amounts of lysis were measured fluorimetrically at 20 minutes, 15 hours and 24 hours. The specificity of the lysis for the immune reaction was also assessed.

Control experiments in which normal sheep serum or
Figure 3.2

Change in Fluorescence Resulting from Complement-Mediated Immunolysis of Albumin-Coated Liposomes

The change in fluorescence with time is indicated, on addition of antialbumin antiserum (right of chart) to a mixture of albumin-coated liposomes and complement. The reaction conditions are described in section 3.4.1. The vertical scale chart gradations are equivalent to 9 arbitrary units. Arbitrary units have been defined in the materials and equipment section.
buffer A replaced the antiserum were performed. In addition, a control experiment in which complement was heat-inactivated (incubated at 45°C for 45 minutes) was carried out. The results are shown in figure 3.3. The entrapment was calculated using equation (5) given in section 2.6, i.e.

\[
\text{fluorophor-release(\%)} = \frac{(\text{final fluorescence} - \text{initial fluorescence})}{(\text{maximum fluorescence (ft)} - \text{initial fluorescence})} \times 100\%
\]

The initial fluorescence was taken as the mean of the duplicate tubes prior to adding the final reagent. For clarity the individual points at zero time are not shown in figure 3.3. None of the points differed from zero by more than 1% entrapment.

When the order of addition of complement and antiserum was reversed (i.e. antiserum added first) a similar if slightly lower fluorescence rise was observed. Antiserum, or its substitute, was added as the final reagent in each control experiment.

The initial rapid dye release only occurred when complement and antiserum were both present. Over longer
Effect of various reaction mixtures on the percentage release of entrapped dye from the albumin-coated liposomes measured by reduction in fluorescence quenching (as described in section 2.6). Symbols are as follows: o antialbumin antiserum added after complement, " complement added after antiserum, - buffer substituted for antiserum, Δ normal sheep serum substituted for antiserum, ◇ complement-heat inactivated, ◦ buffer and liposomes only. All overlaid points represent duplicate measurements.
incubation periods, however, the slower release of dye was observed in several of the control experiments. The latter appears to be mainly though not entirely non-specific since a similar though slightly lower rate of release occurred when antiserum was replaced by either buffer A or normal sheep serum. This secondary rise was shown to be dependent on the presence of complement and was almost absent when the complement was heat inactivated. These results suggest the initial rise was due to complement mediated immune-lysis while the secondary lysis was, at least in part, a non-specific reaction (perhaps of complement with the liposomal phospholipid surfaces).

The effects of varying antiserum, complement and liposome amounts on the initial rapid lysis were examined by incubating the reactants for twenty minutes at room temperature. The total volume was kept constant by varying the amount of buffer. In each case a blank fluorescence was taken immediately prior to adding the final reagent and this was subtracted from the fluorescence increase in order to estimate the amount of lysis.

The immune lysis was found to be dependent on both the amount of antiserum (figure 3.4) and the amount of
Figure 3.4

Effect of Varying Antiserum Amount on the Complement-Mediated Lysis of Albumin-Coated Liposomes

The reaction conditions were as described in section 3.4. The entrapped dye released was measured by the reduction in fluorescence quenching, as described in section 2.6. Overlaid points represent duplicate measurements.
complement (figure 3.5) added. For simplicity of experimental design, complement was added after antiserum in figure 3.4 and visa-versa in figure 3.5. The dye release was calculated from the fluorescence increase after twenty minutes (using equation 2.5, section 2.6). In both cases there was an optimum concentration above which the lysis decreased. This "hook effect" is reminiscent of other types of immunoassay such as immunoturbidimetry. Figure 3.6 demonstrates that both the amount and percentage of dye released were dependent on the amount of liposomes present. (Antiserum was added last).

3.4.2 Spectrophotometric Monitoring of Complement-Mediated Dye Release

The monitoring of quenched fluorescence released when liposomes lyse, as demonstrated above, has been used in immunoassays (see sections 1.4 and 1.6.6) which used fluorophors other than Sulphorhodamine B. The phenomenon of the absorbance change when Sulphorhodamine B is released, described in section 2.8, has not been adopted in an immunoassay previously and would be a novel application. To assess whether the release of dye by
The effects of varying the amount of complement added to the reaction mixture which also contained antialbumin antiserum and albumin-coated liposomes. The reaction conditions are described in section 3.4. The entrapped dye release was measured by reduction in fluorescence quenching as described in section 2.6. Overlaid points represent duplicate measurements.
The volume of albumin-coated liposome preparation added to the reaction mixture was varied as described in section 3.4.1. The reaction mixture also contained antialbumin antiserum and complement. The release in Sulphorhodamine B was detected by a reduction in fluorescence quenching, as described in section 2.6. The increase in fluorescence is indicated □. This was used to calculate the percentage release of entrapped dye ●. Overlaid points represent duplicate measurements.
complement-mediated immunolysis could be monitored spectrophotometrically, reagents were combined as described above (section 3.4.1) and monitored at 565 nm in a spectrophotometer. Changes in absorption analogous to those in fluorescence were indeed detected (figure 3.7). When the order of addition of antiserum and complement was varied similar lysis rates were observed.

3.4.3 Monitoring Entrapped Dye Release Using a Cobas Bio Automated Centrifugal Spectrophotometric Analyser

Although spectrophotometric measurements of dye-release were possible, the manual analysis of large numbers of tubes was found to be impractical. For example, accurate timing of batches of tubes was difficult. Therefore a Cobas Bio Centrifugal analyser was used to assess the potential of the spectrophotometric monitoring of complement-mediated dye release as a routine procedure.

The reactant conditions were optimized using different criteria to those in the manual assays described above. The Cobas Bio uses smaller total volumes and is restricted to two reagent additions so it was necessary
The absorbance changes which occurred when albumin-coated liposomes, antialbumin antiserum and complement were mixed are shown. The experimental conditions have been described in section 3.4.2. Antiserum and liposomes were first mixed (right of chart), followed by complement. In a separate experiment (centre) liposomes and complement were first mixed, followed by antiserum (right). The diluted liposomes had an initial absorbance of 0.344A and horizontal lines (right of chart) indicate absorbance increments of 0.02A.
to modify the reagent volumes and combine some reagents. As the Cobas could monitor reactions accurately over small time intervals shorter incubation times were used since this would contribute to a more attractive routine procedure. Another difference was in the amount of liposomes added to each reagent cuvette. A reasonable absorbance change was desirable but the initial absorbance of the liposomes at the wavelength used was limited by the optical specifications of the analyser. The Cobas is claimed to measure accurately at absorbances up to 3.0. However in practice liposomes were never used at concentrations which would give an absorbance above 2.0 using a 1 cm pathlength; to ensure any procedures would be transferable, in theory, to other spectrophotometric instruments.

The Cobas Bio operates by measuring absorbances vertically through the reaction cuvette contents rather than by the conventional method of reading across the cuvette with a fixed path length (usually 1 cm). This is feasible because the absorbance measurements are made at high centrifugal acceleration which flattens menisci and minimizes the effects of light scatter.
By Beer's law (equation 3.1), the absorbances measured by the Cobas are dependent on the total volume of reactants.

\[ A = ecl \]  (equation 3.1)

\( A \) = absorbance  
\( e \) = molar absorbance coefficient  
\( c \) = concentration  
\( l \) = path length

A volume of 250 ul in a Cobas Bio cuvette was found to be equivalent to a path length of 1 cm. Total reaction volumes of as little as 60 ul could be used on the Cobas. It would have been possible, therefore, to add considerably larger volumes of liposomes in proportion to other reactants without exceeding the optical capabilities of the Cobas, than would be the case if the reaction path length were fixed at 1 cm. However it was decided to restrict the liposome volumes added to those that would give absorbance readings of no more than 2.0 if the reaction volume had a path length of 1 cm. This was to ensure transferability of results to other spectrophotometric instruments, many of which operate using a 1 cm path length.
If reactant volumes other than 250 ul were used, absorbance readings were adjusted by multiplying by a factor of (250/volume). This was to ensure equivalent absorbances would be obtainable with the same reaction conditions if reagents were scaled up in proportion in other instruments which use 1 cm path lengths.

In initial experiments reagents were combined as shown in table 3.1 and absorbances monitored over three minutes. The absorbances obtained at wavelengths of both 565 nm and 530 nm were plotted (figure 3.8). A rapid initial rate of absorbance rise at 565 nm which slowed to a low rate over a few minutes was observed, similar in general to that seen in fluorimetric (section 3.4.1) or spectrophotometric (section 3.4.2) manual experiments. The initial reaction was more rapid, perhaps reflecting either the greater concentration of some reagents or that the Cobas was operated at 37°C rather than room temperature. At a wavelength of 530 nm there was a decrease in absorbance which mirrored the increase at 565 nm. This was consistent with the effects of dye being released from the liposomes, as described in section 2.8. The increase in absorbance at 565 nm was found to be greater than the decrease at 530 nm.
TABLE 3.1

Reaction parameters used initially on Cobas Bio Analyser
Complement-Mediated Immune Lysis

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (reagent 1)</td>
<td>5ml buffer A plus 40ul liposomes 100 ul/cuvette</td>
</tr>
<tr>
<td>sample: antialbumin antiserum (neat)</td>
<td>4ul/cuvette</td>
</tr>
<tr>
<td>diluent: (distilled water)</td>
<td>10 ul/cuvette</td>
</tr>
<tr>
<td>R2 (reagent 2): complement diluted x 2 in distilled water</td>
<td>4ul/cuvette</td>
</tr>
<tr>
<td>Temperature:</td>
<td>37°C</td>
</tr>
<tr>
<td>Printout type:</td>
<td>3 (absorbance measurements)</td>
</tr>
</tbody>
</table>

Assay process: R1, sample and diluent dispensed into cuvettes and mixed. Five minute pre-incubation. R2 added and readings taken at 10 second intervals over 180 seconds (first reading after 3 seconds).

To assess the effect of varying antiserum concentration, the above parameters were modified by increasing the sample volume to 30 ul and monitoring the reaction with a range of antiserum dilutions as samples (in buffer A). The amount of antiserum was calculated as microlitres of neat antiserum present in the reaction cuvette. The
Absorbance changes due to Sulphorhodamine B release from liposomes monitored using a Cobas Bio analyser. Dye release resulted from the reaction of albumin-coated liposomes, antialbumin antiserum and complement. The stated reaction time excludes an automatic initial mixing time of three seconds. The reaction conditions have been described in section 3.4.3.
results (figure 3.9) showed a "hook effect", similar to that seen for fluorescence measurements (figure 3.4). There was a negligible rate when normal sheep serum was used in place of antiserum.

To assess the effect of complement concentration on the reaction rate, complement diluted in buffer A replaced antiserum as "sample" and the antiserum was added as "R2". Again a "hook effect" was observed (figure 3.10) similar to that for fluorescence measurements (figure 3.5). Negligible rate was observed when complement was heat inactivated (see section 3.4.1). When rabbit complement was used instead of guinea-pig complement there was a similar "hook effect" but the absorbance changes were lower. Human complement, examined under the same conditions, (as figure 3.10), failed to show any detectable concentration-dependent lysis and the maximum absorbance change was 2mA.

It was possible to vary the reaction temperature on the Cobas Bio. This indicated the early rates of lysis to be temperature dependent (figure 3.11). However if the total absorbance change over three minutes or more were monitored, rather than initial rate, the temperature had little effect.
Various volumes of neat antialbumin antiserum (○) or neat normal sheep serum (○) were added to the reaction cuvette of the Cobas Bio analyser. The reaction also contained complement and albumin-coated liposomes. The reaction conditions have been described in section 3.4.3. Overlaid points represent duplicate measurements.
The effect of adding various volumes of neat complement preparation to the reaction cuvette, as described in section 3.4.3. The Cobas Bio cuvettes also contained albumin-coated liposomes and antialbumin antiserum. Symbols used refer to: • = guinea-pig complement, □ = rabbit complement, o = guinea-pig complement which had been heat-inactivated at 45°C for 45 minutes. Overlaid points represent duplicate measurements.
Effects of the temperature of the release of entrapped dye measured using a Cobas Bio Centrifugal analyser. Dye release was due to the reaction of albumin-coated liposomes, antialbumin antiserum and complement. (Reaction conditions are described in section 3.4.3). Symbols are: o = 25°C, ▲ = 30°C, - = 37°C.
3.4.4 Inhibition of Complement-Mediated Immune Lysis by Albumin

Competitive complement-mediated immunoassays are based on the inhibition of antibody binding to antigen-coated liposomes by free antigen. To assess whether the immune-mediated lysis of these albumin-liposomes could be inhibited by addition of albumin, various concentrations of albumin were added to reactants and monitored using a Cobas Bio. The buffer used was buffer A containing teleostean gelatin (1g/l) to minimize adsorption onto plastic surfaces.

The conditions shown on table 3.1 were modified so as to mix albumin and antiserum and preincubate before adding complement and albumin-coated liposomes. The volume of the sample (albumin) was also increased to improve sensitivity. Antiserum and complement concentrations were re-optimised to give near maximal lysis under this modified order of addition. However similar dilutions to those found previously to be optimal (figures 3.9 and 3.10) gave maximal rates of lysis. The preincubation period was increased to 15 minutes and the absorbance change over five minutes reaction was monitored. Since temperature had been shown to have only a small effect over this measurement period (figure 3.11) a temperature
of 25°C was used. The revised conditions are shown in table 3.2.

### TABLE 3.2

<table>
<thead>
<tr>
<th>Reaction Parameters to Demonstrate Inhibition of Lysis by Albumin on Cobas Bio Analyser</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1</strong> (reagent 1): Antiserum diluted x 4 in Buffer A + 1 g/l gelatin; 20 ul/ cuvette</td>
</tr>
<tr>
<td>sample: Purified human albumin diluted in Buffer A + 1 g/l gelatin by doubling dilutions to concentrations between 8 mg/l and 4 g/l; 20 ul/ cuvette</td>
</tr>
<tr>
<td>diluent: distilled water; 10 ul/ cuvette</td>
</tr>
<tr>
<td><strong>R2</strong> (reagent 2): Buffer A + 1 g/l gelatin containing liposomes diluted 1/20 and complement (dilution x8); 10 ul/ cuvette</td>
</tr>
<tr>
<td>Temperature 25°C</td>
</tr>
<tr>
<td>Printout mode 2 (absorbance change)</td>
</tr>
<tr>
<td>Assay process; R1 and sample (+ diluent) dispensed and mixed. 15 minute preincubation. R2 added and readings taken at 30 second intervals over 5 minutes.</td>
</tr>
</tbody>
</table>

Under these conditions inhibition of lysis could be demonstrated using samples with albumin concentrations of between approximately 125 mg/l and 4 g/l, as shown in figure 3.12a.
A similar degree of inhibition was obtained when the pre-incubation period was reduced to only 10 seconds (figure 3.12b).

Figure 3.12 demonstrates that in principle inhibition of lysis could be used to measure albumin concentrations. The range of concentrations found in human serum (usually 20 to 50 g/l) might be measurable with appropriate dilution. To test this in practice, human serum was pre-diluted in buffer A (1/40) and assayed against a pure albumin standard curve. The samples were compared to a routine bromocresol green method (obtained by a Bayer Axon analyser). The comparison is shown in figure 3.13. A significant correlation was found (r = 0.96 p<0.01)). The regression equation was:

\[
y(\text{liposomal albumin}) = 1.02x(\text{BCG albumin}) - 1.93
\]

Despite the reasonable regression equation and correlation for serum albumin, the assay could not measure below approximately 125 mg/l (figure 3.12) under these experimental conditions. This was too high to
Effects of Pre-incubation Period on Inhibition of Liposomal Lysis by Albumin

The effects of pre-incubation time on the inhibition of lysis by albumin of albumin-coated liposomes in the presence of antialbumin and complement. The reaction conditions are described in section 3.4.4.
Thirty two human serum samples were measured by both the liposomal immunoassay and by a chemical bromocresol green (BCG) method (as described in section 3.4.4). The measured concentrations are compared.
accurately measure urinary microalbuminuria, for which a limit of detection of approximately 5 mg/l would be desirable.

The effects of decreasing the amount of bound albumin were examined as a means to potentially improve sensitivity. Liposomes were prepared as described in section 3.3.2; except that the amount of PE coupled to SPDP and incorporated into the liposomes was reduced from 2 umol per preparation (66 umol) to 0.4 umol per preparation. This resulted in a reduced protein:lipid ratio of 66 ug albumin:umol lipid (compared to 216 ug/umol using 2 umol PE (section 3.3.3)).

The lysis of these liposomes was monitored using the Cobas Bio procedure described in section 3.4.3. The optimal antiserum dilution was approximately 10 ul per cuvette and the optimal complement dilution was approximately 2 ul per cuvette. These were similar to those for the liposomes prepared using 2 umol PE. However the absorbance change under these reaction conditions was 40 mA, compared to 140 mA found using the previous liposomes (figures 3.9 and 3.10).

Because the small absorbance change it was not feasible to set up a precise inhibition assay.
3.5 Preparation of Antialbumin Liposomes and Effects of Complement

In a further attempt to develop an assay of greater sensitivity; antialbumin-coated liposomes were prepared to assess their response to complement-mediated immunolysis. The liposome synthesis was as described for albumin-coated liposomes (section 3.3.1); except that sheep anti-albumin antiserum (purified IgG fraction, 7.0 g/l) was substituted for albumin and DTP-antialbumin, rather than DTP-albumin, formed.

The protein and cholesterol contents of the final liposome preparation were measured as described in section 3.3.3. The binding was estimated to be 72 ug immunoglobulin / umol lipid.

The stability of the liposomes was assessed by dialysis (as described in section 2.6) and the preparation gave a leakage of 0.3% per day at room temperature and negligible (<0.1%) at 4°C.

The responses of the liposomes to various combinations of complement, antigen, antibody, and also a donkey anti-sheep second antibody, were assessed. Change in fluorescence over a given time was used as an indicator
of dye release (see section 2.7). Typical results are shown in table 3.3, based on the following experimental protocol.

Liposome preparation (10 ul) was mixed with 100 ul of various solutions (see table 3.3) and made up to 2 ml with buffer A. After 20 minutes incubation at 37°C, 20 ul of guinea-pig complement (in buffer A) was added (except for the control) and the fluorescence measured after a further 20 minutes, also at 37°C. Dye release was calculated using the formula derived from section 2.6;

\[
i.e \text{ dye release} = \frac{(\text{final fluorescence} - \text{initial fluorescence})}{(\text{maximum fluorescence} - \text{initial fluorescence})}
\]

The initial fluorescence was 14 arbitrary units and the maximum possible fluorescence was 405 arbitrary units, established by adding 5 ul Triton X-100 to liposomes diluted to the final volume.
TABLE 3.3

Effects of Various Sera and Serum Constituents on Liposomal Dye Release

<table>
<thead>
<tr>
<th>Solution</th>
<th>Fl. Increase (a)</th>
<th>Fl. Increase (b)</th>
<th>Dye Released (%) (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>donkey anti-sheep serum (1/5 dilution)</td>
<td>18</td>
<td>17</td>
<td>4.5</td>
</tr>
<tr>
<td>human serum albumin (800 mg/l)</td>
<td>12</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>human serum albumin (800 mg/l) plus sheep anti-albumin (1/5 dilution)</td>
<td>14</td>
<td>12</td>
<td>3.3</td>
</tr>
<tr>
<td>normal sheep serum (1/5 dilution)</td>
<td>14</td>
<td>14</td>
<td>3.6</td>
</tr>
<tr>
<td>normal sheep serum (1/5) in buffer replacing complement</td>
<td>10</td>
<td>10</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Although a slight dye release was obtained using donkey anti-sheep antibody compared to the normal sheep serum control, no increase in lysis was observed by the addition of serum albumin, with or without free sheep anti-albumin. These results suggested that liposomes coated with anti-albumin could not be used in a complement-mediated immunoassay for albumin.
3.6 Complement-Mediated Immunolysis of Liposomes Coated with Antialbumin Fab' Fragments

3.6.1 Preparation of Fab' Antialbumin Liposomes and Complement-Mediated Immunolysis

Liposomes were prepared coated in Fab' fragments to assess their suitability for complement-mediated immunolysis. The procedure for preparation of antialbumin liposomes was followed (section 3.3), except anti-albumin Fab' fragments (9.6 g/l) replaced albumin in the formation of DTP-Fab' (section 3.3.1). The DTP-Fab' was coupled immediately to liposomes, to reduce any potential cross-linkage with Fab' thiol groups. The binding of Fab' fragments was calculated to be 237 ug protein/umol lipid. The liposomes, like intact antialbumin liposomes, (section 3.5), showed good stability with leakage of less than 0.1% per day at room temperature and at 4°C.

Unlike intact antialbumin liposomes; preliminary spectrophotometric and fluorimetric assays, similar to those in section 3.4.1 and 3.4.2, demonstrated complement-mediated immunolysis when the Fab'-coated liposomes were mixed with human albumin and sheep
antihuman albumin (intact). This was examined using a Cobas Bio (as described for albumin-liposomes in section 3.4.3). The reaction conditions shown in table 3.4 were used to assess the effects of variation in complement concentration on the reaction. These effects are shown in figure 3.14. The lysis was positively related to the complement concentration. No maximum lysis or "hook effect" was observed, in contrast to that seen for lysis of albumin liposomes (figure 3.5).

Table 3.5 indicates the assay conditions used to optimize antiserum concentration. The resulting absorbance changes are shown in figure 3.15.

Using optimized conditions for anti-albumin, at the complement concentration giving near-maximum lysis, the absorbance changes for the reaction was monitored over approximately 30 minutes (assay parameters as table 3.6). Liposomes were, for comparison, combined with complement in reagent 1 (R1) immediately before assay. However, delays between combination of liposomes and complement of up to at least 45 minutes had no observed effect on the reaction rate.
TABLE 3.4

Reaction Parameters used on Cobas Bio Analyser to Assess Effects of Complement Concentration on Liposome Lysis

R1 (reagent 1): buffer A plus liposomes (1/20), 20 ul/cuvette

sample: guinea-pig complement dilutions in buffer A plus 200 mg/l human albumin (pure); 20ul/cuvette
diluent: (distilled water); 10 ul/cuvette

R2 (reagent 2): antialbumin (1/4); 10ul/cuvette

Temperature: 25°C

Printout type: 3 (absorbance measurements)

Assay process: R1, sample and diluent dispensed into cuvettes and mixed. 5 minute pre-incubation. R2 added and readings taken at 30 second intervals over 5 minutes (first reading after 3 seconds).

---

TABLE 3.5

Reaction Parameters used on Cobas Bio Analyser to Assess Effects of Antiserum Concentration on Liposome Lysis

R1 (reagent 1): buffer A plus liposomes (1/20) plus albumin (pure) 200 mg/l; 20 ul/cuvette

sample: antialbumin antiserum dilutions in buffer A; 20ul/cuvette
diluent: (distilled water); 10 ul/cuvette

R2 (reagent 2): complement (neat); 10ul/cuvette

Temperature: 25°C

Printout type: 3 (absorbance measurements)

Assay process: R1, sample and diluent dispensed into cuvettes and mixed. 5 minute pre-incubation. R2 added and readings taken at 30 second intervals over 5 minutes (first reading after 3 seconds).
Figure 3.14

Effect of Varying the Amount of Complement on the Immunolysis of Fab'-Coated Liposomes

The effect of varying the volume of neat complement preparation added to the Cobas Bio reaction cuvette. The reaction mixture also contained liposomes coated with antialbumin Fab' fraction, human albumin and free antialbumin antiserum. The reaction conditions are described in Table 3.4.
The effect of varying the volume of neat antialbumin antiserum added to the Cobas Bio reaction cuvette. The reaction mixture also contained Fab' (antialbumin)-coated liposomes, human albumin and complement. The reaction conditions are described in Table 3.5.
TABLE 3.6
Final Reaction Parameters used on Cobas Bio Analyser for Complement-Mediated Immune Lysis of Fab' Liposomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (reagent 1)</td>
<td>buffer A plus liposomes (1/20) and complement (guinea pig) (1/4); 20 ul/cuvette</td>
</tr>
<tr>
<td>Sample</td>
<td>albumin (e.g. urine or standard); 20ul/cuvette</td>
</tr>
<tr>
<td>Diluent</td>
<td>(distilled water); 10 ul/cuvette</td>
</tr>
<tr>
<td>R2 (reagent 2)</td>
<td>anti-albumin (1/3); 10ul/cuvette</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
</tr>
<tr>
<td>Printout type</td>
<td>3 (absorbance measurements)</td>
</tr>
<tr>
<td>Assay process</td>
<td>R1, sample and diluent dispensed into cuvettes and mixed. 5 minute pre-incubation. R2 added and readings taken at 1 minute intervals over 30 minutes (first reading after 3 seconds).</td>
</tr>
</tbody>
</table>

A more gently plateauing in reaction was observed under these conditions (figure 3.16), compared to earlier reaction curves using albumin-liposomes (cf. figure 3.8). A small decrease in absorbance in the absence of albumin was observed. This was initially attributed to an artifact caused by instrument drift over the 30 minute reaction period. To increase sensitivity, although at some sacrifice in assay speed, a reaction time of 30 minutes was adopted subsequently.

Human teleostean gelatin (1g/l) was added to the Buffer A in order to minimize any absorption of albumin onto
The absorbance changes monitored using a Cobas Bio analyser, due to the reaction of Fab'(antialbumin)-coated liposomes, albumin, free antialbumin antiserum and complement. The reaction conditions have been described in Table 3.6.
the plastic surfaces. The effects of varying albumin concentrations were examined (figure 3.17). This showed a satisfactory overall absorbance changes between 0 and 200 mg/l. Varying the pre-incubation had a small effect on the slope of the curve, but it was considered that insufficient advantage would be gained in increasing the five minute pre-incubation period.

The slope of the standard curve was shallow at low concentrations. For a microalbumin assay a limit of detection below 20 mg/l is required. The poor dose-response at low concentrations was circumvented by including a low concentration of pure human albumin (50 mg/l) in the first reagent (R1). This gave a reasonable slope at low concentrations, while avoiding any plateauing at the higher assay concentrations (figure 3.18). The effect of adding the same concentration of bovine albumin is also shown. Unlike human albumin, this failed to improve the dose-response at low concentrations.
Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of various concentrations (in sample) of albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6; except that the preincubation period was varied as follows: \( \bullet = 10 \text{ seconds, } \square = 5 \text{ minutes, } o = 30 \text{ minutes.} \) Overlaid points represent duplicate measurements.
Effect of Human Albumin Concentration on Complement-Mediated Immunolysis of Fab'-Coated Liposomes with Albumin added to First Reagent

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of various concentrations (in sample) of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6. The first reagent (R1), which contained liposomes, complement and buffer, also included either human albumin (50 mg/l)*, or bovine albumin (50 mg/l)o. Overlaid points represent duplicate measurements.
3.6.2 Assessment of Potential Interferences in Microalbumin Estimation

The effects of variation in the amounts of several urinary components were assessed, including pH; magnesium, calcium, sodium and potassium ions, and globulin concentrations.

The reference ranges for twenty four hour urine excretions are well established. Microalbumin, however, is often measured in untimed early morning samples. To gain an idea of the likely range of interferent concentrations, these were measured in untimed early morning specimens collected for microalbumin estimation. These specimens were obtained from diabetes mellitus patients attending out-patient clinics. The specimens were frozen immediately on receipt, thawed prior to assay, mixed and briefly centrifuged to remove any precipitated material. Aliquots of these specimens were assayed for the concentrations of potential interferents, and for pH, within two days of thawing.

To assess the likely effects of sample pH on the lysis reaction; various proportions of sodium dihydrogen phosphate (0.05M) and disodium hydrogen phosphate (0.05M) were combined to produce solutions of pH between 4.6 and
8.7. Teleostean gelatin was added (1%). These solutions were sampled in the assay procedure, with and without the addition of 75 mg/l human albumin.

The pH which each of these solutions produced in combination with the other reagents in the reaction cuvette was estimated as follows. Each phosphate solution was mixed with distilled water and assay buffer in the proportions of the assay and the resultant pH was measured using a pH meter. Normal sheep serum was added in proportion to replace the antiserum. The sheep serum made a maximum change of 0.1 pH unit. Complement was replaced in proportion by assay buffer. Figure 3.19 indicates the effect of sampling these phosphate solutions on the liposomal assay. The reaction was only slightly pH dependent between estimated reaction pHs of 6.0 and 7.7 (sample pHs of 4.6 and 8.7 respectively).

The pHs of twenty urine samples were measured, both alone and in proportionate mixtures to mimic assay conditions, as described in the previous paragraph. Their urine pHs ranged from 5.5 to 7.0 (mean 6.35, s.d. 0.28), and from 6.2 to 7.2 (mean 6.9, s.d. 0.24) after mixing to mimic assay conditions.
Figure 3.19

Effect of Reaction pH on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. The sample buffer pH was varied as described in section 3.6.2. The reaction pHs were estimated using, as samples, phosphate solutions (0.05M) of the following pHs; (estimated reaction pHs in brackets); 4.6 (6.0); 5.7 (6.65); 6.5 (6.9); 7.6 (7.5); 8.2 (7.6); 8.7 (7.7). The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □. Overlaid points represent duplicate measurements.
To minimize the effect of pH on the response of the albumin standards, human albumin was diluted in pH 6.5 phosphate buffer (0.05M) rather than buffer A (pH 7.5). This buffer resulted in an estimated reaction pH of 6.9. Using these standards a similar dose-response curve was obtained (figure 3.20) to that previously found using buffer A. However, the small negative absorbance change at low albumin concentrations observed using buffer A was not found at this pH.

The potential inaccuracy caused by incomplete buffering of the samples was estimated. Extrapolating the variation in figure 3.19 to the standard curve (figure 3.20), the maximum error due to the observed urine pHs was approximately 2 mg/l at zero concentration and 4 mg/l at 75 mg/l albumin.

The effects of varying phosphate concentration was examined by preparing solutions of various strengths of sodium phosphate buffer, pH 6.5; each containing gelatin (1%), with and without human albumin. Figure 3.21 shows that the reaction was insensitive to changes in these solutions up to approximately 200 mmol/l phosphate concentrations. However at higher concentrations negative absorbance changes were observed. At the highest concentration (500 mmol/l), some precipitation of the
Figure 3.20

Standard Curve for Complement-Mediated Immunoassay Using Fab' Coated Liposomes and Albumin Standards in pH 6.5 Buffer

Standard curve used to estimate urinary microalbumin. The reaction conditions are described in Table 3.6, with the inclusion of human albumin (50 mg/l) in the first reagent (R1). Standards were prepared in pH 6.5 phosphate buffer (0.05M). Overlaid points represent duplicate measurements.
Figure 3.21

Effect of Phosphate Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5). The sample phosphate concentration was varied as described in section 3.6.2. The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □. Overlaid points represent duplicate measurements.
liposomes was evident in the reaction cuvette after the assay.

The observed effects using phosphate buffer could be due to either a specific effect of phosphate or of sodium; or a non-specific effect of high ionic strength. These alternatives were assessed by adding either sodium chloride or potassium chloride in various concentrations to phosphate buffer, pH 6.5 (0.05M) with gelatin (1%). (Phosphate buffer was used to maintain pH.) To these sodium concentrations was added the sodium content of the phosphate buffer, which was calculated to be 69 mmol/l using the Henderson-Hasselbalch equation.

The effect of complete absence of sodium ions from the sample was also assessed using potassium phosphate buffer (0.05M, pH 6.5). This gave very similar absorbance changes to sodium phosphate buffer of the same pH and concentration (98 mA and 95 mA in the presence of 75 mg/l albumin, and 0 mA and 4 mA in the absence of albumin).

Sodium showed minimal effects below approximately 450 mmol/l (figure 3.22). Potassium showed only a slight inhibition up to 400 mmol/l. Both cations showed similar loss of lysis to phosphate at higher concentrations although to a less marked degree at equivalent
Figure 3.22

Effect of Sodium Chloride Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5), to which various concentrations of sodium chloride were added as described in section 3.6.2. The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □. Overlaid points represent duplicate measurements.
concentrations. Unlike at high phosphate concentrations, no precipitation was evident using sodium or potassium chloride.

Twenty urine samples were assayed for sodium, potassium and phosphate to assess whether an error was likely at the concentrations of these ions. The observed concentrations of sodium ranged between 44 and 166 mmol/l (mean 87.0, s.d. 32.8). This is well below the level likely to significantly interfere; (interference over approximately 400 mmol/l).

The range for phosphate of 10 to 37 mmol/l (mean 19.7, s.d. 9.8) would seem also not high enough to interfere; (interference not seen below 200 mmol/l).

For potassium, the observed range was 3 to 99 mmol/l (mean 32.1, s.d. 23.7). Potassium was found not to interfere up to 100 mmol/l (figure 3.23). Slightly higher concentrations might give a small negative bias. However even if much higher concentrations could occur, the error in albumin concentrations would still be small; e.g. at a potassium concentration of 400 mmol/l, less than 2 mg/l at zero albumin concentration and 4 mg/l at 75 mg/l albumin concentration (deduced by extrapolating the absorbance error to the standard curve, figure 3.20).
Effect of Potassium Chloride Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5), to which various concentrations of potassium chloride were added as described in section 3.6.2. The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □ Overlaid points represent duplicate measurements.
To assess the effects of calcium and magnesium, various amounts of the respective chloride salts were added to phosphate buffer, pH 6.5 (0.05M) and sampled. The results are shown in figures 3.24 and 3.25. Calcium showed a small augmenting effect between 0 and 10 mmol/l (equivalent to approximately 1 mg/l albumin at zero albumin concentration and 4 mg/l at 75 mg/l albumin concentration). The observed range for calcium was 0.4 - 4.1 mmol/l (mean 2.2, s.d. 1.1). This is equivalent to a negligible potential error at zero concentration and an error of less than 2 mg/l at 75 mg/l albumin concentration.

The observed range in concentration for magnesium was 0.9 to 4.8 mmol/l (mean 2.3, s.d. 1.1). Magnesium concentrations had little effect over a wide range of concentration (figure 3.25).

The effect of pure human gamma globulin concentration is shown in figure 3.26. Human gamma globulin seemed to have little effect on the absorbance changes up to a sample concentration of 1 g/l.
Effect of Calcium Chloride Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5), to which various concentrations of calcium chloride were added as described in section 3.6.2. The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □. Overlaid points represent duplicate measurements.
Figure 3.25

Effect of Magnesium Choride Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5), to which various concentrations of magnesium chloride were added as described in section 3.6.2. The samples either did not contain albumin , or contained human albumin (75 mg/l). □. Overlaid points represent duplicate measurements.
Effect of Human Gamma Globulin Concentration on Dye Release due to Complement-Mediated Immunolysis of Fab'-Coated Liposomes

Absorbance changes due to dye-release over a thirty minute period measured by a Cobas Bio analyser, caused by reaction of human albumin, Fab' antialbumin-coated liposomes, antialbumin antiserum and complement. The reaction conditions were as described in Table 3.6, except that human albumin (50 mg/l) was also added to the first reagent. Samples were prepared using sodium phosphate buffer (pH 6.5), to which various concentrations of purified human gamma globulin were added as described in section 3.6.2. The samples either did not contain albumin ●, or contained human albumin (75 mg/l) □. Overlaid points represent duplicate measurements.
3.6.3 Urinary Microalbumin Assay Performance

Using the standard curve based on standards in pH 6.5 buffer (figure 3.20), early morning urine albumin samples were assayed. The measured concentrations were compared to a conventional radioimmunoassay (RIA) method which used an overnight incubation and PEG precipitation (NETRIA). The radioimmunoassay tests were assayed in duplicate and compared to a standard curve in the same batch. Samples were assayed in the liposomal assay in the same way. The results are shown in figure 3.27. The correlation (r) was 0.94 and the regression equation was:

\[ y \text{ (liposomal assay)} = 1.09 \times y \text{ (radioimmunoassay)} - 1.54 \]

The within-batch imprecision was assessed in single assay rotors and compared to the within-batch imprecision of the radioimmunoassay. Since the radioimmunoassay used duplicate means, comparison with imprecision of duplicate means in the liposomal assay was appropriate. This indicated less imprecision in the liposomal assay (figure 3.28).
The microalbumin concentrations of early morning urine samples (n=34) were measured, following the procedures described in section 3.6.3, by the liposomal assay using Fab' (antialbumin)-coated liposomes and by a conventional radioimmunoassay (NETRIA).
Comparison of Imprecisions in Measurement of Microalbumin by Complement-Mediated Immunolysis Using Fab'-Coated Liposomes and by Conventional Radioimmunoassay

Comparison of within-batch imprecisions of the liposomal immunoassay and conventional radioimmunoassay (NETRIA) for the measurement of urinary microalbumin. The procedures followed are described in section 3.6.3. The imprecision of the liposomal assay was assessed either using singletons ($n=24$) ■, or using duplicates ($n=12$) ○. The imprecision of the radioimmunoassay was assessed using duplicate measurements ($n=20$) Δ. The microalbumin concentration indicated is the mean of the measurements used to calculate imprecision.
The limit of detection can be assessed from this data in two ways. If it is defined as the concentration giving a C.V.% of 10%, this can be obtained by extrapolation of the curve in figure 3.28. If, on the other hand, it is defined as 3 standard deviations of the results of a value close to zero, this can be approximated from the data for the lowest pool (10.3 mg/l). The limits of detection calculated in these ways are indicated in table 3.7. The limits derived using singleton tubes is shown for comparison. The data indicate that, at least using duplicates, the liposomal assay had similar or lower limits of detection to the radioimmunoassay. The reliability of the limits of detections estimates is questionable because the pool is relatively a little high (10 mg/l) compared to the derived limits of detections. Nevertheless it is clear that the true limit of detection for the liposomal assay, using duplicate means, lies below 10 mg/l. This is acceptable, since the clinically important cut-off is usually taken as approximately 20 mg/l.
### TABLE 3.7

Limits of Detections of Microalbumin Assays (mg/l)

<table>
<thead>
<tr>
<th>method</th>
<th>10% C.V.</th>
<th>3sds*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA (duplic.) (n=20)</td>
<td>&lt;10 (8)**</td>
<td>3</td>
</tr>
<tr>
<td>Liposomal (duplic.) (n=10)</td>
<td>&lt;10 (4)**</td>
<td>2</td>
</tr>
<tr>
<td>Liposomal (sing.) (n=20)</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

*3sds refers to 3 standard deviations of the results for a low sample.

**The concentrations given in brackets are an approximation by extrapolating the respective curves in figure 3.28 to a C.V. of 10%
3.7 Immuno-Precipitation by Antialbumin-Coated Liposomes

Antialbumin-coated liposomes had been prepared but were found, unlike Fab'-coated liposomes, not to be useful in complement-mediated immunoassays (section 3.5). However it was noticed that when the liposomes were mixed with albumin and left to stand overnight a small amount of red, dye-containing precipitate formed. When these liposomes were subsequently centrifuged gently (i.e. 2000g for ten minutes), the liposomes and their entrapped dye sedimented leaving a clear supernatant. The possibility of using antibody-coated liposomes as a solid phase in an immunoassay was examined since liposomes are potentially more easy to pipette than other particulate precipitation reagents (e.g. cellulose), particularly in automatic pipettors. A radioimmunoassay of microalbumin was used as a model assay.

3.7.1 Evidence of Competitive Label Binding

In order for the liposomes to function as a solid phase, there should be competitive binding of the albumin and radiolabelled albumin with the liposomes. Gel filtration was used to establish that this occurred, independently of the immunoprecipitation effect.
Various concentrations of albumin (6 ul) were added to 30 ul of liposome preparation, 6 ul $^{125}$I radiolabelled albumin and 100ul of phosphate buffer which contained 1% normal sheep serum. After four hours incubation at room temperature, the mixtures were each separated using Sepharose 4B. The eluted fractions (0.5 ml) were analysed for radioactivity by a gamma counter. The results are shown in figure 3.29. The amount of radioactive albumin in the void volume (which contained the liposome fraction, as shown in section 2.4) was found to be inversely related to the amount of unlabelled albumin added.

As a control experiment, liposomes were prepared as in section 3.5 but not coupled to antialbumin. These liposomes were incubated with labelled albumin as before. On gel filtration, very little radioactivity was found in the liposomal fraction (figure 3.30). As additional controls, solutions of free albumin alone and also free antialbumin alone both eluted with similar retention times to the free radioactive albumin (as assessed by the Coomassie Blue method). Furthermore, no significant elution of albumin label in the liposomal fraction was observed when free antialbumin was added to labelled albumin prior to incubation and chromatography.
Various amounts of albumin (0 ng x, 10 ng •, 50 ng *, 200 ng +, 2 ug *) were incubated with anti-albumin liposomes and 125I-labelled albumin (as described in section 3.7.1). Mixtures were separated by Sepharose 4B gel chromatography. Fractions (0.5 ml) were collected and measured for radioactivity. The liposomes and bound label eluted in the void volume.
Control experiment showing elution positions of components following gel filtration on Sepharose 4B; after incubation of liposomes, not coupled to antialbumin antibody, with $^{125}$I albumin. Peaks are: (a) = liposome entrapped dye, (b) = free dye, (c) = free $^{125}$I albumin. The Sulphorhodamine B content by absorbance at 565 nm (peaks (a) and (b), +) and radioactivity (peak (c), x) of 0.5 ml fractions are shown.
3.7.2 Use of Liposomes as a Solid Phase

Early morning urine specimens were collected from diabetes mellitus patients and frozen on receipt. Prior to assay they were thawed, mixed and briefly centrifuged to remove any precipitated material.

A standard immunoassay protocol was adopted as follows, but was modified experimentally as subsequently described. Phosphate buffer (100 ul) containing 1% normal sheep serum and 6 ul radiolabelled albumin were mixed in a 4 ml plastic tube with antialbumin liposomes (30 ul) and 6 ul of urine sample or human albumin standard (0 - 200 mg/l). The tubes were incubated at 37°C overnight (16 hours), centrifuged for ten minutes at 2000 g, washed in buffer containing 1% normal sheep serum (2 ml), then centrifuged as before. After aspiration of the supernatant, the precipitated liposomes were counted for 300 seconds per tube using a gamma counter.

Using this assay protocol, no visible sediment formed in control tubes containing either 0 mg/l of 200 mg/l albumin, and using liposomes to which anti-albumin had not been coupled.
The effect of temperature on the immunoassay was assessed. A temperature of 37°C was found to give better sensitivity, in terms of dose-response, than 25°C or 45°C (figure 3.31). At 37°C, the sensitivity (i.e., slope of dose-response curve) increased with an incubation time up to 16 hours, but did not significantly improve further if the incubation were extended to 40 hours (figure 3.32).

The maximum binding of label was found to be dependent on the volume of liposomes added to each tube (figure 3.33). For this experiment the standard protocol was adjusted by reducing the added buffer volume so that the total reaction volume was constant. Using the standard protocol, a maximum binding (zero standard/total counts) of 10-20% was obtained.

A typical standard curve is shown in figure 3.34. Table 3.8 indicates the within-batch imprecision of the liposomal assay, obtained by analysing twenty replicates of two levels of urine pools. The imprecision of a commercial radioimmunoassay which used an overnight incubation and PEG precipitation (NETRIA) (referred to in section 3.6), also obtained using twenty replicates of the urine pools, was compared and found to be similar.
The effect of varying the temperature was examined. The radioimmunoassay which used liposomes as a solid phase is described in section 3.7.2. A 16 hour incubation was adopted in these experiments. In the procedure, 200 mg/l added standard was equivalent to 1.2 ug albumin standard per tube. Temperatures were as follows: 25°C (+), 37°C (x), 44°C (●).
Figure 3.32

Effects of Incubation Period on Dose-Response Curve of Radio-Immunoassay Using Antialbumin-Coated Liposomes as a Solid Phase

Incubations were performed at 37°C. Total counts per minute were 6400 per tube. In the assay protocol, described in section 3.7.2, 200 mg/l added albumin standard was equivalent to 1.2 ug albumin standard per tube. Incubation times were 1 hour (*), 4 hours (+), 16 hours (•) and 40 hours (x).
Effects of varying amounts of liposomes on the observed maximum binding of radiolabelled albumin in the radioimmunoassay using antialbumin-coated liposomes as a solid phase. The assay protocol has been described in section 3.7.2). Total counts per minute were 6500 per tube.
The immunoassay procedure is described in section 3.7.2. Total counts per minute was 6540 per tube. The binding of the zero standard was 15% of the total counts. In the protocol, 200 mg/l added albumin standard was equivalent to 1.2 ug albumin standard per tube.
TABLE 3.8
Within-Batch Imprecision of Liposomal Immunoassay and Conventional Radioimmunoassay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Liposomal</th>
<th></th>
<th>Conventional (NETRIA)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (mg/l) ± S.D.</td>
<td>C.V.%</td>
<td>Mean (mg/l) ± S.D.</td>
<td>C.V.%</td>
</tr>
<tr>
<td>Pool1</td>
<td>20.1 ± 1.6</td>
<td>7.8</td>
<td>23.5 ± 1.9</td>
<td>8.2</td>
</tr>
<tr>
<td>Pool2</td>
<td>46.0 ± 2.3</td>
<td>5.1</td>
<td>49.2 ± 2.6</td>
<td>5.3</td>
</tr>
</tbody>
</table>

When the albumin concentrations measured by the liposomal method and the NETRIA radioimmunoassay were compared (figure 3.35), a good correlation was obtained ($r=0.97$). The regression equation was $y(\text{liposomal}) = 0.83x(\text{NETRIA}) + 1.65$.

3.8 Discussion

This chapter first described the synthesis of albumin-coated liposomes and their use in a complement-mediated immunoassay for the measurement of albumin in human sera. The assay was of the type usually referred to as competitive. Antialbumin antiserum was first preincubated with free albumin in the sample or standard. Albumin-coated liposomes and complement were then added. The
Figure 3.35

Comparison of Urinary Microalbumin Concentrations Measured by Liposomal Radioimmunoassay and Conventional Radioimmunoassay

Thirty nine urine samples were measured by the radioimmunoassay using liposomes coated with antialbumin antibodies, and by a conventional radioimmunoassay (NETRIA). Their measurements of urinary microalbumin concentrations were compared.
complement-mediated lysis was dependent on the amount of antibody bound to the liposomes and inversely related to the amount of albumin in the sample or standard.

The assay using albumin-coated liposomes was not found to be sufficiently sensitive to measure urinary microalbumin. A sandwich type assay was therefore developed as an alternative. In this type of assay, liposomes coated with antibody are incubated with antigen (in this case albumin), free antiserum and complement. A "sandwich" of liposome-bound antibody, antigen and free antibody occurs, which initiates complement-mediated immunolysis. As opposed to a competitive assay; in this type of assay the lysis is directly related to the concentration of antigen in the sample or standard.

The sandwich type assay is theoretically more sensitive than the competitive assay and will tend to have a lower detection limit. This is because in a competitive assay, the amount of antigen must be in excess of available antibody binding sites. (If this were not the case in the complement-mediated liposomal assay there would always be sufficient antibody to lyse all the immunoreactive liposomes.) Conversely, in the sandwich type liposomal assay, the antigen is required not to be in excess of
either the liposomal-bound antibody or the free antibody binding sites. This can enable higher antibody concentrations to be incorporated into reagents.

A sandwich-type assay was developed using liposomes coated with Fab' fragments of antialbumin antiserum. As predicted this had a lower limit of detection compared to the competitive assay, enabling its use in a complement-mediated immunoassay to measure urinary microalbumin.

Liposomes coated in intact antialbumin antibodies were also prepared. These were found not to initiate significant complement-mediated immunolysis and were not used in an homogeneous assay. The possible reasons for the lack of immunolysis will be discussed later. However, an observation was made that after immune reaction, incubation and gentle centrifugation, these liposomes precipitated. This led to their use as a solid-phase in an heterogeneous immunoassay; as demonstrated by a model radioimmunoassay, also for urinary microalbumin.

The development of complement-mediated immunoassays using release of encapsulated Sulphorhodamine B as a colorimetric signal satisfied a major objective of these
these studies. This was in establishing the general applicability of the use of the dye as a marker in liposomal immunoassays, which most frequently have used complement-mediated lysis.

Complement-mediated immunolysis is based on antigen-antibody reaction at the membrane surface, which leads to complex-formation with complement. This in turn is believed to initiate the complement cascade leading to puncturing of the liposome membrane and loss of its contents.

The initially high rate of release of dye by complement, slowing to a near-plateau after a few minutes (e.g. as shown in figure 3.2) has been found previously; including in early studies using glucose release (103). The complement-mediated immunolysis was significantly slower in reaching a near-plateau in the sandwich-type assay, possibly because the immune-complex, which had more components, took longer to form.

Early studies reported the "hook effect" observed at high antiserum concentrations (103). In the present study, the effect was seen in both the competitive and sandwich assays. A hook was also demonstrated for complement in the competitive assay, but not in the
sandwich assay. Unlike the antiserum hook, the hook for complement does not appear to have been previously reported.

In earlier reports which assessed varying complement amounts, a maximum activity of below 20 CH\textsubscript{50} units/ml was used (47,97,111,115,118). The stock complement used in the present study had a high activity (manufacturer's stated activity 2694 CH\textsubscript{50} units/ml) enabling greater activities to be assessed. The hook effect was observed in the Cobas experiments at concentrations above a dilution of 1/12; equivalent to a concentration of 225 units/ml. While it is difficult to compare reaction conditions directly, it is likely that the effect was not shown previously because of lower complement activity.

The nature of either antiserum or complement hook effect is unclear but a number of mechanisms could be postulated; such as electrostatic repulsion at high protein concentrations, steric hindrance, or configurational changes at the liposomes' surfaces.
No evidence was found that human complement might interfere with the reaction. In one previous report, human complement has been shown to react in a liposomal immunoassay (64). In general, however, marked species to species variability occurs in complement reactivity which seems also to depend on the animal species used to raise the antiserum. A relative lack of reactivity of human complement with animal antisera, compared to guinea-pig complement, has also been found previously (169).

As the sandwich assay used the same intact antibody as the competitive assay, it would seem unlikely that human complement would interfere with one assay and not the other. Furthermore, substantial complement activity would seem unlikely to occur in urine, even in the presence of gross proteinuria (e.g. haematuria), since this would require all the components of the cascade to be active. In any case, the presence of frank proteinuria could be screened for prior to assay, as will be discussed later in this section.
Confirmation evidence for the lack of substantial interferences with the complement-mediated reaction was also provided by the good correlation of results on testing patients’ samples with those obtained by the comparison method.

Prior to this study, the use of liposomes coated in Fab’ fragments in a complement-mediated sandwich assay had been suggested (170) but no performance data had apparently been published. Since commencing the study, a report of such an assay has been published, claiming a good correlation with radioimmunoassay for estimation of serum ferritin (171). However, a minority of serum samples showed non-specific lysis which was attributed to complement activity. It was considered that this effect was due to excess unreacted DTP-PE in the liposomes following coupling to Fab’ thiol groups using SPDP. DTP-PE seemed to increase lipid fluidity and susceptibility to complement attack. Use of an alternative coupling method involving bromoacetyl-PE reduced but did not eliminate the effect.
A sensitivity of liposomes containing DTP-PE to human complement was shown previously (169). One factor which may affect the amount of this non-specific lysis is the density of packing of the antigen (or antibody) on the liposome surfaces. At high density, non-specific lysis might be sterically hindered.

Unfortunately it was not possible to compare the conditions used by Ishimori (171) or Okado (169) with the current report, since liposome size distribution and amounts of proteins incorporated were not stated. The liposome preparations were also substantially different to the current study. Neutral MLVs were used, and carboxyfluorescein incorporated. Furthermore, a rabbit antiserum was used by Ishimori (rather than sheep in this report).

If there is a general non-specific complement-mediated effect due to the use of SPDP reagent; it might presumably affect to some degree all liposomal immunoassays which use this coupling method, and not only those involving Fab' fragments. As discussed, interference by human complement was not observed in the current study. A considerable number of other liposomal immunoassays have also been published (see section 1.6) which have not reported this effect. Nevertheless,
possible interference by human complement needs to be carefully assessed before applications of the technique are put into routine use.

The liposomes in this report were coated with protein using the heterobifunctional reagent, SPDP (N-succinimidy1 3-(2-pyridyldithio) propionate). The coupling method is described in section 1.5.2 and summarized in figure 3.1. The method has advantages in that the reagents are commercially available, the method is straightforward and it is not generally susceptible to non-specific cross-linking.

A difference in the coupling reaction of Ishimori who used SPDP (171) was that DTP-PE was coupled directly to the Fab' thiol groups. This has been reported previously (77, 79). In the present study, the Fab' fragments were coupled to liposomes by modification of their amino groups, in the same way as albumin and intact antiserum. The use of derivatized Fab' amino groups instead of Fab'-thiol groups was considered more convenient. Use of thiol groups would seem to require coupling immediately after Fab' synthesis, presumably because of the
susceptibility of the thiol groups to oxidation. To reduce the possibility of some thiol groups cross linking with DTP-Fab', the latter was reduced and coupled to DTP-PE liposomes immediately after synthesis of DTP-Fab'.

A possible reason for the failure of liposomes coated with antialbumin to initiate complement-mediated immunolysis might be that the complex formed at the liposome membrane was too large or too far from the liposome surface, compared to that using smaller Fab' molecules.

There has, nevertheless, been one report of the successful use of intact antibody in a sandwich assay of this type to measure C-reactive protein (115). There were several differences in their approach; for example multilamellar large vesicles were used. The workers also reported species to species variation in antiserum and complement effects. Some combinations failed to initiate complement, while others caused non-specific lysis. These effects may also at least partially explain the lack of lysis in the present study.
A further factor which may account for differences between liposomes bearing intact antibody and those coated in Fab' fragments arises from an estimation of the number of molecules bound per liposome. This can be calculated from protein/lipid ratios.

The coupling of albumin and antialbumin was 54 ug albumin/umol lipid and 72 ug antialbumin/umol lipid respectively. The coupling of Fab' fragments was higher (237 ug/umol lipid). Hutchinson et al. (45) pointed out the difficulty in accurately calculating the number of protein molecules per liposome unless the size distribution is known and is narrow. Nevertheless; as the liposomes in this study were all filtered through a 200 nm filter during preparation, it is possible to calculate the number of protein molecules per liposome based on the approximation of homogeneous 200 nm liposome populations.

Although no accurate size measurements were performed, electron microscope photographs suggested 200 nm to be a reasonable approximate estimate of the average liposome size (as discussed in appendix 1). Furthermore, electron micrographs were previously produced of liposomes prepared by Szoka and Papahadopoulos (18) using a similar reverse evaporation procedure, although a different lipid composition was used (phosphatidyl glycerol: phosphatidyl
choline: cholesterol, molar ratio 1:4:5). After passage through a 200 nm polycarbonate filter, the vesicle size was estimated to be 120 nm to 300 nm by electron microscopy. Other estimates of size include an average size of 260 nm for MLVs extruded through a series of filters culminating in a 200 nm filter (33).

The lack of size homogeneity could be due to several factors. Some liposomes may be less than 200 nm prior to filtration. Some reduction in liposome size may also occur during filtration by electrostatic effects as the liposomes pass through the filter. Countering this; some deformation of the liposomes may also occur allowing some liposomes larger than 200 nm to be squeezed through the filter without their disruption.

Based on the approximation of a homogeneous population of 200 nm diameter, the following formulae were applied to calculate the number of protein molecules per liposome (45);
\[
N = 4\pi R^2 + \frac{4\pi}{a} (R - h)^2 \quad \text{(equation 3.2)}
\]

where \(N\) = number of lipid molecules per liposome

\(a\) = area of lipid molecule in a bilayer (taken as 0.5 nm\(^2\))

\(R\) = radius of liposome (assuming spherical single walled liposomes)

\(h\) = bilayer thickness (taken as 7.5 nm)

\[
P_i = \frac{(P/L)}{N} \quad \text{(equation 3.3)}
\]

where \(P_i\) = number of protein molecules per liposome

\(P/L\) = molar ratio of protein to lipid

The following molecular weights were used in calculating the molar ratio of protein to lipid;

- albumin = 69000 D
- anti-albumin = 150000 D
- Fab' fragment = 52000 D

Applying these formulae, there were calculated to be 366 molecules of albumin per liposome, 233 molecules of antialbumin antibodies per liposome and 2130 molecules of Fab' molecules per liposome.
The larger number of Fab' molecules bound to each liposome, compared to intact antibody, might therefore be another possible explanation for their greater ability to initiate complement-mediated immunolysis.

Hutchinson et al. (45) reviewed several of the reports that have estimated the number of molecules bound to each liposome. The few relating to similar sized liposomes show general agreement to the present study. Intact IgG was bound to 200 nm liposomes at 200 molecules per liposome (74). Two reports describe directly attaching Fab' fragments to similar sized liposomes via their thiol groups (using different reagents). The reports claimed binding of "more than 3000" molecules per liposome and "up to 6000" molecules per liposome respectively (79,77).

In the competitive assay using albumin-coated liposomes; the large number of molecules bound to each liposome is unlike the situation in conventional competitive immunoassays. In a competitive immunoassay such as a radioimmunoassay only one molecule of antigen is usually coupled to each molecule of label. Provided the labelled antigen is immunologically similar to the free antigen, approximately half the labelled antigen tends to be displaced from the antibody when the the free and labelled antigen have equal concentrations.
In a competitive liposomal immunoassay; if each antigen-antibody complex caused an equal amount of complement-mediated lysis, the amount of signal produced would be related to the ratio in each liposome of dye to bound antigen (i.e. the number of marker molecules per antigen). This would be a measure of the amount of amplification of signal produced by the use of liposome-entrapped marker.

The ratio of bound protein to entrapped dye can be calculated in a similar way to the calculation of the bound protein per liposome. The number of lipid molecules per liposome (N) was calculated from equation 3.2. Assuming all the dye in the preparation is entrapped, the number of dye molecules per liposome (D_i) is given by:

$$D_i = (D/L) \times N$$

where $D/L$ is the molar ratio of dye to lipid.

The molarity of dye was deduced from the absorbance of the dye in the preparation (after lysis with ethanol as described in section 2.6, using the absorbance coefficient given in section 2.3).
The number of dye molecules per liposome (assuming 200 nm diameters) was calculated to be 79000. This is equivalent to the following:

216 molecules of dye per molecule of bound albumin
339 .. .. .. .. anti-albumin
37 .. .. .. .. Fab’ fragment

In a complement-mediated immunoassay the situation is more complex than a conventional immunoassay. It is not easy to predict, for example, the ratio of free and bound albumin which would reduce liposome-antibody binding to a sufficient degree as to reduce lysis by 50%. This can be estimated experimentally, however, by comparing the free and liposomal-bound albumin concentrations.

The concentration of albumin bound to liposomes in the reaction mixture was calculated to be approximately 6 mg/l (1/120 dilution of final preparation, section 3.3.3). Albumin at a sample concentration of approximately 1.5 g/l (i.e. a reaction concentration of 500 mg/l) was required to inhibit lysis by 50%. It therefore required free albumin molecules at approximately 83 times the number of bound albumin molecules to halve the lysis rate.
There are several possible explanations for this lack of equivalence. Assuming the free and bound albumin were in true competition for the antibody; liposomal lysis may have occurred when only a small proportion of the liposomal antigen sites were occupied. This would not be surprising since the complement complex formation at only a few sites might initiate the complement cascade leading to lysis. In this model, it would require displacement of the majority of bound antibody molecules to significantly reduce the amount of lysis.

Another possibility is that, up to a certain concentration of free albumin, the liposomal and free albumin might not be in true competition for the antibody. In other words, free albumin may not always displace antibody-albumin complexes at the liposome surface. Instead a proportion might bind to antibody at the liposome surface, creating larger complement-activating aggregates on the liposome membrane. This would be analogous to immune-complex formation in immunoturbidimetry.

Whatever the mechanism, it appears that competitive complement-mediated assays using liposomes may be particularly insensitive compared to other competitive immunoassays (e.g. radioimmunoassays).
There was yet another consideration which was found to hinder an increase in sensitivity of the competitive liposomal assay. In a conventional competitive immunoassay (e.g. radioimmunoassay), provided the signal of the label is intense enough, the sensitivity can be improved by lowering the concentration of labelled antigen. This means that equivalence occurs at a lower concentration. In the liposomal immunoassay there are two ways to attempt this. The first would be to reduce the amount of liposomes. However, this would reduce the absorbance in this colourimetric assay, and hence probably also the amount of measurable absorbance change. In these studies the amount of liposomes was maintained to give an initial absorbance of approximately 1 A. Reducing the amount of liposomes would appear to be less of a problem if a more sensitive label were used (e.g. a fluorescent label).

The second approach would be to reduce the amount of antigen bound to each liposome; so that, at an absorbance of 1A, there would be less labelled albumin in the reaction mixture. It proved possible to reduce the protein coupling by incorporating less DTP-PE into the liposomal membranes (section 3.4.4). The protein content was reduced to a protein:lipid ratio of 17 ug albumin:umol lipid (compared to 54 ug albumin:umol lipid...
found previously). This was calculated to be equivalent to approximately 112 albumin molecules per liposome (compared to 366 in the liposomes incorporating more DTP-PE). The liposome-bound albumin content in the reaction mixture was also calculated to be reduced from 17 mg/l to approximately 3 mg/l.

Unfortunately, although the amount of bound antigen was lower, so was the amount of complement-mediated lysis. The low complement-mediated absorbance change appeared to preclude a sensitive inhibition assay using these liposomes.

Although the competitive complement-mediated liposomal assay would seem to have particular difficulties in achieving sensitivity, the same is not necessarily true of sandwich type complement-mediated assays. In sandwich type assays, one would predict that increasing sensitivity would be achievable by increasing the amount of bound antibody on the liposome. This might have a combined effect of increasing both the sensitivity to small amounts of antigen and increasing the amount of complement-mediated lysis. In other words, the two effects would be predicted to work in tandem, rather than in opposition as in the competitive complement-based assay.
The sandwich type assay using Fab' fragments was optimized to measure urinary microalbumin and was found, as predicted, to be more sensitive than the competitive assay. A limit of detection of below 20 mg/l was achieved, as was required to measure urinary microalbumin.

Adequate sensitivity was found using large unilamellar vesicles in the sandwich type assay for the measurement of urinary microalbumin. Nevertheless, it might be predicted from the previous considerations that small unilamellar liposomes, with their increased surface area to volume ratio, might enable a greater sensitivity in a sandwich type complement-mediated immunoassay. This would be because, for a given amount of entrapped dye, there would be more liposome-bound antibody in the reaction mixture.

The inclusion of albumin (50mg/l) in the first reagent (R1) of the sandwich type assay increased the sensitivity at low concentrations (i.e. dose:response) and lowered the limit of detection. The standard curve was sigmoidal in shape and inclusion of this albumin meant that low concentrations were measured in a steeper region of the standard curve. The reason for the curve flatness at low concentrations may be a requirement for a certain amount
of albumin to effectively initiate complement-mediated lysis.

An alternative explanation could be that, despite the inclusion of gelatin, some non-specific adsorption of albumin onto the plastic sample cups or cuvettes was occurring. The lack of a similar improvement in sensitivity by the inclusion of bovine, rather than human, albumin suggests this was not the case.

A limit of detection of below 20 mg/l is equivalent to 400 ng per reaction cuvette. This is still a relatively insensitive assay compared to many recent immunoassay techniques (10). However; the use of colorimetric dye was examined because of its other advantages, e.g. speed, convenience and ease of automation. The liposomal assay could, for example, be performed in approximately forty minutes; compared to radioimmunoassay which required an overnight incubation.

These studies do not preclude high sensitivity in a sandwich-type complement-mediated immunoassay, but this would probably require the combination with an alternative marker, for example using fluorescence or chemiluminescence. The possibility of greater sensitivity by the use of fluorimetric measurement of
Sulphorhodamine B release was not examined in these studies. The relative strengths of colorimetric and fluorimetric properties of the dye are discussed in chapter 2 and appendix 2. The possible use of SUVs rather than LUVs as another means of improving assay sensitivity has also been discussed.

The study of interfering substances in the microalbumin assay highlighted the careful assessment required of any immunoassay designed to measure urinary analytes. The good correlation with radioimmunoassay suggests that, in the microalbumin assay, potential interferences were largely avoided. Variation in urinary pH caused a small interference which was partially overcome by lowering the buffer pH of the standards. It was elected not to modify the assay buffer (buffer A) in this assay, since this is a buffer commonly used and well characterized in complement reactions. Nevertheless, greater confidence might be achievable by increasing the buffering capacity of the assay buffer.

All urines were frozen on receipt and measured soon after thawing. This probably helped minimize any gradual increase in urinary pH due to bacterial action. The pH range of the urines tested was 5.5-7.0, which is less than a commonly quoted range of 4.5-8.0 (172). Fresh
urine samples should not vary beyond the range 4.5-7 unless there is a urinary tract infection, in which case the pH can become alkaline (pH 7-8). By deduction from the pHs resulting from phosphate solutions, the quoted range would appear likely to cause a significant but probably acceptable interference of up to approximately 2 mg/l at zero concentration and 4 mg/l at 75 mg/l. There would be an option, in any case, of checking the sample pH using a dip-stick test. The dip-sticks used to check for protein (referred to in the next paragraph) usually also measure pH; typically between pH5 and pH9 in pH1 increments (e.g. BM-Test 3, as described in the materials section). It would be feasible to check the pHs of the samples routinely and adjust extreme values with acid or base as required.

The presence of a hook effect at high antigen concentrations is a potential limitation of the sandwich type assay, since it can lead to underestimation of high antigen concentrations. The hook effect fortunately is not a practical problem in this microalbumin assay, since samples with concentrations above the measurable range can easily be detected. All urines can be tested using the dip-stick test, based on a chemical reaction, for the presence of frank proteinuria (i.e. protein concentration greater than approximately 200 mg/l)
This is a useful preliminary check; since, if proteinuria is detected, there is little point in further testing for microalbuminuria. As mentioned above, pH could be checked simultaneously.

The hook effect at high antigen concentrations is also found in other immunoassay techniques, such as enzyme-linked immunosorbent assays (ELISAs) and immunoturbidimetry. It is often circumvented by assaying several dilutions of the antigen. The liposomal sandwich assay is effectively a "one step" assay in that all the reagents are combined in the assay mixture. The hook effect is probably mainly due to saturation of antibody-binding sites by free antigen. Reduction of the hook effect is possible in solid phase assays (e.g. ELISAs) by a washing stage ("two step assays"). However hook effects can still occur, probably due to conformational changes leading to desorption of bound antibody (173).

The use of intact anti-albumin liposomes as a solid-phase arose from incidental observations. Despite the growing popularity of homogeneous and coated-surface assays (e.g. ELISAs), techniques involving separation of the bound and free label by precipitation (e.g. radioimmunoassays and often immunoradiometric assays (IRMAs)) are still widely used.
Other particulate solid-phases such as cellulose are not easily dispensed and settle out rapidly on standing. This has tended to limit their use in automated dispensing systems.

The SPDP method for the coupling of proteins to liposome membranes was at least as simple as most methods used to generate antibody-coated solid phases, using cellulose, Sephadex or latex particles (174,175).

The antialbumin-coated liposomes could be dispensed as easily as a liquid, and showed minimal sedimentation on standing for several days. Considerably greater sedimentation was observed after immunoreaction and gentle centrifugation.

Microscopically, prior to immunoreaction, the liposomes appeared as homogeneous pink spheres (see appendix 1). After immunoreaction and incubation for several hours, the liposomes had clumped into heterogeneous clusters. One possible mechanism for this phenomenon would be immune complex formation by antigen-antibody cross-linkages between liposomes. This would be similar to the reported augmentation of latex agglutination by liposomes (131).
It might be predicted that such complexes would only form with large antigens such as proteins, which have multiple antibody binding sites. Sandwich type assays (e.g. ELISAs, IRMAs etc.) have a similar restricted range of analytes. It is also possible, however, that the net negative charge of the lipids in the liposomes might be modified by immunoreaction at their surface to a sufficient degree to cause sedimentation by electrostatic effects rather than by large immune complex formation.

No noticeable sedimentation was observed during the complement-mediated immunoassay reactions using either albumin-coated liposomes or Fab'-coated liposomes, with the exception of Fab' coated liposomes in the presence of high phosphate concentrations (figure 3.21). This was despite the Cobas Bio centrifugal analyser exerting a centrifugal force during the reaction periods. There were several differences in assay conditions which might have caused this discrepancy; in particular the incubation periods were shorter.

Earlier in this discussion, the molar ratio of dye to bound protein was calculated for the liposomes used in complement-mediated lysis, assuming a homogeneous liposome pool of diameter of 200 nm. Taking albumin-
coated liposomes as an example, the number of bound protein molecules was calculated to be 366 and the number of entrapped dye molecules was 79,000. This is equivalent to a ratio of dye molecules to bound protein of 216.

Further similar theoretical calculations can be used to infer optimum liposome sizes for a range of analytical applications of liposomes.

It is possible to recalculate the ratio of entrapped dye molecules to bound protein for various sizes of liposomes, making the assumption that the only factor affecting incorporation is size. For example, it is assumed that there are no steric or electrostatic factors hindering incorporation. The calculations for small unilamellar vesicles of 50nm and 20nm diameters are shown in table 3.9. Numbers are average molecules incorporated per liposome.

The dramatic reduction in dye entrapment relative to bound protein is due to two factors. Firstly, the liposome volume decreases as the cube of the radius while the area decreases as the square of the radius. Secondly, the lipid bilayer thickness (estimated as 7.5 nm) becomes a significant factor in reducing the entrapped volume of small liposomes.
At a certain diameter (in this case approximately 26 nm) the ratio of entrapped dye to bound protein is unity. Below this size there is no advantage in signal amplification compared to direct coupling of a dye molecule to a protein molecule. There could still be some advantage, however, in the close proximity of antigen or antibody molecules in augmenting an immune-reaction. Furthermore, in small liposomes the total surface area for a given amount of lipid in the preparation will be much higher, so the total amount of bound protein will be significantly greater.

Table 3.9

Effects of liposome size on theoretical incorporation of dye and protein

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Bound Albumin (molecules per liposome)</th>
<th>Entrapped Dye (molecules per liposome)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>366</td>
<td>79000</td>
<td>216</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>535</td>
<td>22</td>
</tr>
<tr>
<td>20</td>
<td>3.7</td>
<td>1.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>
It is therefore incorrect to assume that, in an immunoassay, there will inevitably be an advantage in the use of large liposomes (i.e. LUVs) because of the potential increase in signal per liposome (e.g. on lysis). The optimum size is dependent on the specific application of the liposomes.

Various types of liposomal assays have been described in section 1.6. In some cases, the lysis will be relatively independent of the amount of protein bound and liposomes with a high relative entrapment will be preferable. This type of assay may include solid phase assays, where the amount of antibody or antigen binding to a solid phase will be limited to a small portion of the liposome. Cytolysin assays (section 1.6.6) are not dependent on protein-binding and larger liposomes would also probably be advantageous in these assays.

As discussed earlier in this section, complement-mediated immunoassays benefit from a high amount of surface protein since this increases the amount of complement-mediated lysis. In competitive complement-mediated assays, on the other hand, the limit of detection tends to be increased if the amount of bound protein increases. These conflicting trends pose a problem in optimizing this type of assay. Sandwich type complement-mediated
assays seem to benefit by an increased amount of surface protein, both in terms of limit of detection and amount of complement activity. Small liposomes would seem to have advantages in sandwich-type assays because of their higher surface area.

Small liposomes might also be advantageous in particle enhanced light scatter techniques which depend on surface immune-reactions. Small particles will also move more quickly under the influence of Brownian motion, possibly speeding up complex formation. They also, in general, have an advantage of more uniform size distribution.

Assays using destabilization of liposomes (section 1.6.7) have usually utilized SUVs. Phosphatidyl ethanolamine has a relatively small head group. It may be that the phase change caused by close proximity of unsaturated phosphatidyl ethanolamine is more marked if the surface curvature of the liposomes is greater, since the bilayer structure might be less easily sustained in these circumstances.

Since the number of molecules of dye in 200 nm diameter liposomes has been calculated, another interesting exercise is to estimate the dye concentration within the liposomes. This was calculated to be approximately 0.04
M, despite 0.1 M dye being used in the preparations. There are two possible explanations for this discrepancy. It is possible that the average size of the liposomes is actually less than 200 nm. Alternatively, dye may have been excluded from the liposomes during preparation. The latter explanation seems possible, since a number of factors such as dye concentration and buffer concentration affected entrapment (section 2.9). Furthermore, the maximum entrapment obtained was less than 30%, while entrapments of over 60% have been found in liposomes prepared by reverse evaporation previously (18).
Chapter 4

Liposomal Assays for Anticardiolipin Antibodies

4.1 Introduction

4.1.1 Clinical Applications of Anticardiolipin Antibodies

Cardiolipin is one of a range of phospholipids which are immunogenic. Anticardiolipin antibodies (ACAs) are often grouped with lupus anti-coagulant (LA) as antiphospholipid antibodies (APAs). ACAs and LA can occur together in a range of conditions but they are not identical and sometimes one or other type of antibodies can occur in isolation. In patients with suspected APAs, they are both often measured and can provide complimentary clinical information. APAs, including ACAs, have been reviewed extensively (176-178). ACAs and LAs are measured by different techniques, usually ELISA and haematological anti-clotting tests respectively.

While IgG and IgM classes of ACAs are the APAs most often measured, antibodies can also be present which bind to other lipids such as phosphatidyl serine, phosphatidyl
inositol, phosphatidic acid and phosphatidyl glycerol (179), and also phosphatidyl choline and phosphatidyl ethanolamine (180). In some cases where other antibodies are found, ACAs and LAs can both be undetectable (181). The poor correlation of immunological activity between LA, ACAs and antibodies to other lipids suggests that there are separate specific populations of antibodies, but also populations which cross-react to a variable degree with a range of lipid antigens.

IgG ACAs are found in a subgroup of patients at risk of thrombosis, recurrent foetal loss and thrombocytopaenia. Patients, with or without the autoimmune disease, systemic lupus erythematosus (SLE), who have APAs and associated clinical conditions are included under the term "Antiphospholipid Antibody Syndrome" (APS). When thrombotic features are found together with APAs but without the other clinical features of SLE the condition is referred to as the Primary Antiphospholipid Antibody Syndrome (PAPS). IgM ACAs tend to be associated with a different range of clinical features than IgG ACAs, including livedo reticularis, chorea and haemolytic anaemia.

The antiphospholipid antibody syndrome has been reported to be commonly associated with a range of clinical
conditions including venous thrombosis, arterial thrombosis, recurrent foetal loss, haemocytopaenia, livedo reticularis, cardiac conditions (such as valve lesions) and neurological associations. APAs are also but less commonly found in ischaemic necrosis of bone, pulmonary hypertension, splinter haemorrhages of fingers and toes, and Addison's disease (182). Other recently reported associations include dialysis patients (183), HIV infected patients (184), in polymyalgia rheumatica (with or without giant cell arteritis) (185) and in acute myeloid leukaemia and non-Hodgkin's lymphoma (186).

A number of proposed mechanisms of action of APAs have been suggested, including decreasing prostacyclin levels, fibrinolysis, protein C and thrombomodulin levels, protein S levels, antithrombin levels or an increase in platelet activation and aggregation (182). Recently, B_2 glycoprotein I has been implicated as a co-factor in the binding of ACA (187). B_2 glycoprotein I is an inhibitor of coagulation and platelet aggregation and interference with its action could also be a mechanism for the antibodies' actions.

The establishment of APS has importance for treatment, since low dose aspirin, and possibly anticoagulation if thrombosis is involved, have been claimed to be
effective. Immunosuppressive therapy is generally only required for an underlying condition such as SLE (182).

ACAs occur in SLE, but they are not the commonest type of autoantibody seen. In a follow-up study over five years (188) anti-nuclear antibodies were found in nearly all SLE patients (98%), anti-double stranded DNA in about half (56%), anti-Ro antibodies in 39% and anti-phospholipid antibodies in 38%. A diverse range of other antibodies are found in lower proportions of patients (189). However the association of IgG ACAs with thrombotic episodes, thrombocytopenia and neurological episodes (190,191) largely explains the interest in ACAs in SLE. Screening for antibodies to phospholipids other than ACAs seems to add little additional clinical information, at least in SLE (179). A large prospective study of 500 patients summarised in detail the clinical features associated with APS in SLE (192).

Neurological associations of APAs include multiple cerebral infarctions, transient ischaemic attacks, migraine-like headaches, visual abnormalities and encephalopathy (193,194). Anticardiolipin antibodies have been shown to be an independent risk factor for first ischaemic stroke (195).
In neurological disease associated with SLE, ACAs have been reported to be absent from CSF (196). Thus the neurological symptoms may be associated with cerebral infarcts, and the diffuse encephalopathy of acute central nervous system lupus appears not to be due to APAs acting against neurons directly.

In contrast, ACAs were found in the CSF of a proportion of neurological patients with neurosyphilis and multiple sclerosis and occasionally in Guillain-Barré syndrome, suggesting localised immune reaction can occur within the brain (197).

Serum antiphospholipid antibodies have also been found in a few Guillain-Barré patients (197,198) but this may, at least in some cases, be caused by underlying SLE (199).

Although there appear to be clear associations of the antiphospholipid antibody syndrome with thrombosis (200), the clinical usefulness of ACA measurements in predicting thrombotic events has been questioned, both in SLE (201-203) and in the PAPS (204). While anticardiolipin antibodies seem an important tool in the management of women susceptible to recurrent miscarriages (205,206); the appropriate extent of APA measurements and the most
effective therapeutic intervention are still unresolved (207).

Rote (208) suggested antibodies to phosphatidyl serine should be measured in pregnancy rather than to cardiolipin, since this correlated better with LA. Furthermore, it is easier to postulate a mechanism of action of antibodies during physiological cell fusion and exposure to phosphatidyl serine, than it is to implicate cardiolipin, a lipid which is generally restricted to the inner mitochondrial membrane. (209).

An interesting cardiological application was suggested by Hamsten et al. (210), who found that raised ACAs were associated with a high risk of recurrent cardiovascular events in post-myocardial infarction patients. ACAs were also shown in post-myocardial infarction patients in another small study which also demonstrated the increased presence of anti-cephalin antibodies (211). However, no influence of raised ACAs soon after myocardial infarct on immediate patient outcome was found (212). Further studies are required to reconcile these conflicting findings, which may be due to different selected populations and different lengths of study.
4.1.2 Existing Non-Liposomal Assays for Antiphospholipid Antibodies and Anticardiolipin Antibodies

LA interferes with the conversion of prothrombin to thrombin and the activation of the clotting process, by binding to the phospholipid surface of the prothrombin activator complex (213). The presence of LA is normally established by haematological tests such as the prolonged activated-partial thromboplastin time or by the Russell viper venom test. Serological tests for syphilis may also demonstrate APAs but are not as sensitive as the haematological tests. There are problems in standardizing assays for LA, which makes interpretation and comparison of clinical studies difficult, although there have been attempts to improve this (214).

Radioimmunoassay was introduced in 1983 to measure ACAs (215). Since then ELISA (enzyme-linked immunosorbent assay) has become popular. ELISA, which is based on cardiolipin-coated microtiter plates (216), has enabled analysis of large numbers of sera and elucidation of many clinical associations such as the association of ACAs with recurrent foetal loss.

Considerable international efforts have been undertaken to compare the performance of centres carrying out ELISAs.
and try to improve inter-laboratory agreement. Harris (217,218) proposed a means of quantitating ACAs by comparison to reference materials. Units were defined as the GPL unit (for IgG ACAs) and the MPL unit (for IgM ACAs). The GPL (or MPL) is the unit of activity of a standardized affinity-purified serum from a patient with high IgG ACAs alone (or IgM ACAs alone). One unit is equivalent to the binding activity of 1 mg/l of purified ACA antibody.

Following the distribution of reference standards and controls, a degree of agreement was demonstrated, although results were expressed only semi-quantitatively (i.e. negative, low-positive, medium-positive or high-positive) and not directly in terms of concentration units (217,218). For convenience, high positive was defined as greater than 80 GPL or 50 MPL, medium-positive as 15-80 GPL or 6-50 MPL and low positive as below 15 GPL or below 6 MPL. There is confusion over the definition of negative results since there is no consensus over the cut-off values. Most studies refer to negative as meaning not increased, but this can be taken as above 2, 3, or even 5 standard deviations from the mean of normal samples. This lack of consensus means publications have to be compared carefully and probably contributes to
marked variations in reported incidence of ACAs in various conditions.

ELISA is currently the most popular technique for measuring ACAs; but there are problems affecting the performance of the test which affect agreement of assays and their comparability in clinical studies. Non-specific binding to the microtiter plates can cause problems. This is dependent on the blocking agent used in the buffer (218-220). Cheng et al (221) showed that heat-treating samples for 30 minutes at 56°C caused increased binding in the ELISA assay. This resulted in some samples previously negative for ACAs becoming positive, which was postulated to be due to deactivation of heat-labile inhibitors.

There has been considerable debate about the role of the co-factor, B₂ Glycoprotein I (B₂-GPI) (also known as Apolipoprotein H), since its role in binding ACAs was first suggested by Galli et al (222). Galli’s claim that B₂-GPI was the agent to which ACAs bind, rather than cardiolipin, sparked controversy. Their findings were apparently supported by the detection of APAs using an ELISA with B₂-GPI as antigen (223), but may have been due to trace amounts of phospholipid contaminants in B₂-GPI preparations (224-227). It now appears that B₂-GPI is
indeed required for ACA binding to cardiolipin, but that the antigen is a complex of cardiolipin and B$_2$-GPI (187).

Since ELISAs often employ large pre-dilutions of serum, low B$_2$-GPI concentrations in the reaction mixture could cause variability and underestimation of ACA concentrations. The addition of high concentrations of bovine serum (10%), in which the co-factor is also found, (228), has been employed to reduce the effect of low B$_2$-GPI on ACA detection in ELISAs. However, significant non-specific binding of normal IgG can occur which can lead to inappropriate high results unless the non-specific binding is removed by using control wells (229).

As well as the problems associated with co-factors and non-specific binding, there are a few other practical disadvantages with ACA measurement by ELISA. Large numbers of samples can be easily processed only if sample and reagent dispensing, well-washing and measurements are automated. Each batch is usually restricted to the use of 96 well plates, which either requires batching of samples or is wasteful on plates and possibly reagents. The introduction of eight-well strips has recently allowed smaller and more variable batch sizes.
The practical problems in automating ELISAs, together with a typical assay time of several hours, mean that ELISA measurement of ACAs is difficult to justify in a "district-general" hospital, which may only require a few tests a week. These few samples will usually be referred to a specialist centre, delaying reporting times often to a week or more. At the other extreme, large population studies to elucidate the clinical value of the tests are only feasible in very large centres using automated ELISAs. For these reasons, ACA measurements by ELISA are unlikely to move from specialist centres to other hospitals, let alone to "near-patient" testing in the out-patient or in the general-practice clinic.

4.1.3 Liposomal Immunoassays for Anticardiolipin Antibodies

Flow-cytometry, in which cardiolipin liposomes show an altered light scatter on binding ACAs, has recently been described (130). However, this requires equipment which is not universally available. There are two other reports of the use of liposomes which might form the basis of an alternative immunoassay technique to ELISA. Takashi et al., (142) demonstrated liposomal lysis of cardiolipin-containing liposomes by complement and anti-
cardiolipin antibodies, using glucose release as a marker. However their studies did not extend to potential analytical uses of this phenomenon.

Janoff et al. (108), however, did report a liposome-based test for Systemic Lupus Erythematosus (SLE) antibodies. (ACAs are often a major component of SLE antibodies. The relationships of ACAs and other lupus antibodies, e.g. anti-nuclear antibodies and anti double-strand DNA, were still being clarified at the time of publication.)

The assay was based on destabilization of cardiolipin-containing liposome membranes by divalent cations. This phenomenon was reported by Rand and Sengupta in 1972 (58). Divalent cations cause a phase change of the cardiolipin from bilayer to a hexagonal (II) structure which tends to release the liposomes' contents and may cause precipitation.

Large unilamellar liposomes containing the dye, Arsenazo III, were prepared by the ether-infusion method of Deamer and Bangham (27). Liposomes (200 ul) and serum (20 ul) were placed in the wells of a microtitre plate. After mixing and incubating for an hour, MgCl₂ (10 ul) was added and the mixture left for five to ten minutes. In the presence of non-SLE serum the mixture changed colour.
This was due to the breakdown of the liposomes and release of the dye, which changed from red to blue on complexing with Mg$^{2+}$ ions. Using SLE serum no colour change was observed, presumably because binding of serum antibodies to the liposomes protected them against lysis.

The assay required careful optimization of the liposome concentration and magnesium ion concentration to give the optimal colour change between SLE and non-SLE serum. It was claimed to produce quantitative results by spectrophotometric measurement of the absorbances of the wells at 610 nm. However, the value of this was limited since there was a lack of characterization or quantitation of the antibody present in the SLE serum used as a standard.

4.1.4 Objectives of Anticardiolipin Studies

The aims of these studies were to examine the possibilities for measuring anticardiolipin antibodies using Sulphorhodamine B-containing liposomes; firstly by the use of complement-mediated lysis and alternatively using Mg$^{2+}$ ion-dependent lysis.
4.2 Complement-Mediated Immunolysis of Cardiolipin-Containing Liposomes

Dye-containing liposomes were prepared as described in section 2.9.8, with an incorporation of 30% cardiolipin. They were used to demonstrate complement-mediated immunolysis on incubation with anticardiolipin antibodies and complement.

A Cobas Bio was employed in a manner similar to that for albumin (chapter 3), except patients’ sera samples, including a sample known to contain increased concentrations of anticardiolipin antibodies, were added in place of antialbumin antiserum (table 4.1).

When buffer A alone was added as sample instead of serum, a rapid rate of lysis was observed, even though anticardiolipin antibody was not present (figure 4.1). When complement was replaced by buffer, however, this reaction was not found. This indicated that a non-immunospecific reaction was occurring between complement and the cardiolipin-containing liposomes.
TABLE 4.1

Reaction parameters used on Cobas Bio Analyser to Monitor Complement-Mediated Immune Lysis

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (reagent 1)</td>
<td>Liposomes diluted to initial absorbance of 1A (approx. 5ml buffer A plus 40ul liposomes (100 ul/cuvette))</td>
</tr>
<tr>
<td>sample</td>
<td>Serum (4ul/cuvette)</td>
</tr>
<tr>
<td>diluent</td>
<td>Distilled water (10 ul/cuvette)</td>
</tr>
<tr>
<td>R2 (reagent 2)</td>
<td>Complement diluted X2 in distilled water (4 ul/cuvette)</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Printout type</td>
<td>3 (absorbance measurements)</td>
</tr>
</tbody>
</table>

Assay process: R1, sample and diluent dispensed into cuvettes and mixed. 5 minute pre-incubation. R2 added and readings taken at 10 second intervals over 180 seconds (first reading after 3 seconds).

A similar non-specific lysis occurred whether guinea-pig complement or rabbit complement was used, except the rate was much lower for guinea-pig complement (approximately 20%). The lysis was absent when either complement was heat-inactivated (as section 3.4.1). The non-specific lysis was found whether or not the liposome preparation included PTA. This non-specific lysis, which was confirmed using a spectrophotometer (as described in section 3.4.2), was found to give an approximately linear increase in absorption at 565 nm over at least twenty minutes and to plateau after about an hour. The
Effect of Complement on Dye-Release from Cardiolipin-Containing Liposomes in Absence of Anticardiolipin Antibodies

Non-specific complement-mediated release of dye from cardiolipin-containing liposomes. The reaction conditions are stated in Table 4.1. Symbols indicate: o rabbit complement, ● guinea-pig complement.
relatively linear absorbance change differed from the rapid but slowing initial absorbance change typical of the specific immunolysis for albumin (for example, figure 3.11).

In an attempt to reduce the non-specific lysis, small unilamellar liposomes were prepared as described by Schreier et al. (230). 100 umol of PC were sonicated in 5 ml of distilled water for twenty minutes in an iced water bath. Equal volumes of this preparation and complement (guinea-pig or rabbit) were incubated for 30 minutes at 37 °C. The complement was then used in the reaction as before. Unfortunately no reduction in non-specific lysis could be demonstrated by this technique.

The addition of either normal sheep serum (1%) or normal goat serum (1%) to the buffer markedly reduced the non-specific lysis, but also reduced the lysis in the presence of human sera. Using sheep serum, the effect of human sera samples on rabbit complement-mediated lysis was examined.

Two human sera samples were analysed, a pool of samples taken from patients with no evidence of increased ACA concentrations or immunological disease (normal pool) and a sample of known high ACA concentration. The lysis was
found to be related to the sample dilution. Even though the absorbance changes for both samples were low in the presence of normal sheep serum, consistent differences in lysis rates between the samples were apparent. A dilution of human serum samples of 1:50 seemed to give the largest difference in absorbance between the two samples (figure 4.2).

Using this dilution of serum, the effect of varying the amount of complement was examined. The buffer content of reagent 1 was adjusted to accommodate larger complement volumes. As in the case of complement-mediated lysis of albumin or Fab' anti-albumin coated liposomes, (figure 3.5), a "hook" effect occurred. Figure 4.3 shows that at complement concentrations up to and including the optimal concentration, the high ACA serum gave a higher rate of absorbance change, while at higher complement concentrations the high ACA serum gave a lower lysis rate.

Under these conditions guinea-pig complement also gave a peak lysis at 2 ul/cuvette, although the lysis rate was much lower compared to rabbit complement (approximately 6 mA). No reproducible difference between the ACA positive sample and the pool could be detected at this complement dilution.
Human serum was diluted in buffer A and measured in the sample position of the Cobas Bio analyser, using the conditions specified in Table 4.1. Serum was either a normal serum pool (o) or an anticardiolipin antibody positive serum (●), concentration 1100 GPL units. Overlaid points represent duplicate measurements.
The reaction conditions are as indicated in section 4.2; the amount of complement added to each cuvette of the Cobas Bio being varied. The resultant dye release was measured, in the presence of cardiolipin-containing liposomes, and either anticardiolipin antibody-containing serum (○), or a normal serum pool (●). Overlaid points represent duplicate measurements.
The effect of mixing the ACA positive serum (1100 GPL units) with the normal pool at the optimal for sample dilution (1/50) and rabbit complement (2ul/cuvette) is shown in figure 4.4. A dependence of lysis on the amount of ACA was demonstrated, although the absorbance change was insufficient for a practical assay. The concentrations of ACAs in figure 4.4 were calculated from the assayed value of the ACA serum (see materials), and assumed an absence of ACAs in the normal pool. (This was therefore probably an underestimate since a low concentration of antibodies in the pool was likely).

When liposomes which contained 2.5% cardiolipin, (similar to the cardiolipin content used by Takashi et al (142)) were used, no detectable absorbance change was found, irrespective of whether guinea-pig or rabbit complement was added.
The reaction conditions are as indicated in Table 4.1. The pre-diluted anticardiolipin antibody concentrations of the samples is indicated; obtained by mixing anticardiolipin reference serum (1100 GPL units) with a normal serum pool. The mixed samples were pre-diluted x50 in Buffer A prior to assay.
4.3 \textbf{MgCl}_2-Dependent Lysis of Cardiolipin-Containing Liposomes

The MgCl$_2$-dependent lysis of liposomes was examined as an alternative potential assay to the use of complement.

4.3.1 MgCl$_2$-Induced Lysis Using Manual Spectrophotometer and Fluorimeter

When a solution of liposomes containing 20\% cardiolipin at a dilution giving an absorbance of 1.0 A at 565 nm was mixed with an aqueous solution of MgCl$_2$ at a final concentration of 0.4 mol/l, very rapid lysis occurred with an increase in absorbance or fluorescence of about 15\% within five minutes.
4.3.2 Application of MgCl₂-Dependent Lysis to the Cobas Bio Analyser

The destabilization of the liposomes (as described in section 4.3.1) was investigated using a Cobas Bio analyser. The assay parameters used on the Cobas Bio are shown in table 4.2, modified as appropriate to assess and optimize variables.

| TABLE 4.2 |
| Reaction parameters used on Cobas Bio Analyser To Assess MgCl₂ Destabilization |

R1 (reagent 1): 5ml phosphate buffer plus 40 ul liposomes 100 ul/cuvette
sample: serum 5ul/cuvette
diluent (distilled water): 10ul/cuvette
R2 (reagent 2): 5 mol/l MgCl₂ 10ul/cuvette
Temperature: 25°C
Printout Type: 3 (absorbance measurements)
Wavelength: 565 nm

Assay Process: R1 and sample (+ diluent) dispensed and mixed. 3 minute preincubation. R2 added and readings taken at 20 second intervals over 120 seconds (first reading after 3 seconds)
The ACA positive sample, diluted in normal pool to 80 GPL, gave a higher rate of dye release than the normal pool. When the absorbance was monitored over approximately ten minutes, the difference in rate of dye release between the two samples was found to be greater over the first few minutes (figure 4.5).

Consistent with earlier results involving dye release from liposome lysis (sections 2.8 and 3.4.3), a negative absorbance change at 530 nm was observed. The absorbance change was, as before, greater at 565 nm and this wavelength was used subsequently. The absorbance changes corresponded to those found when reagents in the same ratios were monitored using either a manual spectrophotometer or fluorimeter.

As mentioned in section 4.2, although the normal pool had been prepared from patients without known immunological disorders, a certain amount of antibody might still have been present. This could cause errors if the pool were used as a diluent in preparation of an analytical standard curve. To ensure that the pool had as low antibody concentration as possible, 100 samples from patients without evidence of immunological disorders were collected. The 20 with the lowest lysis by MgCl₂, assessed on the Cobas Bio, were pooled and used.
The assay conditions were as described in Table 4.2. Absorbance changes were monitored using a Cobas Bio analyser. The sample was either a normal serum pool (●) or an anticardiolipin antibody positive serum (○), concentration 80 GPL units. The reaction time excludes an automatic initial mixing time of three seconds.
subsequently as a "negative pool" and as a diluent for the ACA positive serum.

Using dilutions of the reference serum in this new normal pool, the amount of dye release was found to be directly related to the concentration of anticardiolipin antibodies present, as shown in figure 4.6.

This also demonstrates the effect of varying the amount of liposomes, which is indicated by the initial absorbance of the cuvette. The rate of absorbance change increased as the amount of liposomes increased. Although the Cobas Bio can measure higher absorbances than most other analysers because of its ability to use a short path length a maximum initial absorbance of approximately 1A was used subsequently (for reasons discussed in section 3.4.3).

The effect of ACA concentrations on lysis are also shown in figure 4.7. This shows results of figure 4.6 for an initial absorbance of 0.8, which are replotted using a logarithmic scale and include much higher ACA concentrations. The figure indicates the effects of reaction temperatures. Higher absorbance changes were observed at higher temperatures, but there was also greater lysis by the normal pool. As there seemed little
The reaction conditions were as described in Table 4.2, except that the amount of liposomes in the first reagent (R1) was varied; giving the following initial absorbances: $o = 0.4A$, $\bullet = 0.8A$, $\square = 1.6A$. Overlaid points represent duplicate measurements.
Figure 4.7

Effect of Reaction Temperature on Lysis of Cardiolipin-Containing Liposomes by Magnesium Chloride and Anticardiolipin Antibodies

The reaction conditions are described in Table 4.2; except that the reaction temperature was varied as follows: ● = 25°C, □ = 30°C, ○ = 37°C. The points labelled as (x) indicate the absorbance changes in the absence of anticardiolipin antibodies; (absorbance changes increasing with temperature). Overlaid points represent duplicates, with the exception of points labelled * which are singleton measurements.
advantage in using higher temperatures, 25°C was used in subsequent experiments. Figure 4.7 also shows a hook effect at very high ACA concentrations.

The rate of liposomal lysis was shown to be dependent on the amount of cardiolipin within the liposomes (figure 4.8). Although human sera rarely contain IgG anticardiolipin concentrations above 100 GPL units, occasionally concentrations up to 1000 GPL units can be found. Liposomes containing lower amounts of cardiolipin exhibited a "hook effect" at higher anticardiolipin concentrations, but this was absent at up to 1000 GPL units when liposomes containing 40% cardiolipin were used. When liposomes which did not contain cardiolipin were used (PC-Chol 10-2 or PC-Chol-PTA 10-2-1) the lysis rates were very low and not dependent on the amount of anticardiolipin antibody present.

Because of the absence of a "hook" and the fact that much greater absorbance changes were observed, liposomes containing 40% cardiolipin were utilized in further experiments.

No detectable release of dye was observed when liposomes containing 40% cardiolipin were mixed with varying
Effect of Liposomal Lipid Composition on their Lysis Due to Magnesium Chloride and Anticardiolipin Antibodies

The reaction conditions were as described in Table 4.2. In separate experiments, various liposome preparations were used. The symbols refer to the following lipid contents: o = 40% cardiolipin, • = 30% cardiolipin, - = 20% cardiolipin, Δ = 2.5% cardiolipin, □ = PC/PTA/Chol (molar ratio 10-2-1), ■ = PC/Chol (molar ratio 10-2). In the absence of added anticardiolipin antibodies; the absorbance changes were below 0.010A in all cases except 40% cardiolipin, which had a mean absorbance change of 0.018A. Overlaid points represent duplicate measurements, except for points labelled *, which are singletons.
concentrations of anticardiolipin antibody (as figure 4.8) if distilled water was used as the second reagent in place of MgCl₂. In the absence of serum, increasing reaction concentrations of MgCl₂ up to 0.4 mol/l increased the rate of lysis (figure 4.9). No insolubility was observed at this concentration which was adopted subsequently in favour of even higher concentrations, to avoid potential insolubility problems in the latter.

The effect of varying the volume of sera added to the reaction cuvette is shown in figure 4.10. Serum volumes were obtained by setting the sample volume to 20 ul and assaying serial dilutions of sera in buffer. In the absence of normal serum, liposomes were lysed very rapidly by MgCl₂. Normal serum, at sufficiently high concentrations, protected the liposomes almost completely from this lysis. Anticardiolipin antibodies effectively reduced the protective effect of serum, over a limited range of serum concentrations. Undiluted serum, added at 5ul per cuvette, was found to be close to the optimum for differentiating ACA containing serum from ACA free serum.

The effects of adding, in place of serum, purified human albumin and human gamma globulin were also examined (figure 4.11). A protective effect similar to serum, though not as complete, was seen at high albumin
Effect of Varying Magnesium Chloride Concentration on the Lysis of Cardiolipin-Containing Liposomes in the Absence of Serum

The reaction conditions were as described in Table 4.2, except that the concentration of magnesium chloride in the second reagent (R2) was varied to produce the indicated final reaction concentrations.
The reaction conditions are stated in Table 4.2, except that the serum volume added to each cuvette was varied (as described in section 4.3.2). The serum either contained anticardiolipin antibodies (80 GPL units) (○), or was a normal serum pool (●). Overlaid points represent duplicate measurements.
The reaction conditions are stated in Table 4.2, except that human albumin (o) or human gamma globulin (●), each diluted in buffer, was used as sample. The protein content indicated is the concentration in the sample. (The sample volume was 5ul and the total cuvette volume was 125 ul).
concentrations while lysis was less dependent on gamma globulin concentrations.

To assess the effect of Ca\(^{2+}\) ions at near physiological concentrations, CaCl\(_2\) at concentrations equivalent to a sample concentration of 1.0 to 3.0 mmol/l were added in place of MgCl\(_2\). No detectable lysis occurred. A reaction concentration of CaCl\(_2\) of 0.4 mol/l, (equivalent to the concentration of MgCl\(_2\) used above) caused a rapid precipitation of liposomes. A certain amount of dye release was associated with this precipitation. This was found to be variable. In experiments analogous to that of figure 4.10, no anticardiolipin antibody dependence of this lysis was detectable.

4.4 **Estimation of Anticardiolipin Antibodies by Enzyme-Linked Immunosorbent Assays (ELISAs)**

Both normal sera and anticardiolipin antibody positive sera were estimated using both the Cobas method (section 4.3.2 and table 4.2) and by commercial ELISAs (Cambridge Life Sciences "SELISA" for IgG and for IgM anticardiolipin antibodies). The latter were performed according to the manufacturer's instructions.
These ELISA kits were conventional in format. Prediluted standards and samples (100ul, 1 in 100) were added to cardiolipin-coated wells in plastic microtitre plates. After incubation for 30 minutes the plates' contents were decanted, and the plates washed and blotted. Antibody conjugated to enzyme (horse radish peroxidase - 100 ul) was then added to the plates, followed by incubation for 30 minutes, decanting, washing and blotting. Enzyme substrate (100 ul) was added and a colour developed for 10 minutes which was read, after stopping the reaction with sulphuric acid (4M, 50ul), in a microtitre plate reader.

Typical standard curves obtained using the kits are shown in figure 4.12.

4.5 Comparison of Patients' Cardiolipin Antibody Concentrations by ELISA and by MgCl₂-Dependent Lysis

Thirty five samples, on which analysis of anticardiolipin antibodies had been requested, were collected from several hospitals. They were analysed using the Cobas MgCl₂ procedure and by ELISAs for both IgG and IgM anticardiolipin antibodies.
Figure 4.12

Standard Curves for Measurement of Anticardiolipin Antibodies by ELISA Technique

The reaction conditions have been described in section 4.4. Symbols refer to: • = IgG ACAs (GPL units); □ = IgM ACAs (MPL units). Overlaid points represent duplicate measurements.
The relationship between results using the liposomal and the ELISA procedures for IgG ACAs is shown in figure 4.13. The regression equation obtained was:

\[ y \text{ (Liposomal ACAs)} = -1.78 + 1.02 \times \text{ (ELISA IgG ACAs)} \]

The correlation coefficient \( r \) was 0.90.

The examination of any relationship between the Cobas results and the ELISA IgM results was complicated by the lack of ready availability of an IgM reference serum. Furthermore, only four of the thirty-five samples analysed by ELISA gave IgM ACA concentrations above the manufacturer's quoted normal range (0.4 to 5 MPL units).

There was no significant correlation between the Cobas responses (i.e. changes in absorbance) and the ELISA IgM ACA concentrations.

The correlation coefficient \( r \) was 0.20 (probability of null hypothesis \( p \) = 0.27).
Human sera from 35 patients were measured by the liposomal assay, involving magnesium chloride lysis of cardiolipin-containing liposomes, and by enzyme-linked immunosorbent assay (ELISA).
4.6 Establishment of Normal Ranges for IgG ACAs

The manufacturer of the ELISA kits recommended that each user should establish their own normal (reference) range. They quoted a range of 1 to 9 GPL units based on normal blood donors although the mathematical method used to calculate the manufacturer's range was not stated. A local reference range was established using 20 samples of citrated plasma from normal blood donors and also 20 sera samples from ante-natal clinic attenders without evidence of immunological illness. The samples were analyzed using both IgG ACA ELISA and by the Cobas method. Both techniques gave similar arithmetic mean values (table 4.3) and were positively skewed (figure 4.14). A possible bimodal distribution of values was observed which would have contributed to the skew, although the number of samples was not large enough to confirm this.

Logarithmic and square root transformations of the data were compared and the square root transformation proved a better means to normalize the results. The normalized means and standard deviations using this transformation are shown in table 4.4. There was some variation between the normalized mean values for the two types of samples and between these and the combined value. These were not significant using 2 sample t-tests (table 4.5); except
Samples from normal blood donors and from ante-natal clinic attenders were analysed for IgG anticardiolipin antibodies by both the liposomal immunoassay and by ELISA. The distribution of results is shown. The black bars refer to results by the liposomal assay and the white bars refer to results by ELISA. Results are expressed in GPL units, to the nearest whole number.
### TABLE 4.3

Arithmetic Means and Standard Deviations for Normal Samples

<table>
<thead>
<tr>
<th></th>
<th>ELISA IgG ACA (GPL units)</th>
<th>Cobas IgG ACA (GPL units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood Donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>10.9</td>
<td>9.5</td>
</tr>
<tr>
<td>standard deviation (sd)</td>
<td>5.9</td>
<td>5.7</td>
</tr>
<tr>
<td><strong>Ante-Natal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>7.8</td>
<td>7.0</td>
</tr>
<tr>
<td>sd</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>9.4</td>
<td>8.5</td>
</tr>
<tr>
<td>sd</td>
<td>5.2</td>
<td>4.9</td>
</tr>
</tbody>
</table>
for the difference between the ELISA results for ante-
natal clinic samples and blood donor samples which was 
weakly significant (p < 0.05).

The reference ranges calculated after square root 
normalization and also by calculating the 95 percentiles 
are shown in table 4.6. The arithmetic "normal range", 
although statistically invalid due to the skewed results, 
is shown for comparison.

The normal ranges derived from both the ELISA and 
Liposomal methods were found to be divergent from the 
ELISA manufacturer's quoted normal range. The 
latter, therefore, seemed inappropriate to the normal 
samples assayed. The liposomal and ELISA reference 
ranges were similar and an upper limit of normal for the 
liposomal method of 20 GPL units was established based 
on the normalized results from both groups of samples 
combined.
### TABLE 4.4
Normalized Values for Mean and Standard Deviation

<table>
<thead>
<tr>
<th></th>
<th>ELISA IgG ACA (GPL units)</th>
<th>Cobas IgG (GPL units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood Donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean*</td>
<td>10.2</td>
<td>8.4</td>
</tr>
<tr>
<td>sd of roots**</td>
<td>0.82</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Ante-Natal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean*</td>
<td>7.3</td>
<td>6.6</td>
</tr>
<tr>
<td>sd of roots**</td>
<td>0.68</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean*</td>
<td>8.8</td>
<td>7.5</td>
</tr>
<tr>
<td>sd of roots**</td>
<td>0.78</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* mean is defined as the square of the mean of the square roots

** "sd of roots" is the sd of the square roots

### TABLE 4.5
Analysis of Significance of Differences in Means (from table 4.4)

<table>
<thead>
<tr>
<th>Groups Compared</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA v. Cobas (overall)</td>
<td>0.26</td>
</tr>
<tr>
<td>ELISA donors v. Cobas donors</td>
<td>0.62</td>
</tr>
<tr>
<td>ELISA ante-natal v. Cobas ante-natals</td>
<td>0.51</td>
</tr>
<tr>
<td>ELISA donors v. ELISA ante-nataals</td>
<td>0.047</td>
</tr>
<tr>
<td>Cobas donors v. Cobas ante-nataals</td>
<td>0.14</td>
</tr>
</tbody>
</table>
4.7 Comparison of Imprecisions of the Liposomal and ELISA Methods

Pools of patient samples were prepared at various ACA concentrations to compare imprecisions. Since the ELISA normally analyses tests in duplicate the samples were assayed on the Cobas in the same way. The Cobas performs tests using rotors with a maximum capacity of 24 tests per sample tray. The ELISA imprecision was calculated from results obtained from a single microtitre plate.
Samples could be analysed on the Cobas either in a similar way to the ELISA method, i.e. with standards in the same rotor, or using a standard curve in an earlier rotor provided there was little drift in results from rotor to rotor. To assess both these options, imprecisions were calculated within a single rotor and also over three rotors. The results are shown in figure 4.15. The imprecisions were similar, except that over three rotors the Cobas performed considerably worse at low antibody concentrations.

4.8 Calculation of Limits of Detections of IgG Anticardiolipin Antibodies

An estimate of the limits of detections of the methods can be obtained from these data in two ways. The limits of detections could be taken as the concentrations at which coefficients of variations (C.V.s) of 10% were found. These values were obtained from the curves shown in figure 4.15. Alternatively, if the limits of detections were defined as equal to three standard deviations of a value close to zero, these could be calculated from the imprecisions of the lowest pool
Imprecision in IgG anticardiolipin antibody measurement was assessed as described in section 4.7. Symbols refer to: o = ELISA results, duplicates within run (n=16); □ = liposomal immunoassay results, duplicates within run (n=12); Δ = liposomal immunoassay, duplicates over three rotors (n=36).
(approximately 5 GPL U/l). The limits of detections using both approaches (rounded up) are shown in table 4.7.

**TABLE 4.7**

Limits of Detection of Anticardiolipin Assays (GPL U/l)

<table>
<thead>
<tr>
<th>Method</th>
<th>10% CV</th>
<th>3sds*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Liposomal within rotor</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Liposomal over 3 rotors</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

*3sds refers to 3 standard deviations of a low sample

4.9 **Confidence Limits of Anticardiolipin Assays**

The confidence limits of the results obtained using the various procedures were calculated, at various concentrations of antibodies, as (+/- 2 sds) using the imprecision curves of figure 4.15. These confidence limits (rounded up) are shown in table 4.8.
### TABLE 4.8

Confidence Limits of Anticardiolipin Assay Results (GPL units)

<table>
<thead>
<tr>
<th>conc.</th>
<th>ELISA</th>
<th>Liposomal (within run)</th>
<th>Liposomal (over 3 runs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+/- 2</td>
<td>+/- 2</td>
<td>+/- 3</td>
</tr>
<tr>
<td>10</td>
<td>+/- 2</td>
<td>+/- 2</td>
<td>+/- 2</td>
</tr>
<tr>
<td>20</td>
<td>+/- 3</td>
<td>+/- 3</td>
<td>+/- 3</td>
</tr>
<tr>
<td>25</td>
<td>+/- 4</td>
<td>+/- 3</td>
<td>+/- 4</td>
</tr>
</tbody>
</table>

#### 4.10 Discussion

A complement-mediated assay and a Mg\(^{2+}\) ion-dependent assay for ACAs were examined. Both showed a degree of immunospecific lysis although much greater dose-response was obtained in the Mg\(^{2+}\) ion-dependent assay. This was developed to show an acceptable correlation with a conventional ELISA assay for IgG ACAs and similar imprecision.

There was only a small amount of lysis of the PTA-containing liposomes in the absence of anticardiolipin
antibody, and no antibody-dependent change in lysis-rate was found. Like cardiolipin, PTA is a negatively charged lipid. The lack of lysis of PTA-containing liposomes accords with the view that the MgCl₂-dependent lysis is specific for cardiolipin, effected by a phase change from lamellar to hexagonal (II) phase (58).

Although dependence on serum protein, mainly albumin, was observed (figure 4.11); albumin concentrations below about 20 g/l in serum are uncommon. Variations in albumin in patients sera were not found to be a likely major source of analytical interference.

The concentration of Mg²⁺ ions in the reaction was far higher than physiological concentrations (normal range approximately 0.7 to 1.0 mmol/l) and it can be deduced from figure 4.9 that variations in Mg²⁺ ions in patients' sera would not affect the degree of lysis significantly.

The liposomal ACA assay was configured as a two step assay (table 4.2). This provided a check of any liposomal lysis by human complement, which would have resulted in a high absorbance after the pre-incubation relative to the other cuvettes. No such anomalous absorbance changes were noted.
The liposomal assay has many potential advantages over ELISA (as have been described in section 4.1.2). One potential disadvantage compared to ELISA, however, is that the sample dilution is much lower. This means that more standard material is likely to be consumed. However this is mitigated by the small sample volume used on the Cobas Bio.

On the other hand; the use of a greater amount of serum in the assay might have a beneficial effect compared to ELISA, since the concentration of B₂-glycoprotein I is also much greater. It seems probable that adequate B₂-GPI was present in the liposomal immunoassay. The addition of animal serum is necessary in ELISAs which involve large pre-dilutions of serum (as discussed in section 4.1.2). The presence of adequate B₂-GPI is suggested by the reasonable correlation with the commercial ELISA method, which used 10% bovine serum as diluent.

The variation in B₂-GPI concentrations in normal serum has been found to be between 74 and 285 mg/l (mean 179.8 ± 48.7 (s.d.)), and no significant difference was found between ACA positive and ACA negative sera (227).
range of $B_2$-GPI is therefore quite narrow in normal samples. Although possible pathological causes of variation in $B_2$-GPI concentrations may remain to be elucidated, there is little evidence that the liposomal assay would be likely to suffer from the occasional sample which had a gross deficiency or excess in $B_2$-GPI.

There are several potential interferences, other than $B_2$-GPI concentrations, which can affect ELISA assays for ACAs (as discussed in section 4.1). In addition, aspects of the liposomal technique differ markedly from ELISA. For example, the presentation of cardiolipin in the lipid membrane might have a different configuration and orientation to that of cardiolipin coated directly onto a micro-titre plate. Despite these differences, a satisfactory agreement between IgG ELISA and the liposomal assay ($r = 0.90$) was obtained. A similar correlation ($r = 0.91$) was quoted by the manufacturer of the ELISA used in the comparison; when two IgG ACA ELISA methods were compared (Manufacturer’s Technical Leaflet).

On the face of it, the augmentation of liposomal lysis by ACAs is contradictory to the report of Janoff et al. (108). Their assay, described in section 4.1.3, gave inhibition of liposomal lysis by SLE serum. However, both the preparation of liposomes and the reaction
conditions used by Janoff differed. Some of these differences are compared in table 4.9. As the procedures varied so markedly, it is perhaps not surprising that different effects were observed.

**TABLE 4.9**

Comparison of Mg\textsuperscript{2+} Ion-Dependent Assay Conditions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Janoff et al.</th>
<th>This Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>ether infusion</td>
<td>reverse evaporation</td>
<td></td>
</tr>
<tr>
<td>approx size</td>
<td>not stated</td>
<td>200 nm (filtered)</td>
</tr>
<tr>
<td>serum dilution</td>
<td>1:9</td>
<td>1:25 (table 4.2)</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+} conc.</td>
<td>140 mmol/l (approx)</td>
<td>400 mmol/l</td>
</tr>
<tr>
<td>reaction period</td>
<td>5 - 10 minutes</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>

Unfortunately it is not possible to estimate the relative amounts of liposomes used in the techniques, since the liposome dilution of Janoff was not indicated. Nevertheless, a tentative explanation for the apparently different effects of antibody can be put forward based on the relative amounts of reaction components; in particular the amounts of serum and liposomes. If the
amount of serum used by Janoff was much higher relative to the amount of liposomes, the antibody found in normal sera (section 4.6) might be sufficient to augment lysis. The SLE serum, under the conditions used by Janoff, might on the other hand contain sufficient antiserum to inhibit lysis by the "hook-effect" found in this study (figure 4.8).

At sufficient concentration, normal serum inhibited ion-dependent lysis (figure 4.10), an effect that was more dependent on albumin concentration than globulin concentration. This is likely to be due to a non-specific adsorption of protein onto the liposome surface, probably due to electrostatic interaction. The serum protein probably has this effect by covering the liposome surface, impeding the access of Mg (II) ions. At lower serum concentrations the non-specific effect of serum proteins of inhibiting lysis is largely lost and it is at these serum concentrations that ACAs increase lysis.

The effects of divalent cations were elucidated by Rand and Sengupta (58). The cations neutralize the negative charges on the cardiolipin and enable the head-groups to come together, causing a phase change from bilayer to hexagonal (II) phase. The differential effects of Ca (II) ions and Mg (II) ions, observed experimentally (section
4.3) are compatible with Rand and Sengutpa's findings. Ca (II) ions are more able to form hexagonal structures which tend to precipitate. Mg (II) ions tend to form more hydrated structures enclosing larger water volumes and with a larger area per polar head group at the water interface. Cardiolipin is present with other phospholipids in these preparations, and a mixture of lamellar and hexagonal (II) phases probably will have resulted. While no precipitation was observed in these studies, the integrity of the liposomes was compromised sufficiently to allow dye release.

The mechanism of the enhancement of lysis by ACAs is harder to explain. As no significant release of dye was observed in the presence of ACAs but without the addition of Mg (II) ions, (section 4.3.2) the antibody must augment Mg (II) ion-dependent lysis, rather than acting independently.

There are several possible mechanisms for augmentation of ion-dependent lysis. ACAs could perhaps bind specifically to cardiolipin molecules and draw several molecules together, acting as a focus for Mg (II) ion-dependent phase change. This would, however, seem unlikely since IgG antibodies are divalent, each molecule having only two Fab' binding sites. Therefore each
antibody could only bind to, at most, two cardiolipin molecules.

Alternatively ACAs could act by drawing liposomes together. This could perhaps occur by a certain amount of antibody binding non-specifically to a liposomes surface and binding specifically to cardiolipin on an adjacent liposome. Under the influence of Mg (II) ions a hexagonal (II) phase could form, possibly entrapping the antibody. The hexagonal (II) structures can be large enough to include protein molecules. Cardiolipin may contribute to pore formation in this way enabling protein movements across mitochondrial membranes (58).

The latter mechanism could explain an absence of IgM ACA activity, since the IgM molecules could be too large to draw the liposomes together closely enough, or too large for hexagonal (II) structures to form around them.

Another possibility is that the antibody, which at near neutral pH probably will have a net negative charge, attracts Mg (II) ions. On immune-reaction, ions could in effect be trapped in the vicinity of the liposome, creating a localized increase in ion concentration.
Each of these mechanisms could be reconciled with the observed "hook effect" at high ACA concentrations. Either ions could be sterically inhibited from approaching the liposomes, or cardiolipin could have restricted movement, preventing formation of cardiolipin-rich regions able to form hexagonal (II) phases.

The liposomal immunoassay correlates with IgG ACAs measured by ELISA, but there is an apparent lack of correlation with IgM ELISA measurements. It is fortuitous that the main clinical applications of ACAs are related to their thrombotic effects; in particular in recurrent miscarriages, neurological and cardiological associations. These effects are reflected by IgG ACAs more than by IgM ACAs. ELISA is likely to remain the main technique for APA measurement for some time, not least because of the extensive efforts invested to standardize the technique. Nevertheless, because of its simplicity, a liposomal technique could be valuable in rapid screening of appropriate clinical populations.
Chapter 5

Final Discussion

5.1 Summary of Investigations

These studies have examined several aspects of the role of liposomes in immunoassays. A principal aim was to develop a means of performing homogeneous liposomal immunoassays using a colorimetric marker. It was first necessary to find and evaluate a suitable chromogen and a means to generate an absorbance change on its release from liposomes.

Sulphorhodamine B was chosen as likely to fulfil the criteria of a suitable chromogen. It has a large absorbance coefficient, which is at a high wavelength (565nm for the free dye). This will tend to reduce interferences due to light scatter in biological fluids. It also has a high aqueous solubility. The only previous application of Sulphorhodamine B in liposomes appears to be as a marker in an heterogeneous coated-tube assay (48).
The alteration in absorption spectrum which resulted from entrapment of the dye within liposomes was unexpected; although it could be explained by dimer formation at high concentrations, as described in section 2.11. The absorbance change when the dye was released provided the mechanism to develop homogeneous, colorimetric immunoassays.

Another aim of these studies was to examine the use of liposomes in a range of immunoassay techniques and for a variety of analytes. This included proteins, for which there have been relatively few published reports (as described in section 1.6).

Using Sulphorhodamine B as a colorimetric marker, complement-mediated liposomal immunoassays were devised which could measure proteins in serum and urine. Albumin was chosen as a model analyte. Complement-mediated assays are the commonest approach to liposomal immunoassays, which suggests the use of Sulphorhodamine B as a marker could be generally applicable to a wide range of other analytes.

Using this technique, a competitive assay for the measurement of serum albumin was developed. This was followed by a sandwich-type liposomal immunoassay for
urinary microalbumin which made use of liposome-coated Fab' fragments of antialbumin antiserum. The urinary microalbumin assay demonstrated adequate analytical performance compared to an established method (RIA), as described in chapter 3.

Entrapped Sulphorhodamine B was also used as a novel marker to measure anticardiolipin antibodies. The assay was based on the specific effect of divalent cations to destabilize cardiolipin-containing liposomes, which was found to be augmented by ACAs under certain conditions (chapter 4). Comparable performance to an alternative technique (ELISA) was again demonstrated.

The final application in this study employed antibody-coated liposomes as a solid-phase; as shown by another model assay for urinary microalbumin. This was an exception to the other assays in that it was based on immunoprecipitation of liposomes. This application was demonstrated in a radioimmunoassay using liposomes as a solid-phase, and did not rely on dye-release (section 3.7).
5.2 Advantages and Disadvantages of the Use of Liposomes Containing Sulphorhodamine B

The possible advantages of the use of liposomes in immunoassays were described in the introduction (section 1.6). These may include speed of use, sensitivity and ease of automation.

The development of homogeneous colorimetric liposomal immunoassays might enable both chemical assays and immunoassays to be performed on a single spectrophotometric analyser. This would be likely to confer considerable organizational benefits, for example to a high throughput clinical biochemistry department.

It is possible to draw some specific conclusions regarding the advantages and disadvantages of Sulphorhodamine B as a marker in liposomal immunoassays, in comparison to other liposomal chromogen markers.

There have been a few alternative chromogens used as liposomal markers in immunoassays, which have been described in section 1.6. These employ a shift in absorbance due to released chromogen binding with various ions. The reliance on a separation of the ions external to the liposomes from the chromogens inside the liposomes
(or possibly visa-versa) complicates the preparation and optimization of the reagents. It may be necessary either to add the ions to the buffer at the time of assay, or risk reagent instability by the ions diffusing across the liposomal membranes on storage.

The use of Sulphorhodamine B, on the other hand, could be claimed to be inherently simpler, stabler and require less optimization than other known chromogenic markers. This is because the colour change is developed simply by dilution, rather than relying on colour development by a second reagent.

The reagents used in the liposomal immunoassays based on Sulphorhodamine B release were simple to prepare and assays were performed in minutes; while comparative immunoassays (RIAs and ELISAs) required at least several hours. The dye-containing liposomes were very stable when stored at 4°C, loosing at most a few percent of entrapped dye per day at room temperature. No detectable alteration in immunoreactivity was found on storage of liposome preparations for at least six months.

Sulphorhodamine B showed good solubility and a high absorbance coefficient. The shift in absorption between 530nm and 565nm on dye release from the liposomes was
satisfactory for the Cobas Bio which uses a diffraction grating monochromator with a narrow transmitted wavelength range.

Some analysers use a bichromatic approach. Measurements are taken at one wavelength and a blank reading at a secondary wavelength to reduce interferences (e.g. light scatter due to sample turbidity). In these analysers it might be possible to almost double sensitivity, by taking measurements at 565nm (with increasing absorbance) against a blank at 530nm (with decreasing absorbance).

There are also several potential disadvantages of the use of Sulphorhodamine B-containing liposomes, compared to some other immunoassay techniques.

Several instruments still use optical filters which allow a much wider range of wavelengths to be transmitted than diffraction grating monochromators (e.g. 30 nm). These may not be able to adequately differentiate the absorbances at 530 nm and 565nm. Furthermore, the release of entrapped dye showed only a slight visual colour change. Qualitative tests, perhaps for near-patient testing, would also benefit from a more marked colour change.
Another aspect which needs careful thought is the susceptibility of liposome preparations in general to disruption by detergents (chapter 2). This is not an insurmountable difficulty as some automated analysers, such as the Cobas Bio, do not use them. Other equipment, particularly those that clean reaction chambers and cuvettes rather than using disposables, may rely on detergents. Thorough rinsing would be required to eliminate the possibility of interference with the liposomal reagents.

A further difficulty lies in the complexity of liposomes and their varied compositions. These can hinder a systematic prediction of their properties. Even with growing knowledge of liposome properties (e.g. 2-4), liposome preparation and assay optimization still seem to require a degree of trial and error.

This unpredictability is illustrated by apparent contradictions in the present study to earlier reports. For example, anticardiolipin antibodies were found to augment destabilization of cardiolipin-liposomes by
divalent cations. This contrasts with an earlier finding (108) which showed inhibition of lysis by anticardiolipin antibodies, although the difference may be largely due to different assay conditions (as discussed in section 4.10).

Another unpredictable factor lies in the use of complement. As discussed in chapter 3, this is a multi-component mixture whose activity depends on its species of origin and probably also on its preparation and extent of purification.

The liposomes in this report were very stable at 4°C but showed a rate of dye loss of up to several percent per day at room temperature. This is probably acceptable for laboratory applications. For a wider use of liposomal immunoassays, e.g. in "near-patient testing", it would be preferable to have reagents stable for long periods at room temperature.

A further potential problem which was not addressed was the possibility of batch to batch variation. For consistency, the reported results are based on a single
batch of each type of liposome. At least two batches of each type of liposomes were prepared and showed similar immunoreactivity. Extensive studies of the comparability of several batches of liposomes, however, were beyond the scope of this study.

5.3 Areas for Further Study and Future Developments of Liposomal Assays

Further studies could address some of the potential problems described in the previous section. For example, a systematic survey of the optical properties of other members of the rhodamine class of dyes, and indeed other dyes, would seem worthwhile. This might uncover other chromogens with a wider shift in absorption wavelengths on their release from liposomes. In this study, only one other member of the rhodamine family of dyes, Rhodamine B, was examined. This showed a less favourable absorbance coefficient and lower aqueous solubility to Sulphorhodamine B and also, in preliminary experiments, poor entrapment (section 2.12).

It would also be worth examining alternative chromogenic markers and methods to generate a colour change when the chromogens are released from liposomes. pH-dependent
colour change seems not to have been exploited in a liposomal immunoassay, even though liposomes tend to have a low permeability to protons.

As discussed in chapter 2, it would be useful to examine whether the Beer-Lambert law is obeyed at the high concentrations in chromogens which do not exhibit dimer formation. If not, this might provide another mechanism to produce an absorbance change on liposomal lysis and chromogen dilution.

The question of liposome stability has been mentioned in the previous section. It may be possible to freeze-dry liposome preparations and reconstitute them prior to assay by adding a diluent (30). A similar approach has recently been used in the pharmaceutical field for liposomes carrying vaccines (231).

An alternative approach to improved stability would be chemical modification of the liposome membranes. Several such stabilized liposome formulations have been made (232-234). Stabilized liposomes are less likely to be suitable for homogeneous assays involving release of their contents following their lysis. However they could be used in solid phase assays (section 1.6.8).
An improvement in stability and possibly homogeneity might also be achieved by development of novel "synthetic liposomes", i.e. vesicles composed of synthetic, non-lipid amphiphiles. The following two reports suggest this might be possible. Lamellar structures (not closed vesicles), formed from dihexadecyl-dimethylammonium bromide, were used to promote agglutination in the presence of antibody. This involved electrostatic interaction with an anionic antigen \((N, N' \text{ bis}(2,4 \text{ dinitrophenyl})-\text{L-lysine (235)})\). In a second, non-immunological, application; vesicles composed of didodecyldimethylammonium bromide were used in a chemiluminescent assay for free cyanide ions (134).

Also discussed in the previous section was the necessity to minimize the degree of batch variation between preparations if any application were to be developed for routine use. A commercial application would, in addition, require the scaling-up of the reagent preparation. An alternative to the reverse-evaporation method might be easier to scale-up. Various techniques to synthesize liposomes were discussed in section 1.3. The optimum size of liposome preparation for a given application should also influence the synthetic technique used. The effects of size on liposomal immunoassays was discussed in section 3.8.
As far as the applications of liposomal immunoassays studied in this report, the potentially most useful studies are in the measurement of anticardiolipin antibodies. Their clinical value, particularly in cardiovascular disease, remains to be fully established. The liposomal assay which was developed might have a role to play in this research. Its rapid speed and ease of automation would enable the measurement of large number of patient samples. In addition, rapid colorimetric assays are more likely to be usable in the clinic (e.g. in out-patients) where there may be an advantage in a rapid result being immediately available to the physician.

Although assays for urinary microalbumin are common, the liposomal assay has potential advantages over other techniques. The assay is much more rapid than radioimmunoassay. Rapid homogeneous assays for microalbumin are also available using immunoturbidimetry. However the liposomal assay has the advantage that measurements are made at a higher wavelength, so interferences due to non-specific light-scattering from the sample are less likely.

From a wider perspective, there are a range of potential future roles for liposomal assays.

306
The use of liposomes in ultra-sensitive assays was not addressed in this project. Many alternative ultra-sensitive techniques are being developed. New labels which have high potential sensitivity include bioluminescent labels, time resolved fluorescent labels, Fourier transform infra-red analysis of metal carbonyl complexes, and use of phosphors (10). In addition, coupled enzyme reactions can be used to produce an amplified signal. These techniques should not necessarily be seen as competing with liposomal immunoassays. In most if not all cases, these labels could, in principle, be combined with liposomal amplification to create even greater sensitivity.

New applications may arise which make use not only of the characteristics of the marker in the liposomal assay, but also unique properties of the liposomal structure. The possible uses of liposomes in latex agglutination assays has been described in section 1.6.10. Liposomes might also enhance immunoturbidimetry in a similar way, as an alternative to other particle enhancement agents (discussed in section 1.6.1).

As another possible application, Wallace and Wood (236) described the use of semi-permeable nylon capsules containing antiserum as a means to separate radiolabelled
free hormones. Liposomes may provide an alternative to the use of nylon capsules. It might, for example, be possible to develop sensitive assays for free hormones (e.g. free thyroxine), using antibody-containing liposomes incorporating pores of specific restricted size in their membranes.

Recent analytical applications of liposomes, which have been described in chapter 1, include flow injection analysis, flow cytometry and electrochemical detection. Other technical advances are also possibly amenable to the involvement of liposomes. Several applications make use of colloidal gold sols, which absorb between 510 nm and 550 nm and can absorb proteins non-covalently onto their surfaces. These are often used in multi-assay systems.

One such technique is the Ascend Multiimmunoassay (TM Biosite), which utilizes the flow of reagents and sample through a wick which contains discrete areas impregnated with specific antibodies to provide a screen for a panel of drugs of abuse (237). Replacement of the gold sol conjugates by liposomes coated in specific antigens (or antibodies) might improve the clarity of the colour reaction, if intense dyes were entrapped.
A non-immunological application of liposomes which might be developed using a simple colorimetric marker is the measurement of phospholipases. This was described in section 1.7). Another possible non-immunological area for liposomal assay development lies in the use of liposomes to study or measure the effects of lipid peroxidation (also described in section 1.7). Liposomes have been used to study this; however combination with a simple dye marker might enable novel analytical tests of this important biological process to be developed.

Although this project has concentrated on analytical applications, major recent advances in the applications of liposomes have come in the pharmaceutical field. For example, there is currently considerable interest in the use of liposomes to carry genes in the therapy of individuals suffering from genetic disorders. Clinical trials are underway in the treatment of cystic fibrosis using liposomes (238).

Many of the difficulties encountered in the pharmaceutical use of liposomes are not found in analytical applications. For example, the route of liposomal clearance from the circulation is not a consideration. There is nevertheless a certain common ground, since even the most elegant pharmaceutical
application must first be based on carefully optimizing entrapment of often scarce amounts of material. The relationships of liposome size, amount of entrapment and surface binding of proteins were discussed in chapter 3. These seem equally relevant to the pharmaceutical field.

The low cost and the ease of colorimetric detection of Sulphorhodamine B might, in some circumstances, make it a useful preliminary tool in assessing entrapment and stability of pharmaceutical liposome preparations. However it could not replace the use of the pharmaceutical agent to any great extent; since the agent might exhibit differing effects on entrapment and stability from those of the dye, e.g. by differing charge. Furthermore, the safety of Sulphorhodamine B in pharmacological studies would require examination. Perhaps the liposomal entrapment of this dye might be more useful in other fields. For example, it might provide a simple addition to existing methods in the study of membrane properties using liposomes.

Returning finally to the analytical applications of liposomes, these studies have demonstrated some features and applications of liposomal immunoassays. The use of liposomes has some advantages over other techniques, as well as a few potential disadvantages.
It seems unlikely that any one immunoassay technique will replace all others in the near future. The wide range of possible approaches to the use of liposomes and the wide choice of possible labels that can be incorporated have been described. This variety makes it likely that liposomal immunoassay will at least find a place, for specific applications, alongside other recent approaches.
Appendix 1

Light and Electron-Microscopy Photographs of Liposome Preparations

Light-microscope photographs were taken of reverse-evaporation liposomes prepared as described in section 2.4. A droplet of aqueous liposome preparation was placed on a microscope slide, covered by a cover-slip and photographed immediately to avoid drying. The greatest clarity of photographs were obtained using a blue optical filter.

Figure A1.1 shows liposomes fractionated by Sepharose 4B gel filtration but not passed through a 200 nm polycarbonate filter to increase homogeneity (as is the case in the procedure for protein-coated liposomes, e.g. section 3.3.1). The magnification was x 640 (using x 40 objective lens).

Figure A1.2 shows the same liposome preparation using a x 100 objective lens (magnification x 1600). Using this magnification the image was less clear and some distortion of the liposome images is evident due to Brownian movement of the liposomes.
Figure A1.1

Sulphorhodamine B-Containing Liposomes by Light Microscopy

Light microscope photograph of liposome fraction from Sepharose 4B gel filtration, without subsequent passage through 200 nm filter. The magnification was x 640.
Light microscope photograph of liposome fraction from Sepharose 4B gel filtration, without subsequent passage through 200 nm filter. The magnification was x 1600.
The figures show a wide range of sizes of liposomes with a few very large liposomes (i.e. several um diameters). In figure A1.1, for example, 200 nm liposomes would have a diameter of approximately 0.12 mm.

Liposomes which had been passed through a 200 nm polycarbonate filter were visible by eye as small pink dots using a (x 40) objective (magnification x 400). Liposome preparations with surface bound protein (sections 3.3.1, 3.5 and 3.6) and without surface-bound protein (section 2.4) had similar appearances with no visible evidence of clumping. These preparations did not permit clear light-microscope photographic images to be obtained.

Using albumin-coated liposomes (which had been passed through a 200 nm filter, section 3.3.1); clear micrographic images were obtained by the School of Materials Sciences, University of Surrey, using an electron microscope (figure A1.3). The negative staining technique was used (239). A drop of the preparation was placed on the electron microscope grid, left for a minute and excess removed using filter paper. The grid was then floated in stain (2% potassium phosphotungstic acid), dried and viewed in the electron microscope. The magnification was approximately x 8,500.
Albumin-coated liposomes, after passage through a 200 nm polycarbonate filter, were observed by electron microscopy, using the negative staining technique. The magnification in the photograph is approximately 8,500.
The small white dots were believed to be an artifact, probably due to background stain. It is impossible to say whether a degree of clumping was present or whether the apparent aggregations were due to the overlap of discrete liposomes in the field of view.

The objective in obtaining these electron microscope photographs was to demonstrate the presence of liposomes, rather than to estimate their size. To estimate size reliably various dilutions and fields of view would be required to minimize artifactual aggregation (239). The diameter of the original liposome is usually assumed to be less (e.g. 0.71 x the diameter of the photographed disc) to allow for flattening of the disc on drying.

A mean diameter of close to 200 nm was used as a basis for calculations of protein binding and dye entrapment in the discussion of chapter 3. Allowing for flattening, a 200 nm liposome would have an approximate diameter of 2.4 mm in figure A1.3. The photograph suggests this may be a reasonable approximation. However, because of the difficulty in accurately assessing size from a limited number of fields, such an estimate was not attempted.
Appendix 2

Spectrofluorimetric Properties of Sulphorhodamine B

The fluorimetric characteristics of a solution of Sulphorhodamine B (2 x 10^{-6} M in distilled water) were examined between approximately 240 nm and 700 nm. The emission and excitation filter band-widths were both set at 10 nm (except when either was set as unrestricted). Figure A2.1a shows a scan of wavelengths of excitation allowing all emission wavelengths to be open. This indicated that the only significant excitation wavelength was 400 nm. Figure A2.1b is a scan of emission wavelengths in which the excitation wavelengths were unrestricted. This indicated that the only significant emission wavelength was 580 nm. The vertical gradations in figure 2.1a and 2.1b were 3 arbitrary units (as defined in materials and equipment section.)

Figure A2.2 shows the emission at the excitation wavelength of 400 nm. A single emission peak at 580 nm was found. The vertical scale gradations in figure A2.2 were 27 arbitrary units. These excitation and emission wavelengths were used throughout this study. A 10 nm band-width for both emission and excitation filters was also used throughout.

318
Figure A2.1

Fluorimetric Scans to Determine Excitation and Emission Wavelengths of Sulphorhodamine B

Figure (a) is a scan of excitation wavelengths, in which the emission wavelengths are open. Figure (b) is a scan of emission wavelengths, in which the excitation wavelengths are open. The scanned solution was Sulphorhodamine B in distilled water, as described in Appendix 2.
Fluorimetric emission wavelength scan of Sulphorhodamine B in distilled water. The measurement conditions are described in appendix 2.
There was a high amount of emitted light at the excitation wavelength which was attributed to an artifact of the instrument's optical system. This was demonstrated by the apparent emission at the excitation wavelength when the dye solution was replaced by distilled water (figure A2.3).

The minimum scale gradation for the fluorimeter was 1 arbitrary unit and for the manual spectrophotometer in these studies was 1 mA. Under the fluorimeter settings described, the dye gave an absorbance of 1 mA for every 4 arbitrary units of fluorescence. It was established that the minimum scale gradation could be measured reproducibly by identical readings of a water blank over 12 replicate samples. This indicated that fluorescence gave only approximately a four fold increase in sensitivity compared to absorbance measurements under these conditions. A large potential increase in fluorescence sensitivity could, however, be obtained by increasing the filter band-widths for the excitation and emission filters. This is demonstrated in table A2.1 for various filter band-widths. At least a 25 fold increase in fluorescence signal could be obtained compared to 10 nm band-widths. This would have conferred at least a hundred fold increase in sensitivity of fluorescence relative to absorbance measurements.

321
<table>
<thead>
<tr>
<th>Excitation Band-width (nm)</th>
<th>Emission Band-width (nm)</th>
<th>Fluorescence Signal(^+)</th>
<th>Relative* Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>900</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>50</td>
<td>0.055</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>225</td>
<td>0.25</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>228</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>2080</td>
<td>2.31</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>4210</td>
<td>4.68</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>6970</td>
<td>7.74</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>11700</td>
<td>13.0</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>16200</td>
<td>18.1</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>23100</td>
<td>25.7</td>
</tr>
</tbody>
</table>

* relative to 10 nm filter band-widths
+ arbitrary units
Fluorimetric emission wavelength scan of distilled water, indicating a large artifactual light emission at the excitation wavelength. The measurement conditions are described in appendix 2.
References

(3) Gregoriadis G. and Allison A.C. (Eds.) Liposomes in Biological Systems, John Wiley and Sons 1980
(4) Knight C.G. (Ed.) Liposomes: from physical structure to therapeutic applications, Elsevier/ North-Holland Biomedical Press 1981
(7) Gregoriadis G. Liposomes and anti-aging creams: the facts behind the face. Biochemist 1994; 8-11
(16) Monroe D. Liposomal Electrochemical Immunoassay, ICPR.1988; 18-28

324
(21) Bangham A.D. and Horne R.W., Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J. Mol. Biol. 1964; 8: 660-668
(23) Szoka F., Olson F., Heath T., Vail W., Mathew E. and Papahadjopoulos D. Preparation of unilamellar liposomes of intermediary size (0.1-0.2um) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim. Biophys. Acta 1980; 601: 559-571
(33) Olson F., Hunt C.A., Szoka F.C., Vail W.J. and


(41) Verkleij A.J. and deGier J. Freeze fracture studies on aqueous dispersions of membrane lipids. Liposomes: from physical structure to therapeutic applications, C.G. Knight (Ed.) Elsevier/North Holland 1981: Chapter 4:83-103


(44) Borrel J.A. and Sole R.P. Caracterizacion de los liposomas por el metodo de dispersion de la luz. (Spanish) Il Farmaco-Ed. Pr. 1986; 42: 139-148


(46) Van Renswoulde A.J.P.M., Blumenthal R. and Weinstein 326
(66) Scherphof G., Damen J. and Hoekstra D. Interactions of liposomes with plasma proteins and components of the immune system. (in) Liposomes: from physical structure to therapeutic applications (Ed. Knight C.G.) Elsevier/North Holland 1981; Chap. 10: 299-322
(76) Carlson J., Drevin H. and Axen R. Protein thiolation
328


(78) SPDP Heterobifunctional reagent. Technical leaflet; Pharmacia Fine Chemicals AB; Sweden


(82) Leserman L.D., Barbet F., Kourilsky F. and Weinstein J.N. Targetting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or Protein A. Nature 1980; 288: 600-604


(90) Marsh D. and Watts A. ESR spin label studies of liposomes. (in) Liposomes: from physical structure to
therapeutic applications (Ed. Knight C.G.) Elsevier/North Holland Biomedical Press 1981; Chapter 6: 139-188


(97) Urema K. and Kinsky S.C. Active vs. passive sensitization of liposomes toward antibody and complement by dinitrophenylated derivatives of phosphatidylethanolamine. Biochemistry 1972; 11: 4085-4093


(104) Alving C.R., Joseph K.C., Lindsley H.B. and Schoenbechler M.J. Immune damage to liposomes containing

330


(119) Paul A., Madan S., Vasandani V.M., Ghosh P.C. and Bachhawat B.K. Liposomal immune lysis assay (LILA) for


(124) Shiba K. et. al. Thin-layer potentiometric analysis of lipid antigen-antibody reaction by tetrapentylammonium (TPA+) ion loaded liposomes and TPA+ ion selective electrode. Anal. Chem. 1980; 52: 1610-1613


(129) Leserman L.D., Barbet J. and Kourilsky F. Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. Nature 1980; 288: 602-604


(134) Ishii M., Yamada M. and Suzuki S.


(162) Jones M.C. Microalbuminuria and kidney disease. ICPR 1987; 64-71


(178) Makworth-Young C. Antiphospholipid antibodies: more
than just a disease marker? Immunology Today, 1990; 11: 60-65


(190) Gulkó P.S. Reveille J.D., Koopman W.J., Burgard

336


(204) Joss A.L. and Steven M.M. Anticardiolipin antibody. Lancet 1990; 399

(205) Creagh M.D., Malia R.G., Cooper S.M., Smith A.R.,

337
(217) Harris E.N. and Hughes G.R.V. Standardising the anti-cardiolipin antibody test. Lancet 1987 (i), 277
(218) Harris E.N. The second international anticardiolipin standardization workshop/ The kingston Anti-phospholipid antibody study (KAPS) group. A.J.C.P. 1990; 476-484

338


(228) McNeil H.P., Simpson R.J., Chesterman C.N. and Krillis S.A. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: (Apolipoprotein H). Proc. Nat. Acad. Sci. USA 1990; 87: 4120-4124


339
(238) Davies K. E. Where the science may take us. MRC News 1994; (Spring): 17-22

UNIVERSITY OF SURREY LIBRARY
Antibody-coated liposomes as a particulate solid phase for immunoassays

Measurement of urinary 'micro-albumin'

S. J. Frost 1, G. B. Firth 1 and J. Chakraborty 2

1 Clinical Biochemistry Department, Hurstwood Park Neurological Centre, Colwell Rd., Haywards Heath, West Sussex, U.K., and 2 Department of Biochemistry, University of Surrey, Guildford, U.K.

(Received 28 March 1990, revised received 6 August 1990, accepted 9 August 1990)

A novel use of liposomes as a solid phase material achieving separation in immunoassays is described. Antibody-coated liposomes were prepared and used as a particulate solid phase in a radioimmunoassay procedure for urinary albumin. The assay was compared to a liquid phase albumin radioimmunoassay. The potential benefits of liposomes over other particulate solid phases are discussed. The use of liposomes in this manner need not be restricted to radioimmunoassay but should also be applicable to other immunoassays using alternative non-isotopic labels.

Key words: Anti-albumin; Liposome; Micro-albumin; Radioimmunoassay; Solid phase; Sulforhodamine B

Introduction

Liposomes, small synthetic lipid vesicles, have been widely used both as drug delivery systems and as model membranes. There has also been considerable interest in their use and versatility as analytical reagents. Assays usually involve the encapsulation, within antigen- or antibody-coated liposomes, of an aqueous marker, e.g., fluorophor, chromophor or an enzyme. The liposomes can then be used as the 'label' in heterogeneous (O'Connell et al., 1985) or homogeneous assays (Freytag and Litchfield, 1984; Bowden et al., 1986; Umeda et al., 1986). In developing such assays it became apparent that antibody-coated liposomes have the potential for use as particulate solid phases in conventional heterogeneous immunoassays.

Despite the attractions of homogeneous and coated surface assays, techniques which involve separation, particularly radioimmunoassay and immunoradiometric assays, have remained popular. Other particulate solid phase materials (e.g. cellulose) are not easily dispensed and settle out rapidly on standing. This may limit their range of application, in, for example, automated systems. Liposomes, prepared as described here, behave as liquids prior to immune reaction, being easily pipetted and only exhibiting slight sedimentation after standing for several days. It was noticed that...
these liposomes, after reacting with the respective antigen, precipitate more readily, thereby permitting separation by moderate centrifugation e.g., at 2000 × g for 10 min. This phenomenon was used as the basis for developing a heterogeneous immunoassay for urinary albumin (microalbumin) and the results are reported here.

Materials and methods

Materials

Purified lipids, dipalmitoyl phosphatidyl choline (PC), dipalmitoyl phosphatidyl ethanolamine (PE), cholesterol (Chol) and dipalmitoyl phosphatidic acid (PTA), the dye sulphorhodamine B and human albumin standard were supplied by Sigma Chemical Company Ltd., Poole, Dorset. A purified immunoglobulin G fraction of polyclonal anti-human albumin antisera and normal sheep serum (NSS) were obtained from Guildhay Antisera Ltd., Guildford, Surrey. 125I-labelled human albumin (specific activity 16.4 yCi/µg) was supplied by North East Thames Regional Immunoassay, London (NETRIA). Gel filtration materials and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were obtained from Pharmacia Ltd, Milton Keynes, Buckinghamshire. Protein was measured using a manual Coomassie blue assay (Bio-Rad Laboratories Ltd, Watford, Hertfordshire) and cholesterol by a colorimetric procedure (`CHOD-PAP' method, BDH Ltd, Poole, Dorset). Other chemicals and AnalaR grade reagents were also supplied by BDH Ltd. Gel filtration was performed using 0.7 mm × 20 cm ‘Econo Columns’ (Bio-Rad Laboratories Ltd). Dialysis employed 18/32” dialysis tubing (Medicell International Ltd, London). Liposomes were filtered using 200-nm ‘Minisart NML’ filters (Sartorius Ltd, Epsom, Surrey). Sonication was achieved by a Soniprep 150 probe sonicator (MSE Scientific Instruments Ltd, Crawley, West Sussex). An LKB-Wallac Clinigamma counter (Pharmacia Ltd) was used to measure radioactivity. Phosphate buffer, 0.05 M, pH 7.4, was used throughout except for ‘L Buffer’ which contained 10 mM N-[2-hydroxyethyl] pipеразине-Ν’-[2-этилсульфононая кислота] (HEPES) and 145 mM sodium chloride, pH 8.0.

Dithiopyridyl phosphatidyl ethanolamine (DTP-PE)

DTP-PE was prepared by a standard method (Truneh et al., 1987), adapted so that the entire procedure leading to the preparation of liposomes was completed within a working day. PE, 6.9 mg in 300 µl chloroform, SPDP, 3.75 mg in 300 µl methanol and triethanolamine, 2.7 µl, were mixed, solubilised by brief heating to 30°C and then incubated at room temperature for 2 h. The organic phase was then washed once in phosphate buffer and twice in distilled water, centrifuging at 2000 × g for 5 min and discarding the aqueous phase at each stage. Chloroform (1 ml) was added prior to the last centrifugation step to facilitate complete removal of the final aqueous phase without excessive loss of the organic phase. A white precipitate which formed at the solvent interphase could be reduced but not eliminated by addition of a small amount of methanol. This precipitate was discarded after the final centrifugation, leaving a clear organic phase containing DTP-PE. The concentration of DTP-PE formed was estimated by the release of dithiopyridine as described previously (Truneh et al., 1987). An aliquot (25 µl) of the organic phase was gently evaporated to dryness, immediately 1 ml of ‘L Buffer’ was added and vortexed vigorously. The absorbance before and after adding 50 µl dithiothreitol (100 mM) was measured and the molar concentration of DTP-PE calculated as the change in absorbance at 343 nm × 41 (dilution factor)/8.08 × 10⁻³ M.

Liposomes

The DTP-PE was immediately incorporated into liposomes by a reverse phase evaporation method based on that of Szoka and Papahadjopoulos, (Szoka and Papahadjopoulos, 1978). DTP-PE (2 µmol, usually approximately 0.5 ml) was mixed with 28 µmol PC, 9 µmol PTA and 26 µmol chol in 2 ml petroleum ether and chloroform added to a total volume of 4 ml. Sulphorhodamine B, 1 ml 0.1 M in distilled water, was then added so that the liposomes could easily be seen after precipitation in the immunoassay. The mixture was sonicated for 2 min, then 1 ml of phosphate buffer was added and the preparation evaporated at 45°C under reduced pressure until the foaming subsided after about 20 min leaving a liquid. The resulting liquid which contained large unilamellar lipo-
somess (LUVs) was filtered through a 200-nm polycarbonate filter to increase homogeneity, then dialysed overnight against phosphate buffer to remove unencapsulated dye. The DTP-liposomes were coupled to dithiopyridyl-antibody (DTP-antibody) within 2 days.

Formation of dithiopyridyl-antibody (DTP-antibody)

DTP-antibody was formed essentially as described previously (Truneh et al., 1987). The protein content of the antiserum was measured and SPDP added in a small volume of methanol (usually 1 ml) with gentle mixing to give a molar ratio of SPDP:antiserum of 10:1. The mixture was incubated at approximately 23°C for 30 min with occasional stirring. The pH was then reduced to approximately 4.5 using 50 µl of acetic acid, pH 3. Immediately prior to coupling with the liposomes, 50 µl of dithiothreitol (500 mM) were added and the DTP-antibody incubated for 20 min at room temperature. The DTP-antibody was then separated from the other reagents by gel filtration on Sephadex G50 equilibrated in 0.05 M phosphate buffer, pH 7.4. The protein content of the fraction was measured by the Bio-Rad Coomassie blue method.

Coupling of liposomes and antibodies

Equal volumes of the DTP-antibody reagent and the liposome preparation were mixed and incubated overnight at room temperature. The mixture was then separated by gel filtration using Sepharose 4B and 0.05 M phosphate buffer, pH 7.4, the antibody-coated liposomes eluting in the void volume while the uncoupled protein and residual free dye were retarded by the gel. The liposome fractions so prepared have been stored at 4°C over several months without noticeable loss of immunological activity.

The protein content of the preparation was determined by the Bio-Rad Coomassie blue method. The modification of this method for this purpose entailed solubilising the protein by the addition of 50 µl of ethanol to 20 µl liposome preparation. The standard colorimetric procedure was then followed by the addition of 1 ml of saline and 200 µl of Coomassie blue reagent, incubating at room temperature for 15 min and then measuring the absorbance at 580 nm. It was also necessary to subtract a blank for the absorbance of Sulphorhodamine B at the same wavelength. In this case saline replaced the Coomassie blue reagent. Correspondingly, 50 µl of ethanol per tube were also added to the standards to compensate for the small additional effect of ethanol on the absorbance of the reagent.

Measurement of the lipid content of liposomes by simple chemical methods was complicated by the interfering intense absorbance of Sulphorhodamine B. This was avoided by overnight dialysis of the preparations in the presence of a detergent (Triton X-100) against a thousand-fold excess of distilled water. Detergent has the effect of lysing liposomes, allowing the dye to be dialysed while the lipid is retained. Cholesterol was assayed after dialysis (allowing for dilution during dialysis) and total lipid calculated (mol cholesterol x 2.42).

Evidence of competitive label binding

To establish, independently of the immunoprecipitation process, that competitive binding of albumin to liposomes was occurring, various amounts of albumin were added to 30 µl of liposome preparation and 100 µl phosphate buffer containing 1% NSS and [125I]albumin. After 4 h incubation at room temperature the mixtures were each separated using Sepharose 4B and the eluted 0.5 ml fractions analysed for radioactivity. Control studies made use of liposomes synthesised as above but not coupled to anti-albumin antibody.

Specimens

Early morning urine specimens from 39 diabetic patients were frozen on receipt. Prior to assay they were thawed and briefly centrifuged to remove precipitated material.

Immunooassay protocols

A standard liposomal immunooassay protocol was as follows. 100 µl of 0.05 M phosphate buffer, pH 7.4, containing 1% normal sheep serum (NSS) and [125I]albumin were mixed in a 4-ml plastic tube with 30 µl of anti-albumin liposomes and 6 µl of urine sample/standard (0–200 mg/l). The tubes were incubated at 37°C overnight, centrifuged for 10 minutes at 2000 x g, washed in 500 µl of buffer containing 1% NSS and then centri-
fuged again as before. After aspiration of supernatant the precipitated liposomes were counted for 300 s per tube using a gamma counter. This protocol was varied as appropriate experimentally. The assay was compared with a liquid phase immunoassay performed using an established protocol (NETRIA). Briefly, this involved an overnight incubation followed by polyethylene glycol precipitation, centrifugation (2000 × g, 30 min) and counting the pellet for radioactivity. Precision was estimated by replicate single measurements of pooled urine albumin specimens.

Results

The liposomes synthesised were calculated to have a ratio of bound anti-albumin to lipid of approximately 72 µg : µmol. For use in immunoassay these were diluted to an anti-albumin concentration of 43 mg/l. Competitive binding of albumin label was demonstrated by analysis of fractions from gel filtration (Fig. 1). Fig. 2 il-

![Graph](image1.png)

**Fig. 1.** Various amounts of albumin (0 ng X, 10 ng O, 50 ng +, 200 ng *, 2 µg *) were incubated with anti-albumin liposomes and $^{125}$I-labelled albumin (see text) and the mixtures separated by Sepharose 4B gel chromatography. 0.5 ml-fractions were collected and measured for radioactivity. The liposomes and bound label eluted in the void volume.

![Graph](image2.png)

**Fig. 2.** 'Control experiment' showing elution positions of components following gel filtration on Sepharose 4B, after incubation of liposomes, not coupled to anti-albumin antibody, with $^{125}$I-albumin. Peaks are (a) liposome entrapped dye, (b) free dye, (c) free $^{125}$I-albumin. The Sulphorhodamine B content by absorbance at 565 nm (peaks a and b) and radioactivity (peak c) of 0.5-ml fractions is shown.

![Graph](image3.png)

**Fig. 3.** Effect of increasing amounts of liposomes on the observed maximum binding of $^{125}$I-albumin using the liposomes immunoassay protocol (see text). Total counts-min$^{-1}$ was 6500 per tube.
Illustrates, for comparison, the separation of liposomes not coupled to anti-albumin, free dye ($A_{\text{max}}$ 565 nm), and free albumin label under the same experimental conditions as for Fig. 1. Free immunoglobulin and free albumin elute with essentially the same retention time. No significant elution of albumin label in the liposomal fractions was observed when free anti-albumin (20 µg) was also added to the preparation prior to chromatography.

In the immunoassay procedure, the effects of changing the incubation time and temperature were investigated. The sensitivity as indicated by the dose–response curve was greater at 37°C than at either 25°C or 45°C. The effect of incubation time was assessed from the dose–response curves at 1, 4, 16 and 40 h. Sensitivity increased to 16 h but a longer incubation did not cause a significant improvement. Overnight incubation at 37°C was selected as giving the best sensitivity for the assay.

Fig. 3 indicates that the maximum binding of label depends on the amount of anti-albumin liposomes added to each tube. A maximum binding

![Graph](image)

Fig. 4. Typical standard curve using the liposomal immunoassay (for protocol see text). Total counts min⁻¹ equals 6540 per tube. The binding of the zero standard was 15% of the total counts. In the protocol 200 mg/l added albumin standard equals 1.2 µg albumin standard per tube.

![Graph](image)

Fig. 5. Comparison between measured albumin concentrations in 39 urine specimens measured using the liposomal immunoassay and the NETRIA RIA method. ($y = 0.83x + 1.65, n = 39, r = 0.97$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean value (mg/l) + S.D.</th>
<th>n</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine pool, No. 1</td>
<td>20.1 ± 1.57</td>
<td>20</td>
<td>7.8</td>
</tr>
<tr>
<td>Urine pool, No. 2</td>
<td>46.0 ± 2.34</td>
<td>20</td>
<td>5.1</td>
</tr>
</tbody>
</table>

(i.e. zero standard/total counts) in each assay of between 10–20% was achieved using the present protocol.

A typical standard curve is shown in Fig. 4. Within batch precision data for 2 levels of albumin is given in Table I. The correlation of the data obtained with the liposomal method with those from an established radioimmunoassay is shown in Fig. 5 ($y = 0.83x + 1.65, n = 39, r = 0.97$).

**Discussion**

The liposomes incorporated antibody at an antibody/lipid ratio similar to that previously re-
ported (Hutchinson et al., 1989). Sulphorhodamine B dye was encapsulated into the liposomes for several reasons. The dye offered some advantage in the immunoassay itself in that the red immune-precipitate could be readily seen during the aspiration stage. It also permitted, either visually or by absorbance measurement, the identification and quantitation of liposomes during preparative procedures such as gel filtration. Furthermore, dye was included for the development of homogeneous immunoassays, proceeding concurrently with these investigations. Microscopic visualisation of liposomes was also made easier by the inclusion of dye. Prior to immune reaction, the liposomes appeared as mainly discrete spheres with only occasional larger aggregates.

The method we used to synthesise antibody-coated liposomes was at least as simple as most methods for generating antibody-coated solid phases using cellulose, Sephadex or polystyrene, e.g. latex particles (Wide 1981; Collet-Cassart et al., 1983). Coupling of antibody and phospholipid amino groups is achieved using SPDP, a heterobifunctional reagent which avoids protein-protein cross-linkage.

An alternative method of liposome synthesis to the reverse evaporation method we employed, such as detergent dialysis (Lichtenberg, 1988), may be more suited to the production of a large batch of DTP-liposomes which could be coupled, if desired, to a range of antibodies.

The liposomes we prepared showed no loss of immune reactivity over several months indicating an adequate 'shelf-life'. Methods have been employed that increase the stability of liposomes using, for example polymerizable phospholipid analogues (Regen et al., 1982). This approach has the potential to produce very long 'shelf-life' liposomes although the preparation would almost certainly be more difficult.

The assay showed greater sensitivity at 37°C than at either 25°C or 45°C. The phospholipids used undergo a phase transition between 37°C and 45°C, allowing greater membrane fluidity at the higher temperature. However, the data failed to indicate any increase in sensitivity due to this fluidity. A maximum (zero standard) binding of label of 10–20% was achieved under the conditions of the adopted protocol, which is lower than the 50% normally sought in a conventional radioimmunoassay. Our results, shown in Fig. 3, indicate that higher binding might be achieved by increasing the liposome/label ratio. This will be investigated in future studies once the liposome preparation can be scaled up.

Albumin was chosen as the analyte here in order to develop and evaluate the use of liposomes as a solid phase in immunoassay. The present optimised procedure for urinary albumin correlated well with the alternative RIA method within the concentration range of 0-200 mg/l. A concentration of 20 mg/l, or thereabouts, is generally adopted as the cut off point above which "microalbuminuria" would be indicated. A clinically useful assay should therefore have a good dose-response sensitivity at this concentration. Fig. 4 shows that the liposomal immunoassay has a near maximal dose response slope at this level suggesting near optimal sensitivity. The correlation data of Fig. 5 suggests that the liposomal immunoassay and the NETRIA assay have similar abilities to discriminate microalbuminuria patients from other patients using the 20 mg/l cutoff.

Above 200 mg/l, frank albuminuria is considered to be present. There is less interest in measuring these higher concentrations by immunoassay since they are easily measured by less sensitive chemical methods.

It is interesting to postulate the mechanism involved in the increased precipitation of immune-reacted liposomes. Microscopically after immune reaction the liposomes, previously relatively homogeneous pink spheres, clump into heterogeneous clusters. One possible mechanism for this phenomenon would be immune complex formation, which may be similar to the reported augmentation of latex agglutination by liposomes (Kung et al., 1985). It might be predicted that such complexes would only form with large antigens such as proteins, which have multiple antibody binding sites. Immunoradiometric assays and 'sandwich type' enzyme-linked immunosorbent assays are generally similarly restricted to large analytes. However, although composed of a relatively simple mix of components, the surface properties of liposomes can be complex. The liposomes used in this study contain lipids of net negative surface charge. This charge is likely to be modified by
antibody and antigen binding. Liposome clumping has also been reported after incubation with other agents, e.g. high concentrations of calcium ions (Papahadjopoulos and Vail, 1978). Therefore an alternative electrostatic mechanism for the clumping, not dependent on large immune-complex formation, cannot be ruled out.

Acknowledgements

This study has arisen from research supported by grants awarded by South West Thames Regional Health Authority Locally Organised Research Scheme, the British Heart Foundation and the T.V.S. Trust.

References


